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The use of natural genetic variation in *Caenorhabditis elegans* to identify novel polymorphisms that can confer benzimidazole resistance in nematodes

by

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Abstract

Benzimidazoles are important drugs for parasite control. Benzimidazole resistance is widespread in parasites of domestic animals, and an emerging problem in human parasites. *Caenorhabditis elegans* is a powerful model system to study biology of drug resistance. Using natural genetic variation in wild populations of the non-parasitic nematode *C. elegans*, three novel amino acid substitutions and a deletion in the β-tubulin drug target have been identified that confer varying levels of benzimidazole resistance. These residues are different to those previously reported in other organisms including nematode parasites or fungi and provide new candidate polymorphisms to be investigated in parasitic nematode species including human parasites where resistance is poorly understood. They may also represent new residues important for drug binding. In addition, presence of these resistance conferring polymorphisms in wild populations of a free-living nematode may indicate benzimidazole drug residues in the environment having a significant impact on natural fauna. Finally, the presence of null *ben-1* β-tubulin alleles in wild *C. elegans* populations indicates the functional redundancy of this gene in nature.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
WHO	World Health Organisation
HIV	Human Immunodeficiency Virus
BZ	Benzimidazole
ABZ	Albendazole
MBZ	Mebendazole
TBZ	Thiabendazole
β-tubulin	Beta tubulin
α-tubulin	Alpha tubulin
N-terminal	Amino terminal
C-terminal	Carboxy terminal
nAChR	Nicotinic acetyl choline Receptor
P-glycoprotein	Permeability glycoprotein
GABA	Gamma-Amino butyric acid
AAD	Aminoacetonitrile derivative
DEC	Diethylcarbamazin
MDA	Mass Drug Administration
SNP	Single Nucleotide Polymorphism
NGM	Nematode Growth Medium
Bp	Base pair
Mbp	Million base pair/Mega base pair
RNA	Ribo nucleic acid
rRNA	Ribosomal RNA
SL	Splice Leader
UV	Ultra violet
DNA	Deoxyribonucleic acid
VNTR	Variable Number Tandem Repeat
CNV	Copy Number Variation
aCGH	Array Comparative Genomic Hybridization
RAD	Restriction-site Associated DNA
EMS	Ethyl-methanesulfonate
ENU	N-ethyl-N-nitrosourea
SNV	Single Nucleotide Variant
QTL	Quantitative Trait Loci
cDNA	Complementary DNA
CGC	Caenarhabditis Genetics Centre
IVT-MS100	IntelliView Technologies-Microscopy System-100
F1	First Generation
L1	First Larval stage
DMSO	Dimethyl sulphoxide
MSE	Mean Squared Error
PBS	Phosphate Buffered Saline

a.u.	Arbitrary Units
ROUT	Robust Outlier detection method
Q	Maximum desired false discovery rate
PCR	Polymerase Chain Reaction
T_m	Melting temperature
oJG	Oligo John Gilleard
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
CCD	Close Circuit Digital
SD	Standard Deviation
CDS	Coding Sequence
mRNA	Messenger RNA
P	Parental
ns	Not Significant
RNAi	RNA interference
GTP	Guanosine Tri Phosphate
MAP	Microtubule Associated Protein

Chapter 1: General Introduction

1.1 Parasitic Helminths

Helminth parasites include nematode, cestode and trematode species and are capable of infecting humans, animals and plants. They are a serious threat to the health and well being of their hosts, thus having major socio-economic impacts worldwide especially in the developing world (Kaplan 2004; Hotez, Brindley et al. 2008).

1.1.1 Impact of parasitic nematodes infections on humans

The World Health Organisation (WHO) estimates that nearly 3 billion people are infected by parasitic helminths worldwide (Stepek, Buttle et al. 2006; Hotez, Brindley et al. 2008). These pathogens are the cause of seven of the seventeen major neglected tropical diseases listed by the WHO (http://www.who.int/neglected_diseases/diseases/en/); namely, Dracunculiasis/guinea worm disease, food borne trematodiases, lymphatic filariasis, Oncocerciasis or river-blindness, Schistosomiasis, soil-transmitted helminthiases, and Taeniasis or Cysticercosis.

The most common gastro intestinal nematodes of humans are *Ancylostoma duodenale, Necator americanus, Ascaris lumbricoides, Trichiuris trichiura* and *Trichinella spiralis* while *Brugia malayi* and *Wuchereria bancrofti* present themselves as systemic infections. In addition to nematodes, trematodes (e.g. *Schistosoma* and *Fasciola*) and cestodes (e.g. *Taenia solium*) are also of considerable importance in some parts of the world (Horton 2003). These parasites have a worldwide distribution especially in tropical, developing regions such as sub-Saharan Africa, East Asia, India, China and South America (Brooker, Clements et al. 2006; Stepek, Buttle et al. 2006) and are responsible for over 130,000 annual deaths (Bethony, Brooker et al. 2006; Harhay, Horton et al. 2010). Infections are mainly seen in school children and their cognitive capabilities and growth are affected as a result of anaemia and malnutrition caused by the blood-feeding nature of these parasites. In adults, disability caused by heavy infections prevents them from working leading to poverty (Hotez, Brindley et al. 2008). In addition, more than 40 million pregnant women are severely anaemic due to hookworm infections (Diawara, Schwenkenbecher et al. 2013). Affected individuals are also prone to secondary infections such as meningitis, tuberculosis and human immunodeficiency virus (HIV) due to the immune-suppressive effects of gastro-intestinal nematodes (Stepek, Buttle et al. 2006).

1.1.2 Impact of parasitic nematode infections on animals

In the livestock industry, the most common infections are by *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus* spp. in sheep and *Ostertagia ostertagi* and *Cooperia* spp. in cattle. These parasites cause substantial financial loss due to reduced production resulting from factors such as weight loss, anaemia and ultimately, death of the infected animals. For instance, an estimated two billion dollars in production loss per annum is reported to occur in the North American cattle industry alone (Stromberg and Gasbarre 2006), while in Australia the costs to treat infections in sheep and cattle is estimated to be about one billion dollars (McLeod 1995). Other notable helminth infections include cyathostomins in horses, and heart and lung worms in cats and dogs.

1.1.3 Impact of parasitic nematode infections on plants

The most common plant parasitic nematodes are *Meloidogyne* spp. (root knot nematodes) and *Heterodera* spp. (cyst nematodes) (Lamber and Bekal 2002) and affect a range of host species such as cereal crops, sugar cane, banana and plantains and tuber crops like potato and carrots. Estimated loss in crop yields due to nematode infections globally is set at $80 billion (Handoo 1998). The manifestations of plant nematode infections are mainly due to damage to the roots, which results in decreased nutrient and water uptake (resulting in wilting, stunting and poor yields) while also compromising plant anchorage (Nicol, Turner et al. 2011).

Considering the aforementioned factors, it is clear that helminth infections are detrimental to a country's economy if left untreated. This thesis deals with a major challenge towards the success of control strategies-the development of resistance in parasitic helminths towards commonly used chemotherapeutic agents.

1.2 Control of parasitic helminth infections

Although improved pasture practices for livestock and public health measures for humans exist, chemotherapy with anthelmintic drugs is the mainstay for the control of helminth infections. Three major classes of anthelmintics currently exist and are in use: benzimidazoles, imidazothiazoles and macrocyclic lactones. These drugs generally have a remarkable level of safety and tolerability in mammals. The discovery and mode of action of these drug classes is reviewed below.

1.2.1 Benzimidazoles

1.2.1.1 Spectrum of activity and benzimidazole derivatives

This was the first class of broad spectrum anthelmintics to be developed after the discovery that the benzimidazole nucleoside (Figure 1.1A) is a core component of many natural substances including the vitamin B12 structure (Townsend and Revankar 1970). In addition to activity against helminths, benzimidazoles are also used as antifungal and antiprotozoal agents. Benzimidazoles have activity against *Trichomonas vaginalis* and *Giardia intestinalis* but are ineffective against *Entamoeba histolytica, Leishmania* spp., and *Trypanosoma* spp. (Katiyar, Gordon et al. 1994).

The very first member of this class to be introduced was thiabendazole in the 1960s (Brown, Matzuk et al. 1961). Subsequently, other members with varying C_5 substituents in the basic ring structure were introduced into the market (e.g. mebendazole and cambendazole) followed by those with sulphide and sulphoxide substituents (e.g. albendazole and fenbendazole) which had higher efficacy (Figure 1.1 B, C, and D). Finally, in order to increase the solubility and thus the bio-availability of the drugs in host animals, the benzimidazoles were formulated as prodrugs in which the inactive form of the drug upon administration is metabolized by the host animals to the active form. Examples include benomyl (prodrug of carbendazim), netobimin (prodrug of albendazole) and febentel (prodrug of fenbendazole) (McKellar and Scott 1990). All these drugs are selectively toxic towards nematode parasites, while being non-toxic to mammalian cells due to differences in affinity of the β-tubulin drug target (Russell, Gill et al. 1992).

1.2.1.2 Modes of action of benzimidazoles

Benzimidazoles are anti-microtubule agents that disrupt polymerization of β-tubulin monomers during the formation of microtubules (Figure 1.2). Within the cells, microtubules undergo cycles of polymerization and de-polymerization and are found in a dynamic steady state (Dustin 1985; Downing 2000). It has been shown that benzimidazoles competitively bind to the colchicine binding site of the β-tubulin monomer (Friedman and Platzer 1978). The β-tubulin monomers together with α-tubulin monomers form α-β tubulin heterodimers which are the building blocks of microtubules. The binding of benzimidazoles to β-tubulins interferes with the addition of tubulin subunits to the elongating (+) end of the microtubule chain. This results in the disruption of the steady state of microtubule formation and reduction in microtubule levels (Figure 1.2).

Since microtubules are involved in many cellular processes like the formation of mitotic spindles during cell division, formation of the cytoskeleton, maintenance of cell shape, cell motility and intracellular vesicle transport, benzimidazoles can have a variety of effects on parasites. For example, it was shown that benzimidazoles inhibit glucose uptake in intestinal cells causing depletion of glycogen reserves leading to starvation, paralysis and eventually death of the parasite (Bennet and Bryant 1984). In addition to these effects, it has also been shown that benzimidazoles are capable of disrupting secretory vesicle transport in intestinal cells of the parasite (through the primary inhibition of microtubules), which results in the release of digestive enzymes contained within these vesicles into the cytosol leading to auto-digestion of these cells (Jasmer, Yao et al. 2000). This may be explained in terms of conformational changes induced in the C-terminal domain of β-tubulin (to which the kinesins bind) upon exposure to benzimidazoles (see also Section 6.5), which prevents the interaction of these motor proteins and thus disrupts transport processes within the cell (Song, Marx et al. 2001; Robinson, McFerran et al. 2004).

1.2.2 Imidazothiazoles (IMZs)

These drugs fall into the nicotinic agonists category of anthelmintics (Martin 1997) (Figure 1.3A). Examples of this class include levamisole, tetramisole (a racemic mixture of levamisole) and butamisole. They are agonists of nicotinic acetyl-choline receptors (nAChR) located on muscle cell membranes and act by interfering with post-synaptic nerve transmission, which causes spastic paralysis in nematode somatic muscles (Martin and Robertson 2007). Paralysis of muscle leads to the expulsion of parasite from the host due to the inability of parasite to retain its position within the host intestine (Sangster and Gill 1999). Electrophysiological studies show that these drugs bind to nicotinic receptor subunits (mimicking acetyl choline) causing membrane depolarization by altering the membrane permeability (non-selectively) towards cations (Robertson, Bjorn et al. 1999). Work in *C. elegans* confirms acetyl-choline receptors as the target of these drugs showing that mutants of genes coding for subunits of these receptors (e.g. *unc-29*, *unc-38 and lev-1*) and peripheral proteins are resistant to this class of drug (Figure 1.4) (Lewis, Wu et al. 1980; Fleming, Squire et al. 1997; Qian, Robertson et al. 2008).

1.2.3 Macrocyclic lactones

This class includes the avermectins (e.g. ivermectin, abamectin) and milbemycins (e.g. moxidectin) both of which have the same basic ring structure (Figure 1.3B) and mode of action (Shoop, Mrozik et al. 1995). The difference between avermectins and milbemycins is a disaccharide (on the 13^{th} C atom), which is absent in the milbemycins. The avermectins are produced by the soil-dwelling bacterium *Streptomyces avermetilis* and were first identified when a panel of microbial fermentation products was screened for anthelmintic activity in mice (Burg, Miller et al. 1979; Campbell W, Burg R et al. 1984). These drugs act by binding to glutamate-gated chloride channels and increasing the chloride permeability of nematode cell membranes of the pharynx and somatic muscles, which subsequently leads to starvation and paralysis causing death of the parasite (Shoop, Mrozik et al. 1995; Wolstenholme and Rogers 2005).

Genetic evidence that glutamate gated chloride channels are the target of these drugs comes from work in *C. elegans* where it was shown that simultaneous mutations in three genes *avr-14, avr-15 and glc-1* that encode α-subunits of these channels (Figure 1.5) confer ivermectin resistance (Dent, Smith et al. 2000). The selective toxicity of avermectins are attributed to the presence of susceptible channels in the nematodes, which are absent in the mammals (Martin 1997). P-glycoproteins have also been implicated as targets of these drugs owing to the change in expression patterns seen in resistant parasites (Blackhall, Liu et al. 1998; Wolstenholme and Rogers 2005).

1.2.4 Other anthelmintics

Apart from the three major classes discussed above, there are a smaller number of narrow spectrum anthelmintics that fall into several categories depending on their mode of action. They may be nicotinic receptor agonists (e.g. pyrantel, morantel, biphenium and methydrine), acetylcholinesterase inhibitors (e.g. haloxon), GABA receptor agonists (e.g. piperazine), calcium channel blockers (e.g. praziquantel), proton pump disruptors (e.g. closantel, niclosamide etc.), or inhibitors of various metabolic pathways or enzymes (e.g. diamphenethide inhibits malate metabolism, clorsulon inhibits phosphpglycerate kinase and mutase, diethyl carbamazine inhibits arachidonic acid metabolism) (Martin 1997).

1.2.5 More recent discoveries of novel anthelmintic compounds

The history of anthelmintic discovery has been rather disappointing in that the rate at which new anthelmintics reach the market has been slow given the heavy use of these drugs to treat helminth infections. This is because pharmaceutical companies have been rather reluctant to invest in this area of research due to the lack of profitability. However, three narrow spectrum anthelmintics have been introduced to the market in recent years: monepantel, derquantel and emodepside.

Monepantel is a synthetic aminoacetonitrile derivative (AAD 1566) (Kaminsky, Gauvry et al. 2008) that acts via nematode specific nicotinic acetyl choline receptors (specifically the DEG-3 subfamily), leading to continuous flux of ions followed by depolarization of muscle cells resulting in paralysis (Rufener, Mäser et al. 2009; Epe and Kaminsky 2013). Derquantel, a semi-synthetic derivative of paraherquamide (a fermentation product of *Penicillium simplicissimum*) belongs to the spiroindol class of anthelmintics and acts as an antagonist of B-type acetylcholine receptors causing flaccid paralysis (Epe and Kaminsky 2013). Emodepside is a semi synthetic derivative of a secondary metabolite of *Mycelia sterilia* (a fungus which is a microfloral component of *Camellia japonica* leaves). The drug acts by binding to nematode presynaptic lactrophilin receptors facilitating a series of signal transduction events, which leads to the release of a yet unidentified transmitter that induces flaccid paralysis in the pharynx and somatic muscles (Harder, Holden–Dye et al. 2005).

1.3 Anthelmintic Resistance

Anthelmintic resistance is a major concern in the livestock industry and a growing concern in human medicine. It results in significant production loss in livestock industries and challenges treatment and control success in humans (Sangster 1999; Geary 2012; Kaplan and Vidyashankar 2012).

1.3.1 Development of resistance in parasitic populations

It is believed that resistance is not an ‘all-or-nothing’ phenomenon and multiple molecular changes can occur prior to a drug becoming completely ineffective against a parasite (Hastings and Watkins 2006). Parasite populations tend to have an innate level of ‘tolerance’ (or unresponsiveness) to any drug even before they are first exposed to it (Fallon, Tao et al. 1996). Thus, the term resistance can be used to refer to any stage from where a parasite population begins to show a decrease in response to a drug compared to a completely naive population to when the population is completely unresponsive to the drug at the maximum dose tolerable by its host (Coles 2006). This heritable decrease in susceptibility is the result of two (not so) distinct processes: (1) an increase in the frequency of individuals with a pre-existing lack of susceptibility or (2) introduction of spontaneous genetic changes and their fixation in the population. Both these processes are facilitated by the selection pressure incurred upon a population through increased drug exposure. Thus, resistance can be an ‘innate’ or ‘acquired’ state (Fojo 2007; James, Hudson et al. 2009).

The increase in number of individuals in a parasite population carrying favorable molecular polymorphisms in their genome is a result of the beneficial polymorphisms (alleles) being passed on to successive generations under the influence of selection by drug use (Gilleard and Beech 2007). Certain inherent genetic features of the parasite populations facilitate the rapid evolution of resistance among individuals. High mutation rates and large effective populations sizes of parasitic helminths (especially trichostrogylids) lead to high levels of genetic variation (Anderson, Blouin et al. 1998), which provides a reservoir of mutations that can be selected upon by the use of anthelmintics (Gilleard and Beech 2007). This coupled with gene flow due to host movement facilitate the rapid spread of resistance in parasitic populations worldwide (Nadler 1987; Blouin, Yowell et al. 1995; Anderson, Blouin et al. 1998; Grillo, Jackson et al. 2006). A particularly concerning fact is that this resistance once developed is irreversible due to the selective advantage offered to the worms by the resistance alleles that they carry (Kaplan 2004).

1.3.2 Anthelmintic resistance in veterinary parasites

From Section 1.2 it is apparent that the 'armory of drugs available for combat' is very limited given the magnitude of the problem of parasitic helminth infections. Since these drugs at the point of introduction were highly effective and relatively cheap, their extensive and inappropriate use has led to the rapid development of resistance among parasitic populations of veterinary importance worldwide (Kaplan 2004). In the case of sheep parasites, on many farms in Australia, South Africa, Brazil and Malaysia multiple resistance to all major drug classes is already prevalent and threatens the sustainability of the sheep industry in these countries (Waller 1994; Jackson and Coop 2000; Kaminsky 2003; Kaplan 2004). It is now a widely acknowledged truth that wherever chemotherapy is used, subsequent development of drug resistance is an inevitable phenomenon (Sheps and Ling 2007) and the rate at which it develops is an inherent feature of the population in question (Gilleard and Beech 2007).

The first reported case of anthelmintic resistance in veterinary parasites was to the drug phenothiazine in sheep (Drudge, Leland et al. 1957). A similar trend followed for the subsequent anthelmintic classes introduced. Benzimidazoles (thiabendazole) were introduced in 1961 and the first case of resistance was reported just three years later (Conway 1964). Resistance to imidazothiazoles (Sangster, Whitlock et al. 1979) and avermectin-milbemycins (Van Wyk and Malan 1988) was reported in less than a decade after their introduction.

1.3.3 Anthelmintic resistance in human parasites

Taking lessons from veterinary parasites, it is obvious that anthelmintic resistance in at least some human parasites is inevitable (Geerts and Gryseels 2000; Vercruysse, Albonico et al. 2011). Unlike for veterinary parasites, there have not been many reported cases of field resistance in human parasites apart from instances of reduced efficacy. These reports include reduction in efficacy of mebendazole, pyrantel, ivermectin and diethylcarbamazin (DEC) against *N.americanus* in Mali *A.duodenale* in Australia, *Onchocerca volvulus* in Ghana and *Wuchereria bancrofti* in Haiti respectively (Eberhard, Lammie et al. 1991; De Clercq, Sacko et al. 1997; Reynoldson, Behnke et al. 1997; Osei-Atweneboana, Awadzi et al. 2011). However, the methodologies used in some of the above studies were not standardized and therefore the results are debatable and are not definitive evidence for resistance per say (Geerts and Gryseels 2000).

Mass drug administration programs and implications for the development of resistance

Control of helminth infections in humans (Oncocerciasis, lymphatic filariasis, soil-transmitted helminthiases, Schistosomiasis and Cysticercosis) is mainly dependent on yearly or biannual mass drug administration (MDA) programs (Table 1.1). These programs involve a limited number of anthelmintics that have been donated or made available at a low cost by pharmaceutical companies (Prichard, Basáñez et al. 2012). The scenario involved with these MDAs is very similar to the patterns of treatment strategies employed for veterinary helminths (in terms of the repeated dosing) and so the probability of occurrence of resistance is very high due to the strong selective forces offered by this practice (Geerts and Gryseels 2000; Smits 2009).

However, it appears that differences in therapeutic approaches in terms of frequency of treatment and subsequent environmental contamination, and therefore the probability of re-infection, are reduced in humans. Therefore, some consider that the rate at which resistance will develop may also be much less for human parasites (Horton 2003). This complacency is a huge concern since lack of attention can be detrimental upon the unexpected development of resistance and future failure of treatments. For instance, ivermectin has been used for the treatment of 'river blindness' for the past thirty years through the Onchocerciasis Control Program (OCP). However, recent studies have shown reduced treatment efficacy and repopulation of parasite indicating the emergence of resistant populations (Osei-Atweneboana, Eng et al. 2007; Osei-Atweneboana, Awadzi et al. 2011). Since ivermectin is the only drug available for the treatment of this disease under MDAs, these findings are quite concerning. Co-endemicity, polyparasitism, and reinfection, optimization of dose rates, monitoring the effectiveness of these control programs, optimizing the participation and compliance of communities, reduction in donations in the latter parts of the programme are additional challenges apart from the limited availability of effective drugs. All of these factors can potentially contribute to the development and spread of resistance following a period of MDA (Vercruysse, Albonico et al. 2011; Prichard, Basáñez et al. 2012).

1.3.4 Strategies to control the spread of resistance

A common strategy adopted to combat the development of resistance in veterinary parasites is to alternate between anthelmintics with unrelated modes of action or the use of a combination of drugs. Unfortunately, in the case of fungi such a strategy has only led to the accelerated development of resistance to drugs like dodine and benzimidazole (Köller and Wilcox 2001). Koller and Wilcox (2001) show that speed of selection for resistance is accelerated in a population already resistance to another class of fungicide with unrelated mode of action and for which pattern of resistance development is different (i.e. a population with pre-existing resistance adapts quicker compared to a completely susceptible population). If there is potential for this scenario to occur in veterinary parasites it is indeed a serious concern considering the limited number of anthelmintics available for treatment.

Considering that there have not been many new broad spectrum anthelmintics introduced to the market for the past 25 years (apart from anthelmintics like monepantel and derquantel licensed for treatment of infections in sheep and emodepside for cats) and the rapidity with which helminths develop resistance, it is vital to develop non-chemical control measures (James, Hudson et al. 2009) . Alongside this there is a serious need to understand the mechanisms of anthelmintic resistance in order to (1) develop molecular markers for early detection as tools to help control the spread of resistance and maintenance of the efficacy of currently used drugs and (2) develop new or synergistic drugs that can help to overcome the problem of widespread resistance (Gilleard 2006).

The current knowledge on resistance mechanisms for the common anthelmintics used for the treatment of parasitic helminths of livestock and humans is discussed in the following subsections.

1.3.5 Known mechanisms of resistance to anthelmintics

Drug resistance can be seen as evolution leading to 'survival of the fittest' (James, Hudson et al. 2009). This is a manifestation of the exposure of parasites to an adverse environment (drug) leading to changes in genes or their expression. Broadly, drug resistance can be caused by three major mechanisms: (1) changes in the amino acid sequence of or deletion of the gene encoding the drug target, (2) detoxification (involves metabolic enzymes) or (3) efflux (involves P-glycoproteins). Although mutations in the drug target are the more commonly encountered mechanism out of these, there is considerable evidence indicating the presence of alternative modes of resistance. For instance, single nucleotide polymorphisms (SNPs) causing resistance in one species may not necessarily be present in others and the frequency of causal SNPs varies among resistant parasitic populations (Schwenkenbecher, Albonico et al. 2007; von Samson-Himmelstjerna, Walsh et al. 2009). As indicated, and will become evident from the discussion to follow, detoxification and efflux may be the most common alternatives/supplementary mechanisms of resistance. While changes in the target lead to decreased affinity towards drug binding, the latter mechanisms prevent the drug from reaching its target at all. In fact, from reviewing the literature on anthelmintic resistance it becomes apparent that the genetic basis of the resistance phenotype to the main anthelmintics (benzimidazole, imidazothiazoles and macrocyclic lactones) can be complex and several different mechanism of resistance may co-exist in parasitic nematode populations.

1.3.5.1 Benzimidazole resistance

Alterations in the genomic sequence of genes encoding drug targets can lead to amino acid changes in the encoded protein which may then lead to resistance (Gilleard 2006). This has been the case with resistance to benzimidazoles. In fact, the only clear cut causal mutations known to date are those of benzimidazole resistance.

Preliminary evidence for the mechanism of benzimidazole resistance came from studies on mutant strains of the fungus *Aspergillus nidulans* where variation in binding affinity of benzimidazole for β-tubulin from resistant and susceptible strains was demonstrated (Davidse and Flach 1977). Similar differential binding was subsequently demonstrated for helminths (Lacey and Prichard 1986). This led to the suggestion that mutations in the amino acid sequence of β-tubulin which induce structural changes and decrease the affinity towards the drug may be the primary resistance mechanism to benzimidazole anthelmintics. This hypothesis was confirmed by the discovery of a phenylalanine to tyrosine change at amino acid position 200 (F200Y) in resistant populations of trichostrongylid parasites of sheep (Kwa, Veenstra et al. 1994; Elard and Humbert 1999). The functional significance of this mutation was demonstrated by the ability of a benzimidazole susceptible allele of isotype-1 β-tubulin gene from *H. contortus* to restore susceptibility in a mutant *C. elegans* strain (CB3474 [*ben-1(e1880)*]) resistant to benzimidazole (Kwa, Veenstra et al. 1995). Kwa et al (1995) also showed that the introduction of a resistant allele of the same gene (with a tyrosine at position 200 instead of phenylalanine) did not revert the *C. elegans* mutant strain back to susceptibility.

Other resistance mutations have been subsequently identified in *H. contortus.* They are a glutamic acid to alanine change at position 198 (E198A) (Rufener, Kaminsky et al. 2009), a phenylalanine to tyrosine change at amino acid 167 (F167Y) (Ghisi, Kaminsky et al. 2007) and complete deletion of the isotype-2 β-tubulin gene in populations with much higher levels of benzimidazole resistance (Kwa, Kooyman et al. 1993).

The F200Y and F167Y mutations had been associated with resistance in the human parasite *N.americanus* although there are no definitive reports of field resistance due to the absence of these polymorphisms in populations of the parasite that showed reduced sensitivity (Albonico, Engels et al. 2004; Schwenkenbecher, Albonico et al. 2007). Other parasitic helminths where the F200Y mutation is associated with benzimidazoles include *T.circumcincta* (Elard and Humbert 1999), *T.trichiura* (Diawara, Drake et al. 2009) and *W.bancrofti* (Hoti, Subramaniyan et al. 2003).

It should be noted here that F200Y, E198A (also Glu to Gln, Gly, Lys or Val) and F167Y mutations have also been identified in field strains of fungi resistant to benzimidazole (Koenraadt, Somerville et al. 1992; Yarden and Katan 1993; Gafur, Tanaka et al. 1998; Albertini, Gredt et al. 1999; Ma, Yoshimura et al. 2003). In addition mutations at codon 6 (His to Tyr), 50 (Tyr to Cys) and 240 (Leu to Phe) have also been reported in benzimidazole resistant field strains of fungi (McKay, Egan et al. 1998; Albertini, Gredt et al. 1999; Ma, Yoshimura et al. 2003).

On the other hand, mutations at amino acid positions 6 (His to Tyr or Leu), 50 (Tyr to Asn or Ser), 134 (Gln to Lys), 165 (Ala to Val), 198 (Glu to Asp or Gln or Lys), 200 (Phe to Tyr), 241 (Arg to His) and 257 (Met to Leu) have been correlated with benzimidazole resistance in laboratory induced mutant strains of different species of fungi (Orbach, Porro et al. 1986; Jung and Oakley 1990; Jung, Wilder et al. 1992; Koenraadt, Somerville et al. 1992; Li, Katiyar et al. 1996; Albertini, Gredt et al. 1999; Ma and Michailides 2005; Zou, Ying et al. 2006). Although many of these mutations are yet to be identified in benzimidazole resistant nematode species they suggest potential mutations that could confer resistance. On a different note, benzimidazole resistance was not correlated with the F200Y mutation in *Giardia duodenalis* suggesting that this change is not essential for resistance in all species (Upcroft, Mitchell et al. 1996).

Early studies in *C. elegans* suggested that a sub group of β-tubulin in benzimidazole selected strains was altered at the binding site while also demonstrating the presence of 13 protofilament microtubules in the intestinal epithelial cells of these strains but not in the susceptible strain suggesting this protein as the target of benzimidazoles (Enos and Coles 1990). There are six different β-tubulin genes in *C. elegans: tbb-1, tbb-2, mec-7, tbb-4, ben-1* and *tbb-6* (Gogonea, Gogonea et al. 1999). Of these, all mutations that confer benzimidazole resistance were mapped to the *ben-1* locus (Driscoll, Dean et al. 1989), which is one of the closest homologues of *H. contortus* isotype-1 and isotype-2 β-tubulin genes (Saunders, Wasmuth et al. 2013).

Driscoll et al (1989) used ethyl methanesulfonate (EMS) and γ-radiation on wild-type animals to create mutant *C. elegans* strains. These strains were then exposed to benomyl and resistant mutants were isolated and characterized. A total of 28 mutations with a dominant to semi-dominant characteristic (at 25 ^{0}C) were identified in this screen and as mentioned previously, mapped to chromosome III and the locus was designated as *ben-1*. Five of these mutations were partial to complete deletions of the *ben-1* gene and individuals homozygous for these mutations are viable indicating that this gene is not essential under laboratory conditions. This study provided evidence that benzimidazole resistance in *C. elegans* is due to mutations in the *ben-1* locus.

The continuous exposure to toxic substances (xenobiotics) in the environment has led to the evolution of detoxifying mechanisms in all organisms with parasitic helminths being no exception to this rule. Cytochrome P450s, Glutathione S transferases and membrane transport proteins are involved in the detoxifying mechanism of cells. Many studies have demonstrated the existence of detoxifying enzymes and metabolism of xenobiotics including anthelmintics in helminths (Precious and Barrett 1989; Cvilink, Lamka et al. 2009). An early study on *H. contortus* cambendazole resistant and susceptible strains revealed almost a two-fold increase in the expression of Glutathione S transferase enzyme in the resistant strain compared to the susceptible (Kawalek, Rew et al. 1984). A separate study demonstrated both *in vitro* and *in vivo* an increase in glucose metabolism in a *H. contortus* resistant strain upon exposure to thiabendazole (Rew, Smith et al. 1982) while a latter study demonstrated a decrease in thiabendazole tolerance in eggs derived from a resistant strain of *H. contortus* in the presence of glutathione S transferase inhibitors (Kerboeuf and Aycardi 1999). These three studies serve as further examples for the involvement of detoxifying enzymes in benzimidazole resistance.

P-glycoproteins have long been associated with multi-drug resistance in cancer chemotherapy (Zhou 2008). Subsequently, they were also implicated in ivermectin resistance in parasitic helminths. Recently, involvement of P-glycoprotein in benzimidazole resistance was suggested by a study that demonstrated decreased P-glycoprotein allele frequency in a cambendazole selected strain of *H. contortus*. The same allele selected for in the *in vitro* experiment was also detected in an independent filed isolate that had undergone thiabendazole selection (Blackhall, Prichard et al. 2008). Increased expression of P-glycoproteins has been observed in strains of the liver flukes *Fasciola hepatica* and *Fasciola gigantica* resistant to triclabendazole (Kumkate, Chunchob et al. 2008). Although SNPs in the β-tubulin gene are denoted as the primary cause of benzimidazole resistance a recent study proposes a role for a serine to arginine substitution at amino acid 1144 of P-glycoprotein in *F. hepatica* triclabendazole resistance (Wilkinson, Law et al. 2012).

1.3.5.2 Levamisole resistance

The most comprehensive knowledge on levamisole resistance comes from *C. elegans* whose levamisole resistance has been associated with the absence of functional acetyl choline receptors (Lewis, Wu et al. 1980) as a result of mutations in the genes *lev-1, unc-29 unc-38, unc-63* and *lev-8*, which code for their subunits (Figure 1.4) (Lewis, Elmer et al. 1987; Fleming, Squire et al. 1997). In addition, there are many other ancillary proteins that are required for the proper assembly and functioning of the receptor and mutations in any of these genes can lead to varying levels of levamisole resistance in *C. elegans.* These genes include *lev-9, lev-10, tpa-1, tax-6, soc-1, nra-1* and *oig-4* that are directly associated with the receptor subunits, *unc-68* involved in signal amplification, *unc-22* and *unc-11* involved in muscle contraction and *nra-2, nra-4, ric-3, unc-74* and *unc-50* involved in the transport, processing and assembly of the receptor subunits (Maryon, Coronado et al. 1996; Fleming, Squire et al. 1997; Gottschalk, Almedom et al. 2005; Martin and Robertson 2007; Almedom, Liewald et al. 2009).

Due to the observed absence of differential uptake or metabolism of drug between susceptible and resistance strains, in *H. contortus* and *T.colubriformis,* levamisole resistance in these organisms is associated with a reduction in number or drug affinity of nicotinic acetyl choline receptors (Sangster, Riley et al. 1988). A similar observation is noted with levamisole resistant *Oesophagus dendatum* where the absence of a electrophysiological signal peak from a levamisole sensitive receptor subtype (G35) pointed towards the possible reduction in the number of high affinity receptors (Robertson, Bjorn et al. 1999). Although homologues for many of the *C. elegans* genes mentioned above have been identified in parasitic nematode species (especially those that fall into Clade V same as *C. elegans*), there is not much evidence to support the functional significance of these genes apart from the observation that a truncated version of *unc-63* orthologue was consistently expressed in resistant strains of *H. contortus, T. colubriformis* and *T. circumcinta* (Neveu, Charvet et al. 2010; Martin, Robertson et al. 2012). Expression of *Ascari suum* homologues of *unc-29* and *unc-38* in *Xenopus* oocytes demonstrated that ancillary proteins were not required for the function. In addition, varying stochiometric combinations of the two subunits produced two different kinds of receptors with different levels of sensitivity to levamisole proposing the plasticity of resistance mediated by receptor structure in parasitic nematodes (Williamson, Robertson et al. 2009). However, expression of levamisole sensitive receptors in *H. contortus* required the presence of ancillary proteins and the ACR-8, the absence of which led to reduced sensitivity proposing a role for this protein in levamisole resistance (Boulin, Fauvin et al. 2011).

1.3.5.3 Ivermectin resistance

In *H. contortus*, *in vitro* selection experiments pointed to the possible involvement of glutamate and GABA gated chloride channels and P-glycoproteins in ivermectin resistance (Blackhall, Liu et al. 1998; Blackhall, Pouliot et al. 1998). Glutamate-gated chloride channels are made up of α and β subunits that bind to ivermectin and glutamate respectively. Analysis of the genetic variability of the α-subunit gene in *H. contortus* passaged in the presence and absence of ivermectin (and moxidectin) showed elevated frequency (almost four-fold increase in the resistant state) of a specific allele due to selection, implicating a role in resistance (Blackhall, Pouliot et al. 1998). However, the actual mechanism of resistance may not be due to mutations in this allele leading to changes in drug binding since a previous study had shown that ivermectin binds equally well to membrane preparations from both resistant and susceptible strains (Rohrer, Birzin et al. 1994). Thus, it was proposed that resistance may be due to either (1) the inability of the resistant α-subunits to undergo drug induced conformational changes or (2) to combine with β-subunits to form the channel or (3) the presence of mutations in the promoter region that cause reduced levels of α-subunit expression, resulting in a decrease in the number of sensitive channels (Blackhall, Pouliot et al. 1998). Although SNPs were not associated in *H. contortus,* a mutation from lysine to phenylalanine at amino acid 256 (L256F) of the glutamate-gated chloride channel α-subunit of *Cooperia onchophora* has been associated with reduced sensitivity to ivermectin (Njue, Hayashi et al. 2004).

In contrast to parasitic nematodes where an increase in specific alleles could confer resistance, simultaneous mutations in multiple genes (*avr-14, avr-15* and *glc*-1) that code for α-subunits of glutamate gated chloride channels (Figure 1.5) were required for high levels of resistance in *C. elegans* laboratory mutants (Dent, Smith et al. 2000). The researchers further demonstrated that mutations in just any of the two genes (*avr-14* and *avr-15*) leads to much lower levels of resistance while also suggesting that mutations in genes involved in modulation of the ivermectin signaling pathway (*unc-7, unc-9* and *Dyf*) may also lead to resistance. According to this study, mutations in all these genes act additively to provide high levels of resistance. Mutations in *Dyf* genes affect the permeability of the worm cuticle towards ivermectin while the *avr-14* and *glc-1* mutations affect the function of the extra-pharyngeal neurons that transfer the signal to the pharynx where *avr-15* is expressed. UNC-7 and UNC-9 form gap junctions through which the signal is transduced from the extra-pharyngeal neurons into the pharynx. While Dent et al (2000) have used *C. elegans* mutant strains derived in the laboratory, a recent study (more detailed discussion included in Section 1.5) using wild *C. elegans* strains demonstrated that a naturally occurring four amino acid deletion in the *glc-1* gene can lead to avermectin resistance in *C. elegans,* suggesting a difference in the mechanism of resistance in the natural habitat (Ghosh, Andersen et al. 2012).

P-glycoproteins have been associated with ivermectin resistance in both *H. contortus* and *O.volvulus* (Blackhall, Liu et al. 1998; Ardelli, Guerriero et al. 2006; Bourguinat, Ardelli et al. 2008). In the study of *H. contortus* laboratory passage of three strains with ivermectin selection and two without drug exposure was carried out. While the strains not exposed to drug showed high allelic variation at the P-glycoprotein locus investigated, all three strains that underwent selection showed increase in frequency of the same allele (Blackhall, Liu et al. 1998). In *O.volvulus*, analysis of the pattern of linkage disequilibrium of genetic markers associated with the P-glycoprotein locus showed increased linkage disequilibrium and low genetic diversity with continued ivermectin treatment (Ardelli, Guerriero et al. 2006). In addition, laboratory selection of *C. elegans* strains with increasing doses of ivermectin resulted in increased expression of multi-drug resistant proteins (selected strains also showed cross-resistance to levamisole) and P-glycoproteins implicating a role for these proteins in the ivermectin resistance mechanism in these organisms (James and Davey 2009).

1.4 The *C. elegans* model

Where their complex life cycle, lack of in vitro culture systems, genomic resources and genetic tools (albeit progressing) have hampered parasitic helminth research, the free living rhabditid *C. elegans* has proved an attractive model to bridge these gaps (Gilleard 2004). Thus, this powerful model organism has been the organism of choice for this thesis research in the hope that it will be able to provide new insights into mutations or mechanisms conferring benzimidazole resistance within the nematode phylum.

1.4.1 Why is *C. elegans* a good model organism?

C. elegans was first proposed as a model system for study of development and behaviour in 1965 by Sydney Brenner (Brenner 1974). Following Brenner, many scientists employed *C. elegans* as a model organism especially to study development and neurobiology (Riddle 1997). This nematode possesses many attributes that are invaluable for a model organism: A rapid and straightforward life cycle (3 days generation time), small size (adult worm is 1.5mm), ease of laboratory cultivation and storage coupled with ability to produce abundant cultures (300-350 progeny from a single animal by self-fertilisation), hermaphroditic life style (ability to self-fertilise and also outcross with males), and anatomical simplicity (made up of <1000 somatic cells) transparency and consistency in cell number from individual to individual (Wood 1987; Riddle 1997). In addition it was the first metazoan to have its genome fully sequenced and annotated and thus there is a plethora of genetic tools available (C.elegans sequencingConsortium 1998).

In the laboratory *C. elegans* is grown on NGM agar plates seeded with *E. coli*. The progress of its life cycle can be controlled by simply controlling the temperature between 12-26^{o}C. Liquid culturing is also possible for higher yields for protein assays etc., while they can also be grown in well established axenic media where bacterial contamination is less desirable. While starved and overcrowded cultures have the capacity to retain viability for months by entering an alternative developmental pathway called the dauer (which is convenient in case of accidental maintenance failures) worms can also be frozen and stored long-term at -80^{o}C or in liquid nitrogen (for decades).

The anatomy of the worm is simple enough in that an adult hermaphrodite consists of a mere 959 somatic cells (and a male 1031) while being completely transparent. This has allowed the complete cell lineage from egg to adult be described (Sulston, Schierenberg et al. 1983) and the function of each and every cell to be known. Despite the relatively simple anatomy, it possesses fully differentiated tissues comparable to higher organisms allowing its use as a model for these organisms. Furthermore, the complete neuronal network of this animal has been established through electron microscopic studies (White, Southgate et al. 1986). Owing to this the illustration of many behavioral aspects- locomotion, touch response, chemical and temperature sensation, pharyngeal pumping and defecation- and developmental aspects- programmed cell death, sex determination, dauer formation, embryogenesis, vulval development and male tail development etc was facilitated (Riddle 1997) and evolutionary studies have shown the conservation of these pathways across animal phyla (Bürglin, Lobos et al. 1998).

The haploid genome of *C. elegans* is around 100Mbp (about 1/30 the size of the human genome) and is distributed among five autosomes and a sex chromosome (Hodgkin, Plasterk et al. 1995; Blaxter 1998). *C. elegans* was the first metazoan to have its genome fully sequenced and annotated (C.elegans SequencingConsortium 1998). This has not only aided research in the worm itself but also the mapping and sequencing of genomes of other organisms. In addition, availability of the complete genomic sequence has afforded the possibility of elucidating the function of most genes in the animal by using RNA interference (RNAi) (Kamath and Ahringer 2003) as well as genome wide expression profiling (Hill, Hunter et al. 2000).

The ability of the worm to self-fertilize as well outcross with males (androdioecious life style) makes way for convenient genetics. Strains with desirable traits can be generated by out crossing and independent homozygous lines can be derived by picking a single F1 animal and allowing it to propagate by self-fertilization (Brenner 1974) .

There are many web based resources that are easily accessible to researchers. The most prevalent are sites like WormBase (www.wormbase.org) and Worm Atlas (www.wormatlas.org) which serves as a comprehensive repository of biological and genetic information. Comprehensive analysis of the *C. elegans* genome has been undertaken by the modENCODE project and a wealth of information on gene structure, transcription profiles, chromatin structure and regulation along chromosomes and evolutionary conservation has been produced (Gerstein, Lu et al. 2010). More recently, whole genome sequence data for 2007 mutagenised strains and 40 wild isolates has been made available through the "Million Mutations Project" (Thompson, Edgley et al. 2013). In addition, both mutant (created at different research labs) and wild *C. elegans* strains are maintained by and available for ordering at the *Caenorhabditis* Genetics Center (http://www.cbs.umn.edu/research/resources/cgc).

1.4.2 Limitations of experimental work in parasitic nematodes

Considering the desirable characteristics that *C. elegans* possesses it is not surprising that it has been extensively used as a model in many areas of biological and genetic research. In contrast to the *C. elegans* system, parasitic nematodes are challenging experimental systems mainly due to two reasons. Firstly, the lack of *in vitro* culture systems and secondly the limited number of genetic tools available for research. Understanding biological processes requires the ability to culture organisms in the laboratory. However, parasitic nematodes require a host to complete their life cycle. Therefore, research is either limited to the free living stages of the parasite (L1-L3) or when laboratory hosts are used the passaging process can be very expensive. This has limited the direct application of experimental procedures on parasitic nematodes. The comparatively larger genomes sizes and high genetic variability have been the main contributing factors for the slow progression of genome sequencing efforts in parasitic nematodes (Gilleard 2013). However, with the advent of next generation sequencing technology the cost of sequencing is continually declining. Therefore, despite the relatively large sizes of parasitic genomes, which range from anywhere between 50-300 Mb (Leroy, Duperray et al. 2003), efforts are continuously being made to make available whole genome sequences for parasites through projects like the "50 helminth genomes initiative" (http://www.sanger.ac.uk/research/initiatives/globalhealth/research/helminthgenomes/) and '959 nematode genomes' (http://www.nematodes.org/nematodegenomes/index.php/959_Nematode_Genomes).

Published parasitic nematode genomes include those of *H. contortus* (Laing, Kikuchi et al. 2013), *Brugia malayi* (Ghedin, Wang et al. 2007), *A. suum* (Jex, Liu et al. 2011) , *T. spiralis* (Mitreva, Jasmer et al. 2011) and *M. incognito* (Abad, Gouzy et al. 2008), although the ability to make use of this information directly for *in vitro* research is limited by the experimental challenges described above.

1.4.3 Application of *C. elegans* to parasitic nematode research

It is estimated that nematode species can range anywhere from between 40,000 to 10 million of which only 25,000 are described to date. Therefore, *C. elegans* has the potential to represent a diverse and ubiquitous group of animals (Blaxter 1998; Sommer and Streit 2011). However, it should be noted that *C. elegans* is believed to have gone through accelerated genomic evolution (Blaxter 1998; Bürglin, Lobos et al. 1998). Therefore, although nematode specific features may be mutual, careful considerations of phylogenetic relationship and degree of biological conservation between *C. elegans* and parasitic nematodes of interest must be made when extending genetic and biological knowledge acquired through research using the round worm (Geary and Thompson 2001; Gilleard 2004).

1.4.3.1 Phylogenetic relationships between parasitic nematode species and *C. elegans*

18S rRNA based classification of helminths (Figure 1.6) shows that *C. elegans* belongs to the same nematode clade (Clade V) as many of the parasitic nematodes that show rapid development of anthelmintic resistance (e.g.: *H. contortus*, *Ostertagia* spp.) and those that infect humans (e.g.: *A.duodenale* and *N.americanus)* (Dorris, De Ley et al. 1999). Thus, *C. elegans* is likely to be a suitable model system for conducting studies on possible mechanisms of drug resistance and development of novel anthelmintics at least for clade V nematodes. It is also predicted *C. elegans* homologues exist for >40% genes from *O.volvulus* and *B.malayi* (clade III nematodes) indicating potential for expansion into use as a model for clade III (Hashmi, Tawe et al. 2001) although in comparison to clade V the applicability of *C. elegans* as a model system to this and other clades is likely to be much less.

1.4.3.2 Conservation of biological and genetic features among nematode clades

There are several biological and genetic features conserved across the nematode phyla. For instance, while the cuticles of parasitic nematodes and *C. elegans* have significant differences, there are still biochemical and basic structural features that are extraordinarily similar (Politz and Philipp 1992). The dauer developmental pathway, which enables long range dispersal and long-term survival in *C. elegans* is analogous to the infective larval stage in parasitic nematodes (Félix and Braendle 2010). Many mechanisms involved in the dauer pathway are believed to be conserved in parasitic nematodes (Ogawa, Streit et al. 2009). The conservation of synteny, or gene order, among nematodes throughout evolution is seen with the some gene clusters (Aboobaker and Blaxter 2003). Such preservation will facilitate the identification of orthologous genes between *C. elegans* and parasitic nematodes (especially those belonging to clade V) owing to the extensive genomic rearrangement seen within the nematode phylum and prediction of gene function despite high degrees of sequence divergence (Guiliano, Hall et al. 2002; Gilleard 2004; Hillier, Miller et al. 2007). Trans-splicing is a feature seen in 70% *C. elegans* genes (Blumenthal 2005). Two splice leader sequences (SL1 and SL2) are involved in this mechanism and the SL1 sequence and the mechanism itself is highly conserved and widespread among parasitic nematodes such as *H. contortus, B. malayi* and *T. spiralis* (Blaxter and Liu 1996; Bürglin, Lobos et al. 1998), which simplifies many PCR based approaches (Sommer and Streit 2011).

Function, regulation and expression patterns of parasitic genes can be ascertained by identifying and studying its homologue in *C. elegans* with immuno-localization or RNA *in situ* hybridization (Hope, Albertson et al. 1996; Tabara, Motohashi et al. 1996). Also since nematode parasites are hard to grow *in vitro,* and genetic approaches are not well developed, their genes can also be microinjected into and often, heterologously expressed in *C. elegans* for functional analysis (Mello, Kramer et al. 1991; Bürglin, Lobos et al. 1998). Another approach to studying gene function is through the creation of knock out mutants. This approach has been successfully employed in *C. elegans* through the use of the CRISPR system (Friedland, Tzur et al. 2013; Frøkjær-Jensen 2013), transposon insertion into (Zwaal, Broeks et al. 1993) or chemical/UV mutagenesis of genes (Brenner 1974). This is particularly relevant for parasitology in the case of nematode specific genes (58% of *C. elegans* genome) such as those that are involved in fatty acid synthesis, formation of the cuticle or expression of surface proteins (Blaxter 1998) and anthelmintic resistance (Kwa, Veenstra et al. 1995).

Homologues have been identified in *C. elegans* for surface proteins like the six cysteine (SXC) domain found in the parasites *Toxocara canis*, *Ascaris* spp., and *Brugia* (Blaxter, Page et al. 1992) as well as for nematode antigen genes such as *cut-1* belonging to *Ascaris* spp., *O.volvulus* and filarial nematodes (Hope 1991; Politz and Philipp 1992; Lewis, Hunter et al. 1999). Additionally, *C. elegans* homologues were identified for many *B.malayi* genes (two evolutionarily distant nematodes) which indicates nematode specificity of these genes such as neurotransmitter receptors and those involved in infective larvae formation (Bürglin, Lobos et al. 1998). *C. elegans* homologues also exist for protein degrading enzymes like Cathepsin-B found in hookworms, filarial nematodes and flukes (Larminie and Johnstone 1996; Tort, Brindley et al. 1999). All aforementioned helminth proteins are seen as potential drug targets as they are important for the establishment of infection and survival within the host and interaction with environment. Therefore, the identification of homologues in *C. elegans* which can be extensively researched is rather fruitful for nematode parasitologists with regard to novel anthelmintic and vaccine development (Hashmi, Tawe et al. 2001).

1.4.3.3 Successful use of *C. elegans* in parasitology research

C. elegans has been successfully used as a heterologous expression system for parasite genes in many instances allowing comprehensive analysis. Kwa et al. (1995) showed that susceptibility can be restored by heterologous expression of the β-tubulin isotype-1 gene from *H. contortus* in *C. elegans* resistant to benzimidazole. Tissue specific expression patterns of parasite gut genes have been studied by using the relevant parasitic promoters in *C. elegans* and shown to have the same spatial (but not temporal) patterns (Britton, Redmond et al. 1999). Further it was shown that for instance *H. contortus cpr-1* gene is spliced and expressed properly under the *C. elegans* promoter (Redmond, Clucas et al. 2001) and that ectopic expression of the *H. contortus elt-2* gene in *C. elegans* is able to direct proper differentiation (Couthier, Smith et al. 2004). These examples demonstrate the functional conservation and interchangeability owing to conservation of regulatory aspects between these species (Hashmi, Tawe et al. 2001; Gilleard 2004). In a slightly different approach, over-expression of the *O. volvulus* glutathione-S-transferase gene in transgenic *C. elegans* helped to infer that protection from oxidative stress may be a potential function of this gene (Kampkötter, Volkmann et al. 2003), demonstrating the possibility of even testing functionally divergent genes, or those for which a *C. elegans* homologue does not exist (Gilleard 2004; Glendinning, Buckingham et al. 2011).

As discussed earlier, *C. elegans* has been instrumental in studies of drug mode of action, drug resistance mechanisms and discovery of novel drug targets. It is commonly used as a platform for drug screens and toxicity testing (Simpkin and Coles 1981; Artal-Sanz, de Jong et al. 2006). An example is the Macrofil Chemotherapy project hosted by the WHO for rational drug discovery. Through the project homologues for cyclophins and nuclear hormone receptors that regulate developmental and reproductive processes in parasites have been identified in *C.*

elegans and their suitability as drug targets investigated which will aid in the development of novel anthelmintics (Dornan, Page et al. 1999; Lizotte-Waniewski, Tawe et al. 2000). In keeping with the theme of this thesis, Sections 1.2 and 1.3.5 illustrate the invaluable contribution that research in *C. elegans* has made to the elucidation mode of action and of resistance mechanisms to the common anthelmintics benzimidazoles, ivermectin and levamisole.

Therefore, it is apparent that *C. elegans* is a great choice for my project to investigate novel polymorphisms that can confer benzimidazole resistance, and the results of this investigation are likely to provide valuable insights for Parasitology research. Furthermore, the availability of hundreds of wild isolates of *C. elegans* populations facilitates the approach that I propose to use in this thesis work. As discussed in the section that follows, this is because, these wild *C. elegans* populations can serve as a rich source of natural genetic variations that are likely to influence the sensitivity of these populations to benzimidazole drugs.

1.5 Natural genetic variation among wild *C. elegans* populations

Since the main theme of this thesis is to look for polymorphisms that confer benzimidazole resistance in populations of *C. elegans* ‘in the field’ here I will review the current status of knowledge regarding the genetic variation of natural populations of *C. elegans.*

1.5.1 General characteristics of *C. elegans* populations

C. elegans is a cosmopolitan nematode found mostly in temperate and rarely tropical regions. Although for many years it was considered as a ‘soil nematode’, it predominantly thrives in human-made ‘semi natural’ habitats such as compost heaps and more natural microbe rich habitats such as decaying plant material- fruit, stems and flowers (Félix and Braendle 2010). To date 200 wild strains of *C. elegans* have been isolated from 58 collection locations (distributed across 6 continents) from sources such as compost heaps, rotting fruit, soil, mushroom farms and snails (Table 1.2) (Andersen, Gerke et al. 2012).

From nature *C. elegans* is primarily isolated as hermaphrodites. Males arise very rarely (0.21%) even under laboratory conditions through spontaneous X chromosome non-disjunction (Barrière and Félix 2005). In nature, males contribute to a mere 1-2% rate of out crossing (Barrière and Félix 2005). A population structure that reflects high local genetic diversity and low global diversity (a meta population) is seen for *C. elegans* due to the primarily selfing mode of reproduction coupled with dauer migration and ‘boom-and-bust’ life style through the use of ephemeral resources (Hodgkin and Doniach 1997; Haber, Schüngel et al. 2005; Félix and Braendle 2010). The global genetic diversity in *C. elegans* is about 10^{-3} per base pair, which is 1/30 the rate seen for a dioecious species. This is due to a combination of factors such as low out crossing rates, population bottlenecks (due to depletion of resources), background selection (against deleterious alleles) and chromosome scale selective sweeps, all of which lead to low effective population sizes (the number of individuals in a populations contributing to its genetic diversity) (Charlesworth, Nordborg et al. 1997; Keller and Waller 2002; Barrière and Félix 2005; Félix and Braendle 2010; Andersen, Gerke et al. 2012). In fact, the effective population size is said to be 50% less in selfing species such as *C. elegans* compared to out crossers or gonochoristic species (Pollak 1987). The estimated effective population size for *C. elegans* in this regard is only 200-44,000 globally (Sivasundar and Hey 2003), despite having millions of individuals in a population (Cutter and Payseur 2003) which is orders of magnitude less than for out crossing species like *C. remanei* for which the number is around 1,000,000 (Cutter, Félix et al. 2006).

1.5.2 Geographical distribution and genetic diversity of *C. elegans*

Generally, widespread species with large population sizes are expected to have high genetic diversity within a population (i.e. large effective population sizes). However in *C. elegans* the selfing mode of reproduction does not allow this. This is because the apparent lack of recombination leads to removal of polymorphisms by background selection. The factors that reduce diversity in a selfing population such as background selection and selective sweeps are expected to increase the inter population diversity, another feature not encountered with *C. elegans*. This is possibly due to a lack of heterosis or hybrid vigour and high degree of gene flow between *C. elegans* populations due to migration events (Johnson and Hutchinson 1993; Sivasundar and Hey 2003). The gene flow hypothesis is further supported by genome wide single nucleotide polymorphism (SNP) studies which show that a Hawaiian strain (CB4856) which is apparently isolated from the rest of the global *C. elegans* populations harbors a large number of unique SNPs (Koch, van Luenen et al. 2000; Wicks, Yeh et al. 2001). Compared to the canonical N2 strain the Hawaiian strain has a SNP rate of 1/840bp (Wicks, Yeh et al. 2001).

Thus, it is clear that *C. elegans* is a paradoxical species in that although globally distributed, it exhibits relatively low intra and inter-population genetic diversity exhibiting very low population structure (Sivasundar and Hey 2003).

In the sections that follow I discuss three different types of approaches undertaken to date that employ wild *C. elegans* strains from different parts of the world to investigate the genetic diversity and phylogenetic relations among global populations of this species (Table 1.2). Overall, these studies demonstrate that despite being a globally distributed species no significant correlation is seen between genetic diversity and geographic distance among *C. elegans* populations.

1.5.2.1 Use of microsatellite markers

Microsatellites are DNA segments with di, tri or tetra nucleotide repeat sequences found throughout the genome of eukaryotic organisms. These regions are also known as variable number tandem repeats (VNTRs) and are found in non-coding regions of the genome. Populations that do not interbreed will have unique combinations of these microsatellites and therefore these regions can be used to genetically characterize populations and infer phylogenetic relationships between populations, if any. In this section I will focus on two studies that used microsatellite markers to characterize global *C. elegans* populations.

The first of these studies included 23 wild *C. elegans* isolates and used 20 genome-wide di-nucleotide microsatellite loci for their characterization (Sivasundar and Hey 2003). The researchers found the genetic variation between populations was low at these markers. Out of the 20 loci that were characterized only 10 were variable while the rest were monomorphic (the state of having just a single allele) across all 23 strains. Furthermore, although they observed some degree of population structuring, the geographic distance by which strains were separated did not seem to have an effect on the genetic distance between them. As mentioned earlier, these observations are paradoxical given the global distribution of *C. elegans.* Although the within population variation is expected to be reduced due to the primarily selfing mode of reproduction, this same phenomenon is expected to lead to genetically distant groups over geographically separated locations. On the other hand, such a pattern is not entirely surprising given probability of long-range dispersal and re-colonization of this species as discussed earlier (Félix and Braendle 2010). Therefore, Sivasundar and Hey (2003) predict that the lack of differentiation between *C. elegans* populations is due to reduction of polymorphisms resulting from increased background selection (due to lack of recombination given the selfing mode of reproduction) and continuous gene flow between populations (Sivasundar and Hey 2003).

The second study involved genotyping 10 genome-wide microsatellite loci containing >12 repeats of tri and tetra nucleotide sequences across the genomes of 58 natural *C. elegans* strains (Haber, Schüngel et al. 2005). Three main findings precipitated from this study: (a) Genetic differentiation was observed with geographic distance and period of isolation, (b) Migration events were implicated by the observation of co-existing diverse genotypes in a population from Northwest Germany and (c) evidence for occasional out crossing was detected although selfing is the main mode of reproduction. Unlike the study by Sivasundar and Hey (2003), where only 10 of the 20 microsatellite loci investigated were polymorphic; in this study all 10 loci investigated were polymorphic (between 2-11 alleles). In addition, the data from this study demonstrated significant correlations between genetic and geographic distance. The discrepancy between the two studies may be due to the nature of the microsatellite loci chosen or due to the number of strains contained within each geographic location included in the analysis. Sivasundar and Hey (2003) employed di nucleotide loci whereas Haber et al (2005) used loci with >12 repeats of tri or tetra nucleotide sequences which are expected to enable better distinction of genotypes (Schlötterer 2000; Haber, Schüngel et al. 2005). In addition, Sivasundar and Hey (2003) included geographic locations from where only one strain was isolated (e.g. Hawaii, Australia), whereas Haber et al. (2005) only include locations from where considerable strains are available (Europe, North America) in the geographic distance-genetic diversity correlation analysis. Haber et al (2005) believe that the use of only geographic locations from where multiple strains are available eliminates any bias introduced by the unbalanced groups (Australia and Hawaaii) included in the previous approach. Furthermore, results from Haber et al (2005) also demonstrated high local genetic diversity consolidating the migration hypothesis, where *C. elegans* dauer larvae spread by means of association with mammals or birds.

Overall, considering the outcomes of these studies, it appears that the inferred evolutionary relationship between *C. elegans* populations depends on the type of microsatellites that are used and the nature of the strains included. Since Haber et al (2005) have utilized a more unbiased approach, it seems that *C. elegans* populations may exhibit some degree of genetic differentiation based on geographic locations. More informed conclusions could be made with the inclusion of more strains from distant geographic locations.

1.5.2.2 Use of copy number variation (Maydan, Lorch et al. 2010)

Copy number variations (CNVs) are variations in the number of copies of segments of DNA, where these segments may include one or more genes. This variation is afforded by insertions (duplications or amplifications) and deletions of DNA segments relative to a reference strain and is capable of contributing to genetic variation in the eukaryotic genomes. Thus, a recent study used this feature to infer genetic diversity among 12 natural isolates of *C. elegans* (Table 1.2). Using array comparative genomic hybridization (aCGH) with exon-centric probes, the researchers were able to find: (1) deletions in 5% of the genes within the *C. elegans* genomes studied (with respect to the canonical N2 strain), (2) complex relationships between the isolates with different genealogies represented by the various parts of the genome due to recombination events and (3) deletions are more common than insertions and are over-represented in autosome arms.

The advantage of this study relative to the previously discussed microsatellite based studies is that the number or density of genome-wide markers used is higher. About 26% of the *C. elegans* genome is intronic and 47% intergenic (C.elegans Sequencing Consortium 1998) which leaves the coverage for this exon centric study at 27% and it covers 94% of the exons of the published *C. elegans* genome (of N2 Bristol). A total of 510 distinguishable types of deletions were identified among the genomes studied with a median indel (insertion-deletion) length of 2.7kb. The CB4856 and JU258 strains had the most number of indels (more genes deleted) and therefore were the most divergent in the set compared to the reference N2 strain, while CB3191 (also shown by Haber et al 2005) and RW700 were the least divergent (Table 1.2). One of the interesting findings of this study is that many of the indels are shared by the strains except that the CB4856 strain is the most divergent with 66% unique deletions. This is also in agreement with data from previous population genetic studies using SNPs (Koch, van Luenen et al. 2000).

Overall this study demonstrated considerable CNV in the genomes of *C. elegans* wild isolates and suggested that this genetic variation should contribute to phenotypic variation- especially in chemosensory behavior and innate immunity considering that the highest number of deletions were recorded for these gene families.

1.5.2.3 Genome-wide SNP analysis

In this section, I will discuss two different studies that employed genome wide SNPs in *C. elegans* to infer phylogenetic relationships between strains from different geographic locations. The first study involved genotyping 23 wild *C. elegans* strains based on genome-wide SNP distributions (Koch, van Luenen et al. 2000). For this particular study, substitutions, deletions or insertions of 1-3bp in length were considered SNPs. The researchers found a total of 366 SNPs relative to the N2 sequence in all the wild strains studied and most of these SNPs were shared among the strains. Thus, no clear correlation was seen between genetic and geographic distance from the data from this study except in the case of the CB4856 strain from Hawaii which is thought to have been reproductively isolated from other strains over a long period of time (Wicks, Yeh et al. 2001). However, they also point out that the inferred relationship between strains from different geographic locations could vary based on the genomic regions under investigation.

The second study investigated genetic variation among 200 wild *C. elegans* strains isolated to date using EcoR1 restriction site associated DNA (RAD) tagging which covered 8% (8Mb) of the whole genome (Andersen, Gerke et al. 2012). More than 40,000 SNPs were identified across all genomes, and the variation among strains was dominated by a set of haplotypes in the Mbp range on four (I, IV, V and X) of the six chromosomes. Out of the 200 strains 47 had unique isotypes (this term is used to define a unique genome-wide haplotype) with the rest falling into 50 sets of almost identical strains. As in the previous microsatellite and SNP based studies (Koch, van Luenen et al. 2000; Sivasundar and Hey 2003), apart from the identification of CB4856 from Hawaii to be a highly divergent haplotype, weak to no apparent geographic clustering was observed. The results of this study were also in agreement with SNP and CNV based studies which showed that genetic diversity varies along the length of the chromosome (Koch, van Luenen et al. 2000; Maydan, Lorch et al. 2010) with the mutation rates being in the order of 10^{-3} in the arms and 10^{-5} in the centers (with an average of 10^{-4}), with the exception of the X chromosome where variation was almost uniform across the length. This pattern of variation is consistent with the notion that background selection is a major contributing factor in shaping the genome of this species (Félix and Braendle 2010). The study concluded that chromosome scale selective sweeps at loci that incur fitness advantages especially during long range dispersal and survival in human associated habitats with ephemeral resources driven by positive selection largely attributed to human activity have reduced the variation on a global scale in *C. elegans* populations.

1.5.3 Natural and laboratory induced genetic variation and phenotypic variation in *C. elegans*

Early studies on gene function, drug mode of action and drug resistance employed laboratory induced artificial mutant *C. elegans* strains (Brenner 1974; Driscoll, Dean et al. 1989; Dent, Smith et al. 2000). This involved the generation of mutant strains through chemical or UV mutagenesis (of a reference strain) after which mutants with desirable phenotypes were selected and the causal genetic loci mapped. While these studies have been instrumental in elucidating many biological phenomena, the mutations causing different phenotypes identified by these studies are not necessarily representative of field situations and both direct and 'off-target effects' (e.g. unnecessary genetic interactions) can take place which can have negative biological or fitness consequences in a natural habitat (Sangster and Gill 1999).

This theory applies to parasitic helminths too- in that the genetic features resulting from *in vitro* selection are different from those resulting from selection *in vivo* or in field situations.

One of the reasons for this is the differences in the dose regimens (gradual or suboptimal vs. full therapeutic dose) used in these situations which may allow for selection to take place on different subsets of genes (Gilleard and Beech 2007).

C. elegans can go through about 100 generations per year under laboratory conditions, but in the natural habitat it is estimated to be only about 2-6 generations per year (Barrière and Félix 2007; Cutter 2008). This difference in time scale can have a considerable influence on the mutation load experienced by the genome in that laboratory cultured strains are likely to harbour far more mutations than those propagating in a natural habitat. This in turn will have an impact on the response of these strains towards various stimuli and their biological characteristics.

In the laboratory *C. elegans* is maintained on NGM agar plates seeded with the uracil auxotroph *E. coli* OP50 and is maintained at stable temperatures. This is far from what the animal encounters in the field where it lives in a changing environment associated with a plethora of microbial and fungal food resources, other nematodes competing for these food resources, pathogens (including viruses), predators (fungi, mites and other nematodes) and invertebrates or carrier organisms (Félix and Braendle 2010). Hence, the habitat of wild *C. elegans* is ever-changing in terms of biota and physical properties. Thus, an animal with chemically induced mutations that survives under ideal conditions in a laboratory may not necessarily do so in the wild, as such mutations may not have had the opportunity to undergo purifying or balancing selection and thus may incur severe fitness costs (Cutter, Dey et al. 2009).

Many of the mutagenesis experiments to date have been carried out solely in the N2 background (strain isolated in the 1950s from a mushroom farm compost heap in Bristol, UK) thus limiting the genetic diversity that could have an influence on the processes studied. This strain is also known to have accumulated mutations during its many generations of propagation in the laboratory before frozen stocks were made (Félix and Braendle 2010). Furthermore, microsatellite based studies have shown differences in 2 loci of the N2 strain used to that of the published genome possibly indicating laboratory accumulated mutations (Haber, Schüngel et al. 2005). Further evidence suggests that evolution of microsatellites in the genome varies under laboratory and natural conditions where certain factors may bias the distribution of alleles (Degtyareva, Greenwell et al. 2002; Sivasundar and Hey 2003; Haber, Schüngel et al. 2005). Therefore, in order to get a better understanding of biological processes, it is more appropriate to use natural genetic variation to study the underlying changes in phenotypes rather than artificially introduced mutations (Kohler 2001).

Insights from The Million Mutations Project

Further support for the value of using natural genetic variation to study phenotypic variation comes from the findings of the Million Mutations project (Thompson, Edgley et al. 2013). All the data from this project is available from a dedicated website at: http://genome.sfu.ca/mmp/. The whole genomes of 40 wild isolates were sequenced alongside those of 2007 EMS or ENU (N-ethyl-N-nitrosourea) mutagenised strains and the distribution of variation in these two data sets is quite illuminating (Table 1.3). From the table, it is apparent that the genetic variation and distribution seen in the wild type isolate set is markedly different from that of the mutagenised set.

Firstly the total number of single nucleotide variations (SNVs) in the mutagenised set was 840, 429 (98% unique SNVs), uniformly distributed across the genome (1/120bp). This result differs dramatically for the wild strains where although 3,780,728 SNVs were detected, only 630,541 (17%) were unique and the distribution was more frequent in the chromosome arms compared to central regions with abundant conserved genes (Thompson, Edgley et al.

2013). This observation is supported by a previous study that looked at the evolution of the nuclear genome in mutation accumulation lines where it was shown that the distribution of mutations across the length of the chromosome is uniform, whereas in wild *C. elegans* strains SNPs are concentrated in the chromosome arms and there is high linkage disequilibrium in the centers (Denver, Dolan et al. 2009). This differential distribution across wild and mutagenised strains is attributed to forces such as genetic hitchhiking, background selection, selective sweeps, and higher recombination rates in the chromosomal arms (Rockman and Kruglyak 2009; Andersen, Gerke et al. 2012).

A similar pattern was seen for the indels detected in the sequenced *C. elegans* genomes relative to the reference strain (N2). The mutagenised strains had about 86% unique indels once again distributed uniformly along the length of the chromosomes while in the wild strains set only 18% of the indels were unique (although the number of indels themselves were higher than in the mutant set) and as with the SNVs were more frequent in the arms where rapidly evolving genes are found (Thompson, Edgley et al. 2013).

Whereas 20% of the mutations that fall within protein coding regions alter the amino acid in the polypeptides of mutant strains, this occurs only for 10% in the wild isolates. Furthermore, in the wild isolates nonsense and missense mutations are reduced by 90% when compared to expected numbers whereas in the mutant set there is only a 45% reduction indicating that such mutations in several genes although compatible for life in the laboratory environment, are not so in the wild (Thompson, Edgley et al. 2013). In fact, a reduction in variability was seen even in non-essential genes in the wild isolates set suggesting selection pressures acting on their genomes which is not evident with mutant strains (Cutter, Dey et al. 2009). The reduction in mutations in the wild strains compared to the mutant set suggests that selection occurs against many of the mutations that arise within the genome of *C. elegans.* This indicates the drawback of using mutant strains in studies since the identified causal mutations possibly would have very little probability of occurring in nature.

To this end *C. elegans* is an attractive option owing to the fact that wild *C. elegans* isolates display a high degree of natural genetic variation (Maydan, Lorch et al. 2010; Andersen, Gerke et al. 2012). Although the out crossing rate is relatively low in *C. elegans* compared to some of its sister species, it is still offers a rich source of naturally occurring sequence variations to study phenotypic variations (Cutter, Félix et al. 2006; Thompson, Edgley et al. 2013).

1.5.3.1 Natural genetic variation and anthelmintic resistance

Despite the apparent lower-than-expected levels of genetic diversity on a global scale (Koch, van Luenen et al. 2000; Sivasundar and Hey 2003; Haber, Schüngel et al. 2005), many studies illustrate that the natural genetic variation in wild *C. elegans* populations can precipitate heritable phenotypic variation in traits such as social feeding behavior (de Bono and Bargmann 1998), copulatory plug formation (Hodgkin and Doniach 1997), out crossing rate (Teotónio, Manoel et al. 2006), response to pathogens (Schulenburg and Ewbank 2004; Schulenburg and Muller 2004; Chang, Paek et al. 2011), chemosensory behavior or stress response (Jovelin, Ajie et al. 2003; Davies, Bettinger et al. 2004; Harvey and Viney 2007), longevity (Gems and Riddle 2000; Gems and Riddle 2000; McCulloch and Gems 2003; Caswell-Chen, Chen et al. 2005) and sensitivity towards cues (temperature, pheromones and availability of food) for entering the dauer formation pathway (Félix and Braendle 2010) etc.

The natural genetic variation in *C. elegans* is also likely to influence responses to drug exposure and in fact, a recent study very elegantly demonstrated just this and is worthy of some detailed discussion (Ghosh, Andersen et al. 2012). The researchers identified that CB4856, a wild *C. elegans* strain from Hawaii is resistant to avermectin when compared to the reference N2 strain. Quantitative trait loci (QTL) mapping in 210 recombinant inbred advance intercross lines (RIAILs) generated from N2 and CB4856 strains allowed the identification of 2 loci that had significant association with the resistance trait. One locus on chromosome V accounted for 26% of the variation while the other on chromosome III accounted for 6%. Further genotyping with additional markers and transgenic rescue of CB4856 with N2 DNA fragments alongside genetic complementation with mutant alleles for the gene narrowed down the causal locus to the *glc-1* which codes for an α-subunit in glutamate gated chloride channels. This subunit has been associated with ivermectin resistance in the parasite *H. contortus* as well (Blackhall, Pouliot et al. 1998). Sequencing of the *glc-1* locus from N2 and CB4856 showed the presence of 32 non-synonymous substitutions and a four amino acid deletion in the CB4856 locus. Based on results from transgenic experiments using N2 cDNA with a subset of the polymorphisms, it was confirmed that the 4 amino acid deletion alone was sufficient for resistance.

Sequencing of the *glc-1* locus in 53 wild isolates identified 16 that carried the CB4856 allele (with the deletion). Some of these isolates were sensitive, while others that carried N2-like alleles were resistant indicating the possible existence of other resistance conferring loci in these isolates which is also supported by the fact that when 6 of the wild isolates carrying CB4856-like alleles (and were resistant) were crossed to N2 the F1s from two of the crosses resulted in F1s that werre resistant. Taken together this study concluded that while the naturally occurring 4 amino acid deletion in *glc-1* is significantly correlated with resistance other causal loci may also be involved demonstrating the complex multigenic nature of avermectin resistance. This observation is also supported by backcross experiments in *H. contortus* isolates resistant to ivermectin which suggested that multiple loci may be involved in the trait (Redman, Sargison et al. 2012). Hence, it is evident that using natural genetic variation is an important approach in studying phenotypic variation since such studies reflect the complex nature of genotype-phenotype relations and demonstrate the multi-factorial nature of many phenotypes (Lehner 2007).

1.5.4 Strains used in this study

Following from the discussion above, it is clear that using natural genetic variation for the study of phenotypic variation is a more suitable approach. Wild *C. elegans* strains, although they share a common ancestry have substantially diverged and exhibit a high degree of polymorphisms in their genomes (Koch, van Luenen et al. 2000). Maydan *et. al* (2010) using an array comparative genomic hybridization study (aCGH) showed that a high degree of copy number variation (CNV) exists between 12 wild strains of *C. elegans.* These strains have been isolated from different parts of the globe and have many indels in their genomes. Eleven of these strains (AB1, CB3191, CB4853, CB4854, CB4856, CB4858, JU258, JU263, JU322, KR314 and MY2) were chosen for my study assuming that the genetic diversity among these strains and the many gene deletions in their genome (Table 1.2) may lead to differences in response to benzimidazole treatment. In addition to this, 5 strains (GXW1, JU088, JU1171, MY14 and MY16) that contain non-synonymous polymorphisms in the β-tubulin gene *ben-1* (Thompson, Edgley et al. 2013) were also chosen for this study to assess if these polymorphisms can give rise to phenotypic resistance to benzimidazoles. Collectively a phylogenetic study based on RAD associated markers showed that these strains exhibit high degrees of haplotype diversity (Andersen, Gerke et al. 2012). As such these strains are considered a fairly representative sample of the global *C. elegans* population (Figure 1.7).

1.6 Chapter 1: Figures and Tables

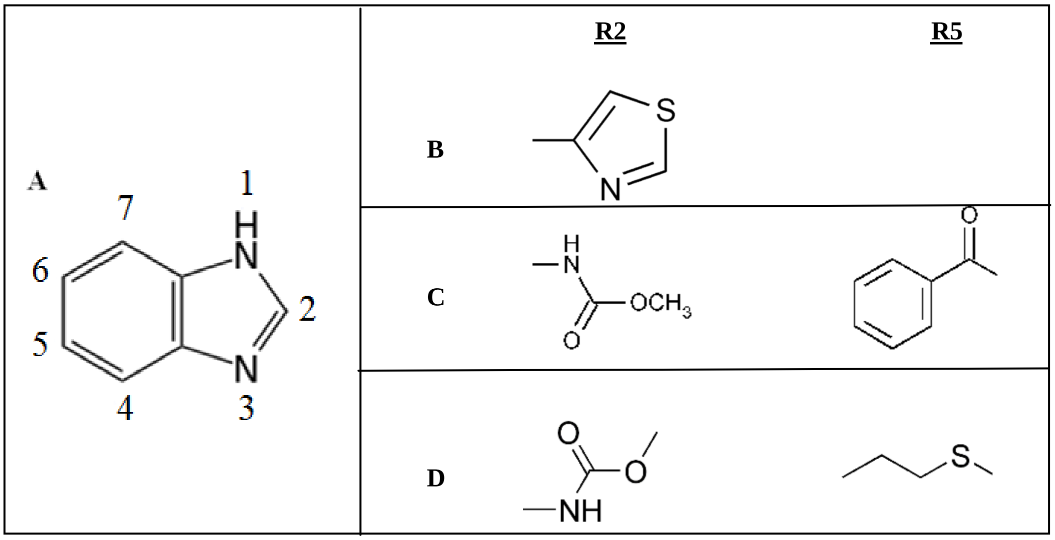


Figure 1.1 Benzimidazole anthelmintics. Various substitutions on the core benzimidazole ring (A) produce thiabendazole (B), mebendazole (C) and albendazole (D).

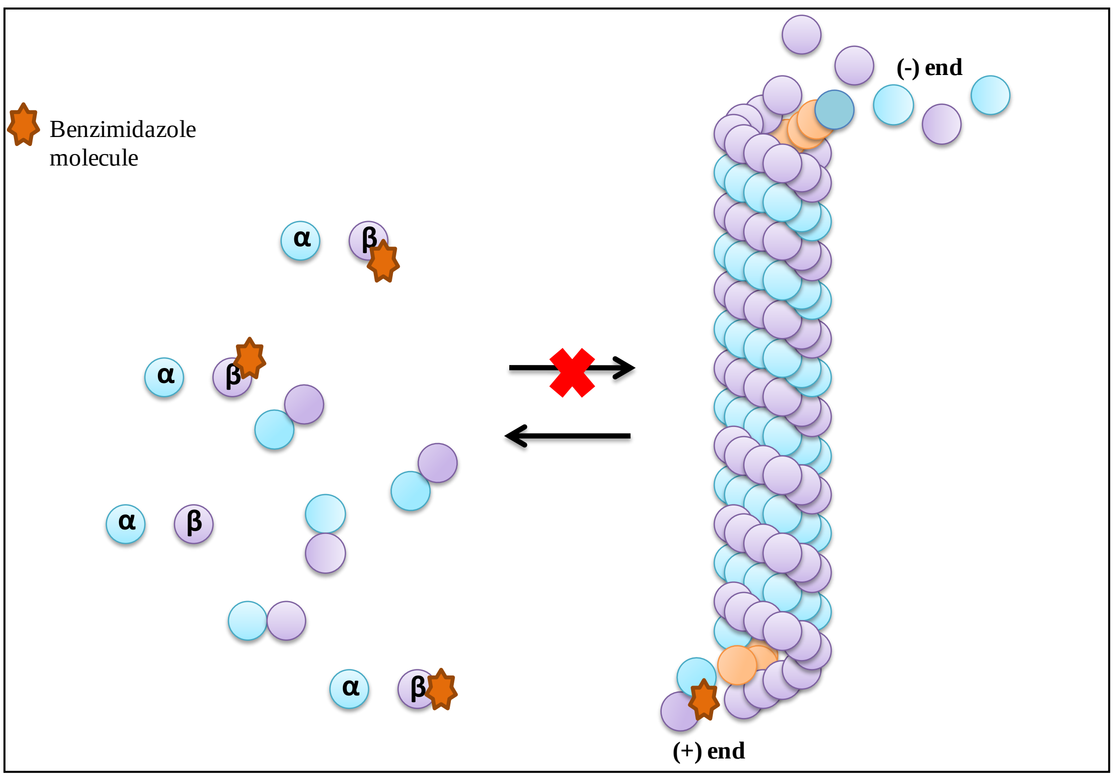


Figure 1.2 Disruption of microtubule dynamics by benzimidazoles. Microtubules are found in a dynamic steady state with αβ heterodimers being added to the (+) end and removed from the (–) end. Benzimidazoles bind to the β-tubulin monomers and disrupt the equilibrium leading to depolymerisation of the microtubule.

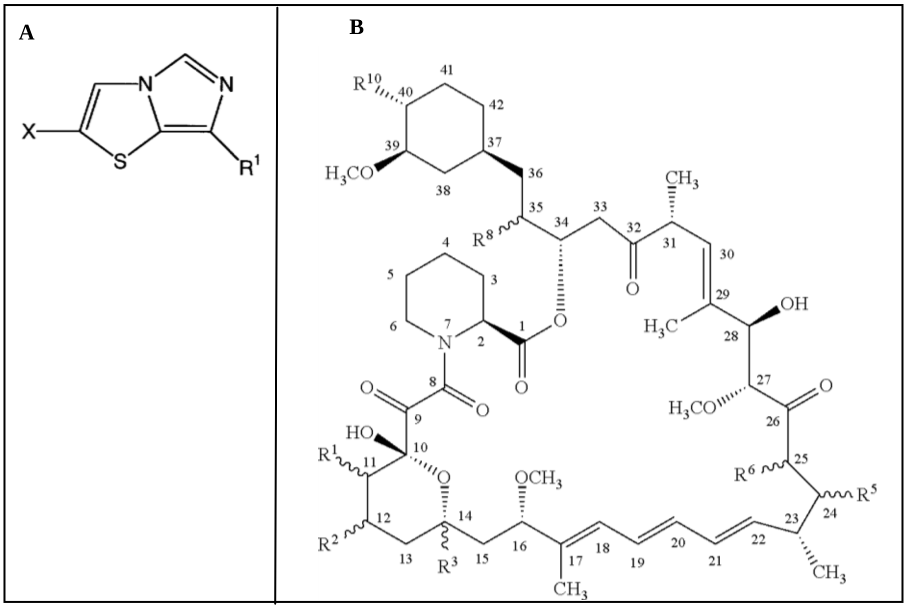


Figure 1.3 Basic ring structures of Imidazothiazoles (A) and Macrocyclic lactones (B).

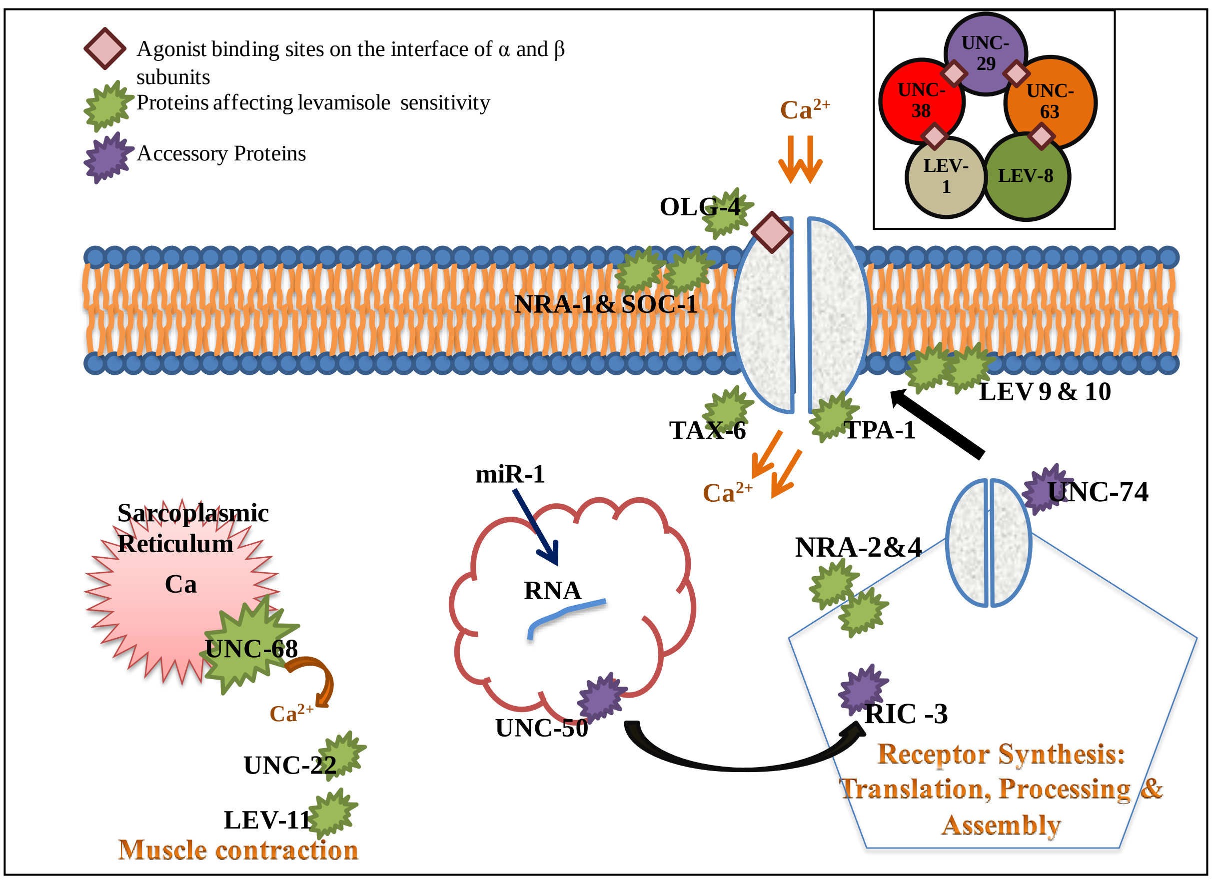


Figure 1.4 Receptor and proteins associated with levamisole response in *C. elegans*. Inset shows cross section of the levamisole receptor which consists of five subunits (UNC-38, UNC-63 and LEV-8 are α subunits and UNC-29 and LEV-1 are non-α (β) subunits). Levamisole binding sites are located at the interface of α-β subunits. Figure adapted from Martin et al (2012).

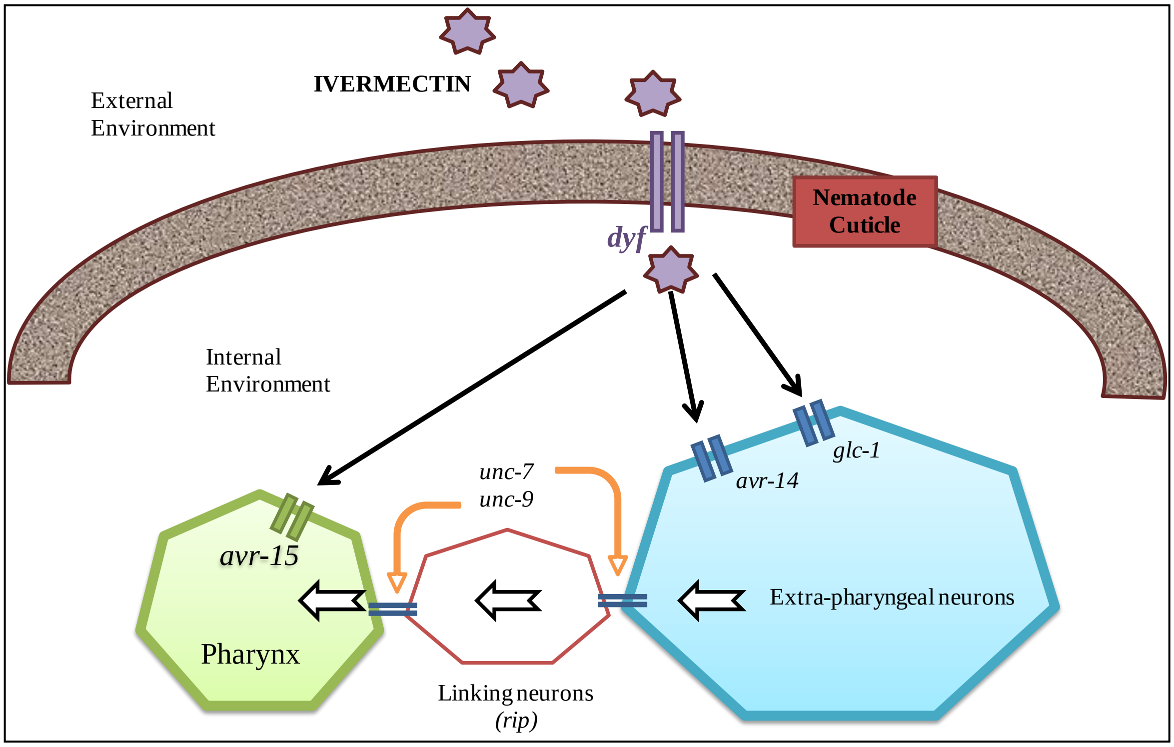


Figure 1.5 Interaction of ivermectin with *C. elegans* receptors. Genes *avr-15*, *avr-14* and *glc-1* code of α-subunits of glutamate-gated chloride channels. *Dyf* genes regulate the permeability of the *C. elegans* cuticle while *unc-7* and *unc-9* code for gap junctions through which signal transduction occurs from extra-pharyngeal neurons to the pharynx. Figure adapted from Dent et al. (2000).

Table 1.1 Mass Drug Administration (MDA) Programs-completed and ongoing

Program	Strategy	Timescale
Oncocerciasis Control Program (OCP)	Annual IVM administration	1974-2002
National OCP-W.Africa	Community directed IVM administration	2002 onwards
Oncocerciasis Elimination Program for the Americas (OEPA)	Biannual IVM administration	1992-2012
African Program for Oncocerciasis control (APOC)	Community directed IVM administration	1995-2015
Global Program to Eliminate Lymphatic Filariasis (GPELF)	Annual administration of IVM+ABZ, DEC+ABZ or DEC	1999-2020
Partners for Parasite Control (PPC), Deworm the world (DtW), Schistosomiasis Control Initiative (SCI)	ABZ or MBZ administration 1-3 times/year	2001 onwards
Joint Research Management Comminttee (JRMC), SCI	Annual administration of PZQ to high risk groups	1992-1999, 2001 onwards

IVM=Ivermectin; ABZ=Albendazole; MBZ= Mebendazole; DEC= Diethylcarbamazin; PZQ=Praziquantel (Prichard et al. 2012).

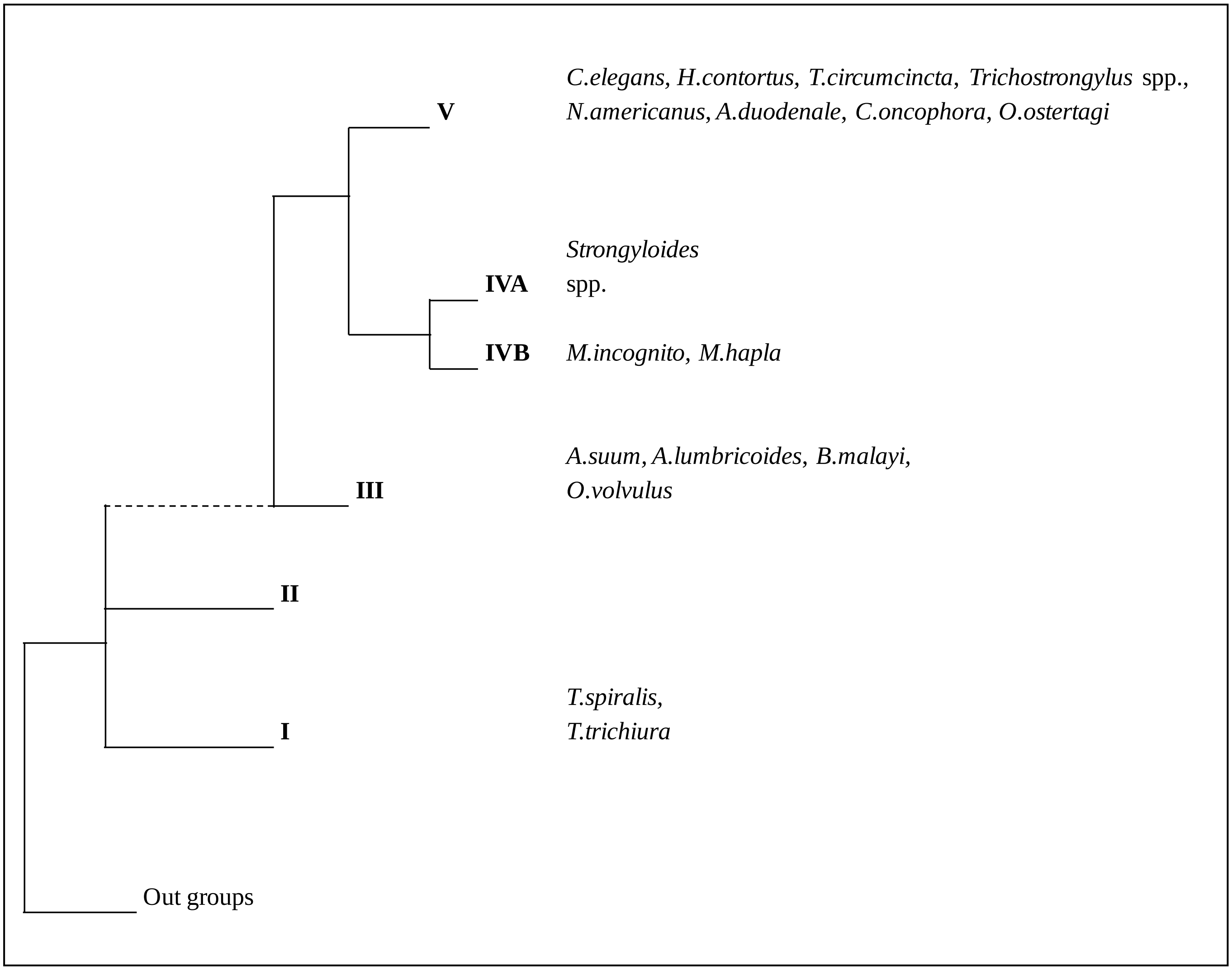


Figure 1.6 Nematode clades. Phylogenetic relationship between nematodes based on their 18S rRNA sequences. Dotted line leading to Clade III indicates several splits after the common ancestor of Clades II and III. Figure adapted from Blaxter et al. (1998).

Table 1.2 Genetic diversity among strains used in this project

Strain	CNV * (genes deleted)	Microsatellite Genotype	Inferred Isotype	Location of Isolation	Source	Isolation date	Received at CGC
AB1	147	AUS1	AB1	Adelaide, Australia	-	1983	1984
CB3191	1	N2	-	Altadena, California, USA	Garden	1973	1997
CB4853	237	NA2	CB4853	Altadena, California, USA	Vegetable Garden (organic garden)	1974	1991
CB4854	122	NA3	CB4854	Altadena, California, USA	Vegetable Garden	1974	1991
CB4856	517	PAC	CB4856	Oahu, Hawaii, USA	Hawaiian island pineapple field	1972	1991
CB4858	211	NA3	CB4858	Pasadena, California, USA	Caltech flowerbed	1971/73	1991
ED3021	-	-	ED3005	Edinburgh, United Kingdom	Compost bin in public vegetable garden	2005	2008
GXW1	-	-	-	Wuhan City, Hubei Province, China	Soil under kiwi fruit tree in botanical garden	2010	2013
JU258	671	ATL	JU258	Ribeiro Frio, Madeira, Portugal	Path through an orchard	2001	2002
JU263	145	EU12	JU310	Le Blanc, France	Vegetable garbage pile in a vegetable garden	2001	2002
JU322	174	-	JU323	Merlet, France	Helix snail on the trunk of a mulberry tree	2002	2005
JU360	-	-	JU360	Franconville, France	Compost heap in garden	2002	2007
JU1088	-	-	JU1088	Kakegawa, Japan	Soil	2007	2007
JU1171	-	-	MY23	Concepcion, Chile	Compost sample	2007	2007
KR314	417	NA8	KR314	Vancouver, British Columbia, Canada	-	?	1987
MY2	301	EU2	MY23	Roxel, Munster, Germany	Compost heap	2002	2004
MY6	-	EU4	MY18	Roxel, Munster, Germany	Compost heap	2002	2004
MY14	-	EU2	MY23	Mecklenbeck, Münster, NW Germany	-	2002	2004
MY16	-	EU6	MY16	Mecklenbeck, Münster, NW Germany	-	2002	2004
Reference	Maydan et al (2010)	Haber et al (2005)	Andersen et al (2012)	Andersen et al (2012)	Andersen et al (2012)	Andersen et al (2012)	

Strains have been characterised based on tri-nucleotide microsatellite loci and RAD markers (Haber et al. 2005; Andersen et al. 2012).
*CNV=Copy number variation: number of genes deleted compared to the canonical N2 genome (Maydan et al 2010).
Hyphens are used where strains were not included in a study or if particular information is not available.

Table 1.3 Genetic variations in artificially mutagenised and wild *C. elegans* strains

Characteristic	Mutants	Wild strains
N	2007	40
Unique SNVs	800000 (98%)	630000 (17%)
Distribution of SNVs	Uniform:1/120bp	Biased towards arms
Percentage of non-synonymous substitutions	21%	10%
Indels + CNVs	16000	220000
Distribution of Indels + CNVs	arms	>arms
Nonsense alleles		90% reduction*

N is the number of strains in each set whose whole genomes were sequenced; SNV=Single Nucleotide Variation; Indel=Insertions and deletions; CNV=Copy Number Variation (generally a deletion or insertion which is >1kbp)
*90% reduction in nonsense alleles compared to the mutagenised set.

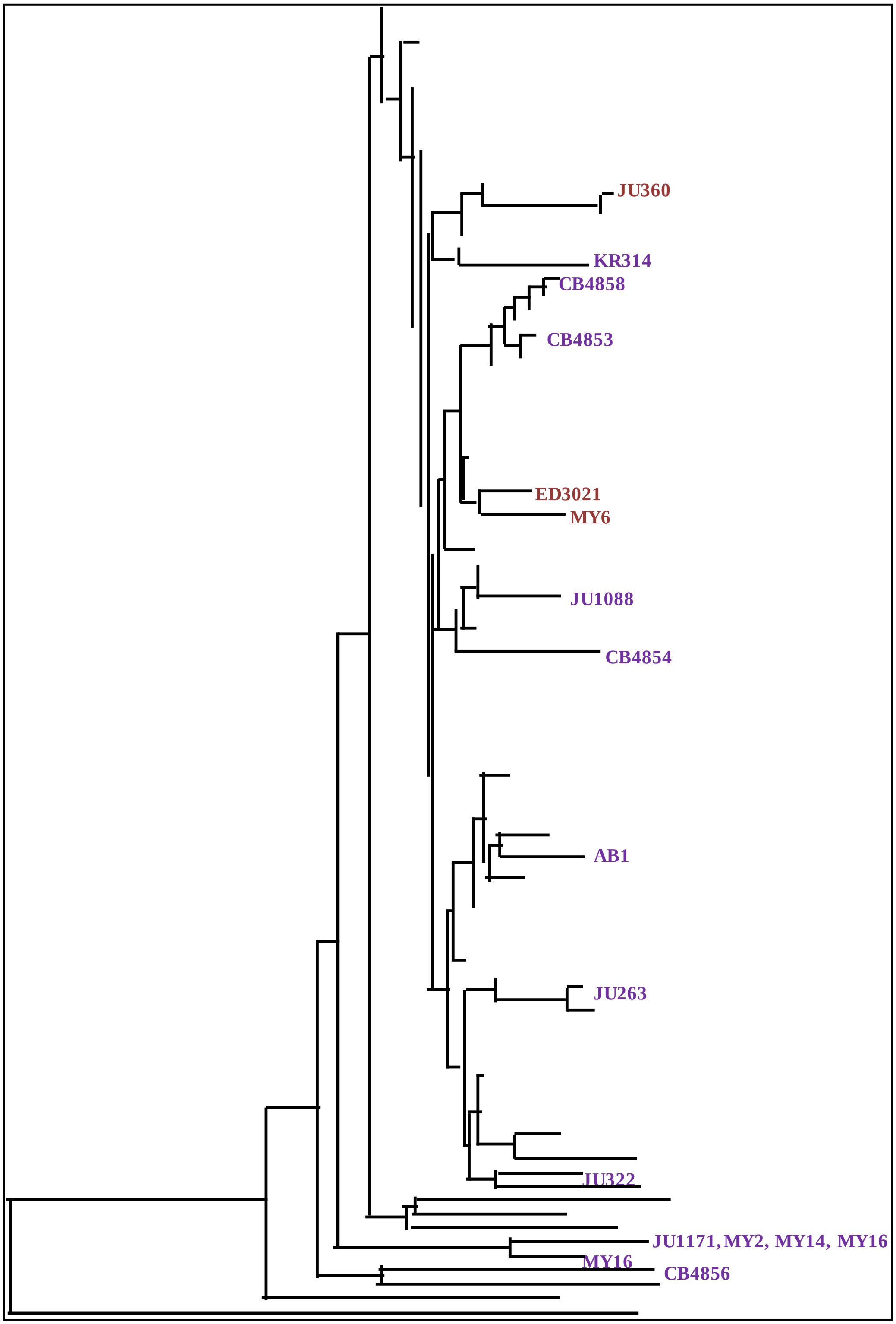


Figure 1.7 Phylogenetic relationships between wild *C. elegans* strains mentioned in this thesis. Phylogenetic relationships are based on haplotype groups assigned by variation in genome wide RAD markers. The strains whose benzimidazole phenotype was characterised are shown in purple and those not characterised are shown in brown. Figure adapted from Andersen et al (2012).

1.7 Hypothesis and Goals of the thesis

1.7.1 Hypothesis

The work in this thesis aims to test two hypotheses:

Hypothesis 1: Natural genetic variation in *C. elegans* can give rise to benzimidazole resistance

Hypothesis 2: Genetic characterization of wild strains that are resistant to benzimidazoles will identify novel benzimidazole resistance mutations

1.7.2. Objectives

The specific objectives of the work presented in this thesis are:

Objective 1: Identify wild *C. elegans* strains that are phenotypically resistant to benzimidazoles compared to the reference N2 strain.

Objective 2: Identify polymorphisms in the *ben-1* gene (that encodes the β-tubulin drug target) or elsewhere in the genome that may be responsible for benzimidazole resistance in wild strains.

Objective 3: Perform genetic complementation experiments to determine the functional significance of the candidate polymorphisms identified in objective 2.

Objective 4: Perform bioinformatic analysis to investigate whether polymorphisms identified in objective 2 are likely to change the BEN-1 protein structure and affect drug binding.

Chapter 2: Materials and Methods

2.1 *C. elegans* strains

The following wild *C. elegans* strains (used as test strains) had already been acquired from the Caenorhabditis Genetics Center (CGC) and were available in the lab as frozen stocks: AB1, CB3191, CB4853, CB4854, CB4856, CB4858, JU258, JU263, JU322, KR314 [*mab-23 (e2518)*] and MY2. The strains N2 (Bristol) and CB3474 [*ben-1(e1880)*], which are used as benzimidazole susceptible and resistant reference strains respectively throughout this project were also available as frozen stocks. One cryo-vial of each of these strains was fast- thawed, placed onto Nematode Growth Medium (NGM) Agar plates seeded with ampicillin resistant *Escherichia coli* strain OP50 and allowed to fully recover at room temperature overnight. The following day the plates were transferred into a 20^0C incubator and the worms were allowed to develop. Once the majority of worms reached adult stage, 10-12 adults were picked onto individual NGM agar plates and allowed to grow over several generations. Passaging of these strains provided working stocks for all subsequent experiments.

Wild *C. elegans* strains JU1088, JU1171, MY14, MY16 and GXW1 were obtained from the CGC during the course of this project. Upon arrival, the agar on the starved CGC stock plates was chunked onto NGM agar plates seeded with ampicillin resistant *E.coli* OP50 and worms allowed to crawl out of the agar and allowed to develop into adults. After the worms had grown through several generations, worms from some of the plates for each strain were used for -80^0C stocks and the rest of the plates were used to establish working stocks to derive worm populations for subsequent assays.

2.2 Exposure of *C. elegans* strains to benzimidazoles drugs

Two benzimidazole class anthelmintics, albendazole and mebendazole were used for the bioassays. Albendazole was used to test the sensitivity of wild *C. elegans* strains and mebendazole was used to test the sensitivity of F1 progeny resulting from genetic complementation crosses. In both cases, strains were subject to chronic exposure alongside reference susceptible N2 and reference resistant CB3474 [*ben-1(e1880)*] strains as controls.

2.2.1 Exposure of wild *C. elegans* strains to albendazole

The benzimidazole sensitivity of sixteen wild *C. elegans* strains (AB1, CB3191, CB4853, CB4854, CB4856, CB4858, GXW1, JU258, JU263, JU322, JU1088, JU1171, KR314 [*mab-23(e2518)*], MY2, MY14 and MY16) was assessed by chronic exposure to albendazole.

2.2.1.1 Production of synchronised worm populations for drug exposure

Eggs were extracted from gravid adult worms using basic hypochlorite treatment as follows: gravid adult worms were washed into a 50mL centrifuge tube from 5-6 densely populated plates using distilled water. The worms were allowed to settle by gravity and supernatant was aspirated leaving the worms in a final volume of 5mL. 5mL of a 2:3 mixture of 5M NaOH and sodium hypochlorite (bleach) solution (Clorox®) was added to the worm suspension and vortexed vigorously for 2-3 minutes to lyse the worms. In order to remove the worm debris, the suspension was diluted with 40mL of M9 buffer and filtered through a 40µm sieve into a 50mL tube (the filtrate contains the eggs). The eggs were then washed 4 times in distilled water and finally with M9 buffer by repeated centrifuging at 1435rpm (300rcf) for 3 minutes. After the final wash the pelleted eggs were re-suspended in ~10mL of M9 buffer and allowed to hatch overnight at 20^{0}C on a rocking platform within an incubator. This resulted in a population of arrested first stage larvae (L1) which were used to set up the assay. The concentration of L1s was estimated by counting their number in three 1μL droplets under a microscope and taking the mean (n) of the counts, expressed as nL1/μL.

2.2.1.2 *E.coli* food source

An overnight culture of ampicillin resistant *E.coli* OP50 was centrifuged at 4539rpm (3000rcf) for 20minutes, the resulting pellet re-suspended in M9 buffer and washed once by centrifugation under the same conditions. The pellet was then weighed and a 10% suspension was prepared by addition of the appropriate volume of M9 buffer. Usually from a 1L overnight culture a pellet of 1.8-2.0g of bacteria was harvested which were then re-suspended in about 18-20mL of M9 buffer.

2.2.1.3 Preparation of albendazole solutions

A 10mM stock solution of albendazole (Sigma-Aldrich; A4673) was prepared by dissolving 0.0026g of powder in 1mL of DMSO (aMRESCO®;0231). Working concentrations of albendazole (0.125mM, 0.25mM, 0.5mM, 0.75mM and 1mM) were then made by appropriate dilutions with DMSO.

2.2.1.4 Preparation of assay plates

A master mix (16mL) of L1s, NGM broth, *E.coli* and streptomycin was prepared for each strain to be tested (Table 2.1). Specific values for the individual assays set up for wild strains are included in Appendix B.

The master mix was vortexed well to ensure the larvae were homogenously suspended and 2.5mL aliquots (~2500L1s) were dispensed into each well of a 6-well plate. Well A1 was used as the control well (to which 15μL of DMSO was added) and into each subsequent well 15μL of increasing concentrations of albendazole working solution was added (Table 2.2). The plates were then wrapped with parafilm to minimize evaporation and incubated at 20^0C on a rocking platform for 2.5 days by which point the worms are expected to reach adult stage.

2.2.2 Exposure of *C. elegans* F1 progeny from complementation crosses to mebendazole

2.2.2.1 Mebendazole stock solution

A 50mM stock solution of mebendazole (Sigma-Aldrich; Product number: M2523) was prepared by dissolving 0.15g of mebendazole in 10mL of DMSO.

2.2.2.2 Preparation of drug plates

NGM agar plates containing a final concentration of 0.3mM or 0.0045mM mebendazole were prepared by adding the appropriate volume of the 50mM stock solution into molten agar (the agar was allowed to cool to ~40^0C before adding the drug solution). The plates were allowed to dry at room temperature overnight. The following day the plates were streaked with an overnight culture of ampicillin resistant *E.coli* and once again kept at room temperature overnight to allow the development of the bacterial lawn. The plates were used the following day or stored at 4^0C if they were to be used later.

2.2.2.3 Assay set up

A single adult hermaphrodite was allowed to lay eggs on a mebendazole containing plate overnight at 20^0C. The following day the adult worms were picked off the plate and the eggs were allowed to develop to young adult stage at 20^0C (2.5-3 days).

2.3 Assessing the sensitivity of *C. elegans* strains to benzimidazoles

2.3.1 Digital imaging equipment used to measure drug exposure phenotypes

Two systems were used to study the effect of benzimidazoles on *C. elegans* strains: (1) NemaRx digital imaging system, developed by NemaRx Pharmaceuticals Inc., Calgary, AB (Porter and Koelle 2010) and (2) IntelliView automated microscopy system IVT-MS100, developed by IntelliView technologies Inc., Calgary, AB (Shan, Rahman et al. 2013). During the initial stages of the project, the IVT-MS100 was not fully developed and optimised and hence I used the NemaRx system for the initial round of screening of the wild strains and then used the IVT-MS100 in the experiments carried out later on.

2.3.1.1 The NemaRx digital imaging system

The NemaRx digital imaging system (Figure 2.1) used here is similar to that used by (Porter and Koelle 2010). Porter and Koelle (2010) used the equipment for a thermal response assay which involved measuring the movement of individual worms following a thermal stimulus using a laser. For my experiments, the laser stimulation was not used and worms were allowed to move without any external stimuli.

The NemaRx system sequentially scans 21 sections (8x8mm non-overlapping frames) of an agar plate three times (Figure 2.2) to maximize the number of data points collected. Each of the 21 sections is scanned for 3 seconds which means that the complete triple traversal of the plate requires a total of 3 minutes. The software detects individual worms, records their co-ordinates and tracks the movement of each worm as they glide across the solid agar surface. The change in co-ordinates of each worm during the scan period is recorded and used to compute the speed and size (in arbitrary units) of each individual worm tracked. Since the plate is scanned there times in total, single animals may be scanned multiple times (Porter and Koelle 2010).

2.3.1.2 The IVT-MS100

In the IVT-MS100 (Figure 2.3) worms are screened in liquid media in multi-well plate format. Instead of tracking individual worms the system records the total movement within each well due to the thrashing motion of worms in liquid. A series of images is acquired for each well within a 3 second scan period. The mean squared error (MSE) value is used to represent the sum of grey scale changes within each pixel of successive images.

The grey scale of each pixel is represented by values ranging from 0 to 225. Over the course of 3 seconds the microscope acquires 9 images of each well and the associated software then computes the total grey scale changes for all pixels between successive images. In the output files 8 MSE values representing grey scale change between the 9 successive images are produced for each well.

The basic equation for MSE is given as,

$MSE = \sum_{1}^{n}(\mathrm{Ci} - \mathrm{Pi})^2/\mathrm{n}$, where Ci stands for intensity of the current image and Pi for intensity of previous image, n is the number of pixels in each image

The MSE values can range from 0^2 (no movement) to 225^2 (maximum movement).The values are squared in order to remove the negative sign. The more movement of worms there is in a well the greater the number of pixels that change between successive images so the higher the final MSE value.

2.3.2 Assays performed on the digital imaging equipment

2.3.2.1 Assays using the NemaRx digital imaging system

After the 2.5 day incubation period, worms in each well of the 6 well plate (in case of wild *C. elegans* strains) or from mebendazole containing NGM agar plates (in the case of F1 cross progeny) were transferred to glass test tubes. Once the worms settled (after ~ 2-3 minutes) excess supernatant (~2mL) was carefully removed taking care to minimize worm loss. The worms were then washed three times with X1 PBS (3mL) to remove excess food/drug that may have adhered to their surface during drug exposure and left in a volume ~200µL of PBS after final wash.

Non-seeded (no bacterial lawn) NGM agar plates were placed on a pre-defined template (Figure 2.2) and five drops (5µL each) of the worm suspension were placed onto the agar surface in positions 1-5 noted on Figure 2.2. The plates were left at room temperature to allow the dispensed liquid to dry into the agar and the worms to migrate away from the positions on which they were dispensed on the plate. After 1 hour, each plate was screened on the NemaRx digital imaging system where speed and size of individual worms recorded.

2.3.2.2 Assays using the IVT-MS100

Only the wild *C. elegans* strains KR314 [*mab-23(e2518)*] and MY2 were assayed using this system. In contrast to the NemaRx system, readings on the IVT-MS100 are taken in 96-well plate format. A 100µL aliquot from each well of the 6-well plate was directly dispensed into a well of the 96-well plate in triplicate (Figure 2.4). Before an aliquot was removed the culture in the 6-well plate is mixed well (by moving the plate in a circular motion) in order to make it homogenous. A standard sample loading plan for the 96-well plate is shown in Figure 2.5.

2.3.3 Analysis of data from the digital imaging equipment

2.3.3.1 Analysis of data from the NemaRx system

The raw data output from the NemaRx system was in the form of .txt files. This data was first imported into excel (Figure 2.6). In the excel work sheet, the first five rows are not necessary for further analysis so they are deleted. What data then remains in Column J corresponds to speed measurements and Column S the corresponding sizes of those individual worms tracked. This data table was then sorted according to size (Column S). As recommended by the NemaRx developers, only data that falls within the size range 25-95 arbitrary units (a.u.) inclusive are used for subsequent analysis (rest of the data rows are discarded). This is because values below and above this range are considered artefacts and not produced by a single worm (Dr. Andreas Wissman Pers. Comm.). This means that only objects within the size range 25-95 a.u. are considered individual ‘worms’.

2.3.3.1.1 Analysis of speed data

The remaining speed data (Column J), was then imported into GraphPad Prism software v 6.0 (purchased from http://www.graphpad.com/) for further analysis. First the speed readings were subject to an outlier test using the ROUT method which combined robust regression and outlier detection (Motulsky and Brown 2006) with Q (maximum desired false discovery rate) set at 1% (GraphPad recommended). The outlier test was performed to remove a small number of data points indicating extremely high speeds that were biologically implausible. The cleaned data was then tested for normality using the D'Agostino-Pearson Omnibus Test (GraphPad recommended). Median speed was used for subsequent analysis.

In order to account for the inherent differences in movement speed between the strains in the absence of drug and also to normalise the data set across the concentration range for individual experiments, the median speed at each concentration was transformed and expressed as relative speed where,

Relative Speed = (median speed in test concentration/median in DMSO) X 100

2.3.3.1.2 Analysis of size data

Since lower and upper thresholds were already applied to the size data a further outlier test was not performed on this data set. However, the same normality test was performed and median size and relative size was calculated similar to that done for the speed data set where, Relative Size = (median size in test concentration/median size in DMSO) X 100

The relative speed and relative size data was used to create the dose-response curves on GraphPad. According to this formula, the relative speed and size of worms exposed to DMSO was always considered 100%.

2.3.3.1.3 Comparisons of groups

Since the data sets for each group was not normally distributed, non-parametric tests were used for inter-group comparisons (different concentrations within the same strain or same concentration between strains). When multiple groups were compared by a Kruskal-Wallis Test followed by Dunn's Multiple Comparisons Test the significance level was expressed in terms of an adjusted P value based on the number of comparisons. Where only two groups were compared a Mann-Whitney test was used and the significance level is expressed in the lower case (p).

2.3.3.2 Analysis of IVT-MS100 data

A sample data sheet (Excel format) from this system is shown in Figure 2.7 (only MSE values are shown for clarity). There are 8 MSE readings for each well and the average of these values is first calculated to provide a single MSE value for each well. As outlined in Section 2.3.2.2 the samples (for each drug concentration) are loaded onto the 96-well plate in triplicate (Figure 2.4) and therefore the mean of the three replicates was calculated for each strain at each concentration. As with the NemaRx system, the data was normalised by calculating relative MSE values to account for inherent movement differences between the different strains (in the absence of drug) using the following formula:

Relative MSE= (average MSE in test concentration/average MSE in DMSO) X 100

According to this formula, the relative MSE for wells with worms exposed to DMSO was always considered 100%. The relative MSE data was then transferred to GraphPad software and used to create the dose-response curves.

The MSE values for each well were normally distributed and thus parametric tests could be used for inter-group comparisons. Comparisons were carried out using two-way ANOVA with strain and concentration as factors and significance level is expressed in lower case (p).

2.4 Genetic Complementation

For each cross 7-10 L4 stage hermaphrodites of one strain and 12-15 young males of another were placed on a mating plate (NGM agar plate with a small lawn of OP50 bacteria) using a platinum wired worm tool. The worms were allowed to mate at 20^0C overnight and the hermaphrodites were then singly picked onto mebendazole infused plates for egg-laying (Section 2.2.2.3 and Figure 2.8). F1 progeny resulting from a successful mating was confirmed by presence of 50% each of males and hermaphrodites.

2.5 Molecular Characterisation of the β-tubulin drug target

2.5.1 Complementary DNA (cDNA) preparation

Total RNA was prepared from *C. elegans* strains using the ambion¦ RNA by life Technologies™ TRIzol® Reagent protocol for whole RNA extraction from tissues. Worms grown to adult stage in 15mL liquid culture (~15,000 worms) tissue culture flasks were transferred into 15mL falcon tubes and washed with distilled water ~3 times by centrifugation at 1435rpm (300rcf) for 3 minutes. After the final wash, supernatant was removed leaving the worms in a final volume of 1mL. The worm pellet was re-suspended and 500µL aliquots of this suspension were transferred into 2.5mL eppendorf tubes. 1.5mL of Trizol reagent was then added to each of these eppendorf tubes and stored at -80^{0}C until ready to extract RNA.

cDNA was prepared from this RNA using Invitrogen™ cloned AMV first-strand cDNA synthesis kit (Catalogue number: 12328-040) following the manufacturer's protocol and stored at -20^{0}C until ready to use.

2.5.2 Genomic DNA extraction

DNA was extracted from *C. elegans* strains (N2, CB3474 [*ben-1(e1880)*], KR314 [*mab-23 (e2518)*] and MY2) using lysis buffer (50mM KCL, 10mM Tris-pH 8.3, 2.5mM $MgCl_2$, 0.45% Nonidet p-40, 0.45% Tween-20, 0.01% gelatin) and 20mg/mL proteinase K (Invitrogen™). A master mix of lysis buffer (500 µL) and proteinase K (2.5µL) was prepared. 30µL aliquots were dispensed into 0.5mL PCR tubes after which 20-30 adult hermaphrodites were singly picked using a platinum wired worm tool into each tube. The tubes were then placed at -80^{o}C for ~15mins and then transferred into a thermo cycler (Applied BioSystems PCR System 9700) for the overnight lysis cycle (28X (60^{o}C, 30min) then 85^{o}C, 60min).

2.5.3 Primer design

Primers for the amplification of the *C. elegans ben-1* locus were designed using the online Primer 3 tool version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). Primers were 20-25 nucleotides in length with melting temperature (T_m) between 65-70^{0}C in all cases and as much as possible I ensured that primer pairs had matching T_ms. In addition, to facilitate better annealing stability, I tried to design the primers such that they will have a G/C at the 3' end.

One set of primers (oJG 211, oJG212) was designed to encompass the complete *ben-1* mRNA sequence (NM_065327). In addition, 8 sets of primer pairs (oJG 216/217, 218/219, 220/221, 222/223, 240/243, 241/242, 244/247, 245/246) were designed to amplify the *ben-1* genomic locus as a series of amplicons with at least 60-70bp overlap at each end (Figure 2.9 and Table 2.3). The genomic sequence was obtained from the *C. elegans* cosmid C54C6 (Z77131) along with ~500bp of 5` and 3` flanking sequence. Later on in the project, additional primers (oJG 465, oJG 466, oJG 467, oJG 468, oJG469) were designed for the genomic locus to cover gaps in the sequences obtained from the earlier primers. Table 2.4 shows expected sizes of products amplified from *C. elegans* cDNA and genomic DNA for each primer pair.

Primers were ordered from Integrated DNA Technologies (IDT) and were supplied as desiccated powder. X100 stocks and X10 working solutions were prepared by adding the appropriate volume of Ultra-Pure™ DNase/RNase-free distilled water (Invitrogen™) for use in PCR.

2.5.4 Sequencing of the *C. elegans ben-1* locus

cDNA/genomic DNA was used as template for PCR amplification using the appropriate primers (Table 2.4) in an Applied Biosystems Veriti® thermal cycler. Reaction components and amounts used per reaction are shown in Table 2.5. A master mix (without template) of the different PCR components was prepared based on the number of reactions that were to be carried out. For a 50µL reaction, 48µL of this master mix was used with 2µL template in a 0.5mL PCR tube.

The following cycling parameters were used: Initial denaturation 95 ^{o}C, 5mins; 30-35 cycles of (95 ^{o}C, 2mins; 56 ^{o}C, 45sec, 72 ^{o}C, 1 min 30 sec) and final extension 72 ^{o}C, 7min. (Annealing temperature is set about 10 ^{o}C less than the melting temperatures (T_m) of the primer pairs.

An aliquot (8µL) of amplicon was mixed with 2µL loading dye (X5) containing SyberGreen and analyzed on a 1.5% agarose gel by electrophoresis at 120V for ~45min. The bands were visualized using BioRad Molecular Imager® Gel Doc™ XR+ System with Image Lab™ software. The remaining aliquots of PCR products (~40µL) in each reaction were purified using Omega Cycle Pure™ Kit and micro elute spin protocol in preparation for sequencing. Purified PCR products were sent for sequencing at either Plate-forme d'Analyses Génomiques de l'Université Laval (http://pag.ibis.ulaval.ca/seq/en/index.php) or University Core DNA Services, University of Calgary (http://www.ucalgary.ca/dnalab/sequencing).

2.5.6 Sequence analysis

cDNA and genomic sequences of the *ben-1* locus of the wild strains were compared to that of the N2 strain sequences originally used for primer design using Geneious Pro version 5.6.3 created by Biomatters (available from www.geneious.com).

Sequence from the University of Laval or University of Calgary websites were imported into Geneious software and poor quality 5' and 3' ends were trimmed from each fragment. Ambiguous base calls were corrected by manual examination of the chromatographic sequence traces. For cDNA, the forward (oJG211) and reverse (oJG212) fragments were *de novo* assembled and then aligned to the N2 reference sequence. Due to lack of overlap after trimming, genomic sequences could not be *de novo* assembled and therefore these sequences were mapped (or assembled) onto the reference N2 sequence using the Geneious assembly function. These alignments were used to identify polymorphisms in the *ben-1* sequence of wild *C. elegans* strains compared to the reference N2 strain.

The nucleotide sequences of the *ben-1* genomic locus of the N2 and wild *C. elegans* strains were translated using the Geneious translation tool. The amino acid sequences of the β-tubulin drug target of the N2 and wild *C. elegans* strains were then aligned using the Muscle alignment tool incorporated into Geneious software (using default settings). These alignments were used to identify non-synonymous changes in the amino acid sequences of the wild *C. elegans* strains compared to that of the N2 strain.

2.5.7 Analysis of Million Mutations Project data

This was a two-year long whole genome sequencing project for *C. elegans* strains derived by chemical mutagenesis as well as isolated from the wild (also see Section 1.5.3.1). In total 2007 chemically mutagenised strains and 40 wild isolates have had their genomes sequenced (Thompson, Edgley et al. 2013). Since I only sequenced the *ben-1* locus of wild strains KR314 [*mab-23(e2518)*] and MY2, I made use of the data from this project to find out if there were mutations in the *ben-1* locus of the rest of the wild strains that I had phenotyped (AB1, CB3191, CB4853, CB4854, CB4856, CB4858, JU258, JU263 and JU322) as well as any other strains that carry non-synonymous mutations in their *ben-1* locus. I accessed these data sets from the dedicated website for this project at http://genome.sfu.ca/mmp/.

2.5.7.1 Other wild strains with non-synonymous mutations in the β-tubulin drug target

When I first learned about the Million Mutations Project, I contacted Dr. Donald Moerman and requested for any sequence information on the *ben-1* gene of the wild *C. elegans* strains, a summary of which he kindly provided (summary data for polymorphisms in 27 wild isolates was provided to me in the form of a text file). I imported this data into excel and looked for wild strains with non-synonymous polymorphisms in the *ben-1* gene.

2.5.7.2 Variations in genes close to the *C. elegans ben-1* locus on chromosome III

According the map of *C. elegans* chromosome III available from WormBase I chose to look at variations in a subset of genes on either side of *ben-1* include *unc-45, par-2, dpy-1, smg-6, let-805, mec-12, unc-93, pal-1, lon-1* and *sma-3*. I performed a search for non-synonymous changes in these genes of the wild *C. elegans* strains JU1171, MY2, MY14 and MY16 on the dedicated website for the Million Mutations Project and downloaded data into an Excel file for subsequent use.

2.5.8 Secondary structure predictions

The Geneious software secondary structure prediction tool was used to build models of the β-tubulin protein of the N2 and wild *C. elegans* strains for comparison with each other. The complete amino acid sequences of the N2 strain and a particular wild *C. elegans* strain of interest were aligned using the MUSCLE alignment tool after which the secondary structure prediction tool was used on this aligned protein sequences so that structural changes (points of conflict) can be easily identified.

2.6 Chapter 2: Figures and Tables

Table 2.1 Components used in the master mix for the albendazole exposure assay

Component	Volume used (μL)
L1 suspension	x*
10% OP50 suspension	960
Streptomycin (20mg/mL)	16
NGM Buffer	16000-(x+960+16)

*x=16000/n, where n is the number of L1s in 1μL suspension

Table 2.2 Albendazole (ABZ) concentrations used for the assay

Well	ABZ Concentration (mM)	Volume(μL)	ABZ concentration in well (mM)
A1	DMSO	15	0
A2	0.125	15	0.00075
A3	0.25	15	0.0015
B1	0.5	15	0.003
B2	1*	15	0.006
B3	188.33*	15	1.13

*For some assays concentrations used were as shown, but for others 0.75mM was used for well B2 and 1mM for well B3

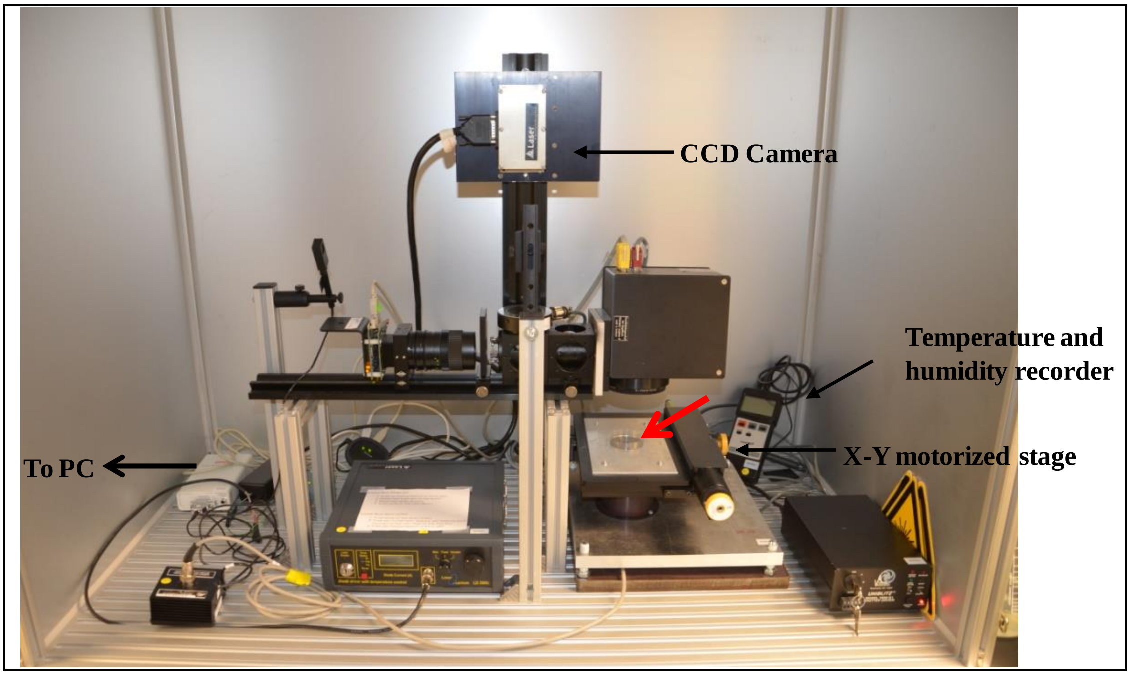


Figure 2.1The NemaRx digital imaging system developed by NemaRx Pharmaceuticals Inc.. The position of the sample plate (15x60mm petri plate containing non-streaked NGM agar) is indicated by the bold red arrow. Behavioural (speed) and morphological (size) data of individual worms on a sample plate are recorded by triple traversal of each plate by the Closed Circuit Digital (CCD) camera attached to a Leica M420 dissecting microscope. Light from a red LED light is used to illuminate the sample plate from below.

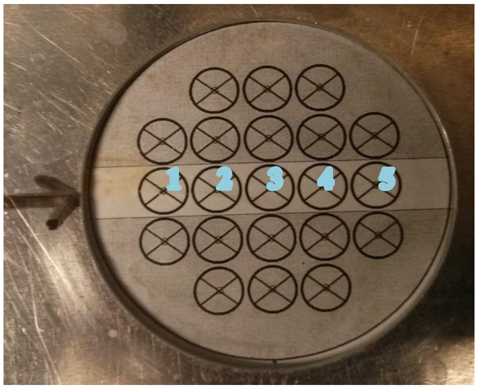


Figure 2.2 Template for application of clean worm suspension onto agar plate. Points 1-5 indicate the initial points of application of worm suspension. The black arrow indicates the orientation in which the plate should be placed on the stage of the NemaRx system to ensure a common origin from which the worms would start to move. The checked circles mark the 21 areas (8x8mm frames) the microscope/camera would scan from top left to bottom right during a single traversal of the sample plate.

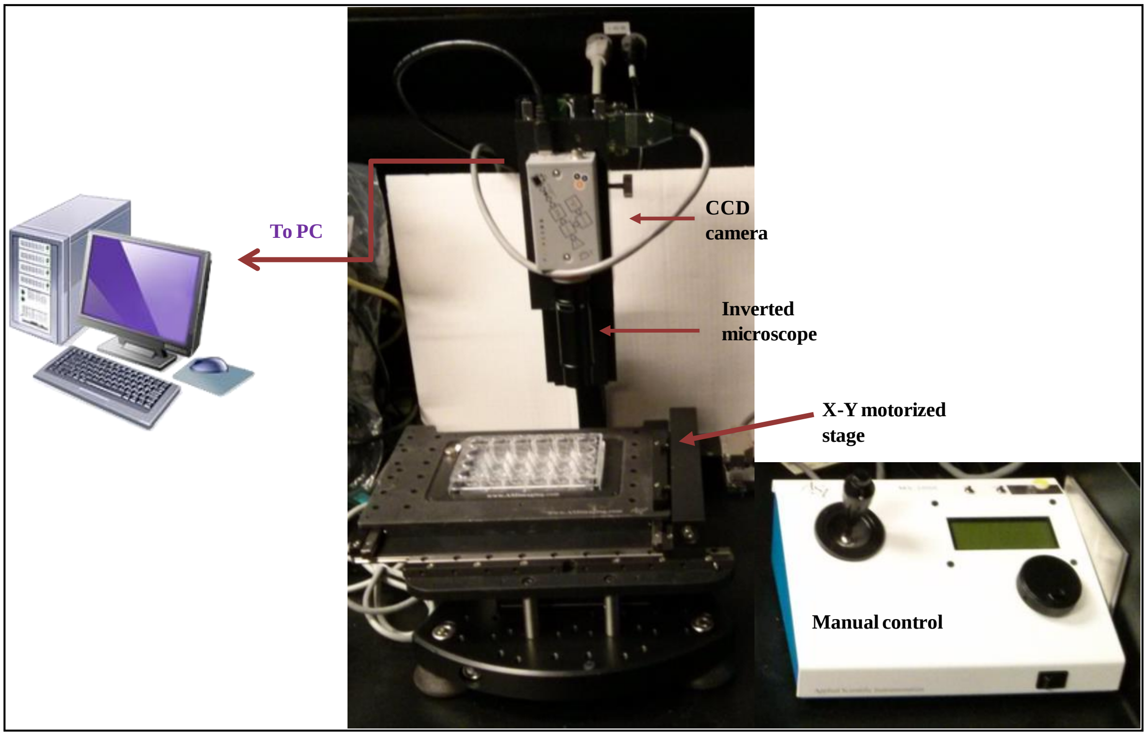


Figure 2.3 The IVT MS-100 system developed by IntelliView Technologies Inc. The multi-well sample plate is placed on the X-Y motorised stage. An inverted microscope/CCD camera combination records and total movement of worms within each well represented as Mean Squared Error (MSE) values in the output files. The microscope focusing and stage movement are automated, but they can also be controlled manually.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1: N2	A1: CB3474	A1:T1	A1:T2	A1: N2	A1: CB3474	A1:T1	A1:T2	A1: N2	A1: CB3474	A1:T1	A1:T2
B	A2: N2	A2: CB3474	A2:T1	A2:T2	A2: N2	A2: CB3474	A2:T1	A2:T2	A2: N2	A2: CB3474	A2:T1	A2:T2
C	A3:N2	A3:CB3474	A3:T1	A3:T2	A3:N2	A3:CB3474	A3:T1	A3:T2	A3:N2	A3:CB3474	A3:T1	A3:T2
D	A4:N2	A4:CB3474	A4:T1	A4:T2	A4:N2	A4:CB3474	A4:T1	A4:T2	A4:N2	A4:CB3474	A4:T1	A4:T2
E	A5:N2	A5:CB3474	A5:T1	A5:T2	A5:N2	A5:CB3474	A5:T1	A5:T2	A5:N2	A5:CB3474	A5:T1	A5:T2
F	A6:N2	A6:CB3474	A6:T1	A6:T2	A6:N2	A6:CB3474	A6:T1	A6:T2	A6:N2	A6:CB3474	A6:T1	A6:T2
G												
H												

Figure 2.4 Loading pattern for a 96-well plate to be screened on the IVT-MS100 system. A1-A6 corresponds to wells of the 6 well bio assay plate from which the 100µL aliquot was taken. T1 and T2 correspond to the wild strains being tested alongside the reference strains N2 and CB3474 [*ben-1(e1880)*]. Rows G and H are empty in this case. As an example A1:N2 means, aliquot is taken from well A1 of the 6 well bio assay plate set up for the N2 strain. The samples are loaded in an alternating pattern to avoid any influence of time on final readouts.

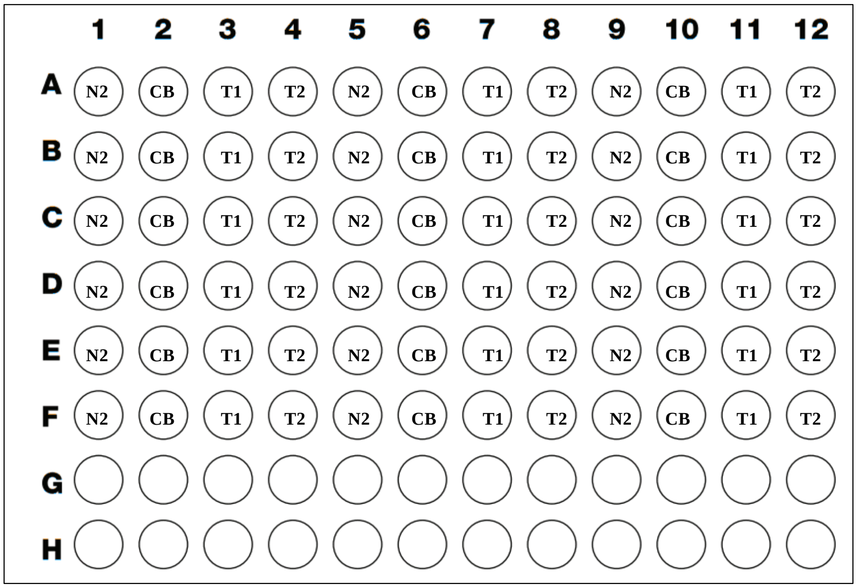


Figure 2.5 Template for loading samples onto a 96-well sample plate. Each sample is loaded in triplicate and notations within the wells indicate the strains N2 (N2), CB3474 [*ben-1(e1880)*] (CB), Test strain/wild isolate 1 (T1) and Test strain/wild isolate 2 (T2). Samples in Row A would be taken from well A1 of the 6-well bio assay plate of each corresponding strain, samples in Row B from well A2 of the 6-well bio assay plate and so on (also see Figure 2.4). Rows G&H would be empty and therefore not scanned by the IVT-MS100 system.

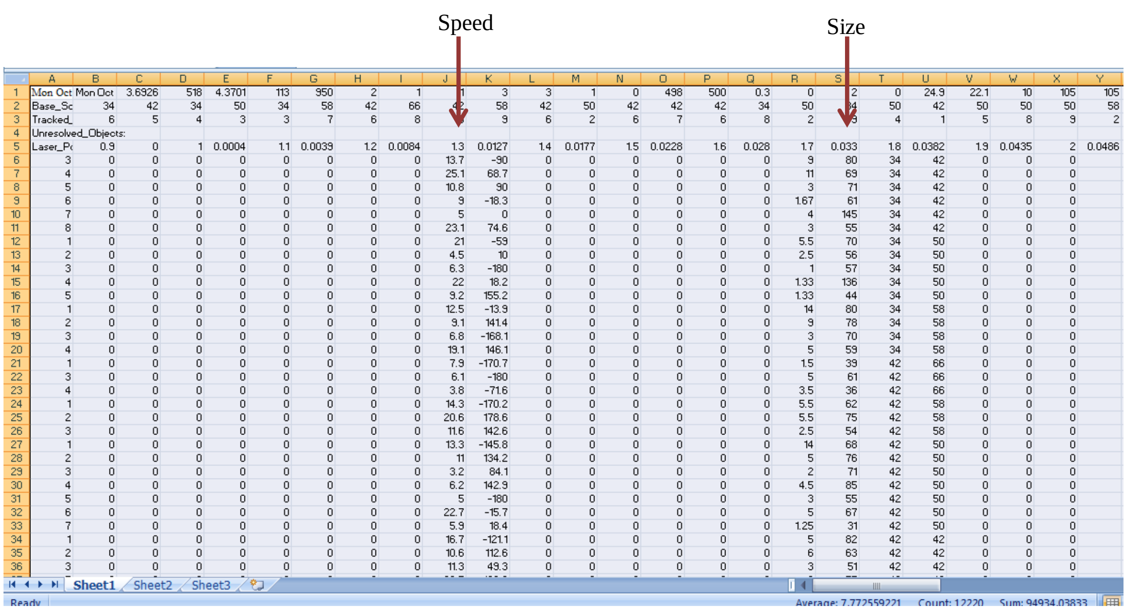


Figure 2.6 Data output from the NemaRx digital imaging system for a single sample plate. Raw data in Text file format is copied and pasted on to an excel work sheet (only part of it is shown). Rows 1-5 are removed since they contain information about the experiment. Once this is done, Column J contains the speed of each of the individual worm tracked by the system and column S will contain the corresponding size data for these worms (since each plate is scanned thrice, the speed and size data may contain multiple values for the same individual).

Well	1	1	1	1	1	1	1	1	Avg/Well
MSE	112.8509	99.34784	101.4851	98.84255	95.8888	99.46177	108.7993	96.43591	101.639
Well	2	2	2	2	2	2	2	2	
MSE	75.32333	84.76096	85.45497	82.90542	88.46766	81.36635	80.4	80.75244	82.42889
Well	3	3	3	3	3	3	3	3	
MSE	79.77063	89.7968	86.61526	85.44768	93.67611	89.40816	88.78357	89.79794	87.91202
Well	4	4	4	4	4	4	4	4	
MSE	71.41487	80.06099	89.42528	90.73408	86.80365	84.62863	87.73956	78.67807	83.68564
Well	5	5	5	5	5	5	5	5	
MSE	73.68719	75.13488	78.90271	74.38361	79.61404	73.13306	81.38465	80.17351	77.05171
Well	6	6	6	6	6	6	6	6	
MSE	80.04863	84.48173	91.46095	87.67143	82.7842	86.31055	88.23231	90.42803	86.42723
Well	7	7	7	7	7	7	7	7	
MSE	77.53502	84.76928	88.33653	86.63377	90.10072	90.71511	92.37413	84.43263	86.86215
Well	8	8	8	8	8	8	8	8	
MSE	45.69449	51.09818	49.24705	49.42873	50.97216	52.51514	51.34924	52.35708	50.33276
Well	9	9	9	9	9	9	9	9	
MSE	71.32303	74.31485	73.02071	71.55478	78.31541	74.91956	80.72796	78.77109	75.36842
Well	10	10	10	10	10	10	10	10	
MSE	64.82578	71.91371	72.51606	67.59641	71.41938	68.1159	70.04577	63.75074	68.77297
Well	11	11	11	11	11	11	11	11	
MSE	70.2334	87.44898	81.56927	78.0064	73.26986	88.83677	77.30004	78.82145	79.43577
Well	12	12	12	12	12	12	12	12	
MSE	43.68615	46.73569	47.67543	47.8517	48.11886	49.0242	47.10777	49.41577	47.45195

Figure 2.7 Data from the IVT-MS100 system for the first 12 wells of a 96 well plate. The 8 MSE readings for each well correspond to differences between 9 successive images of the well contents taken within a 3 second scan time. The averages of the 8 MSE readings are computed in Excel to reflect total movement within each well.

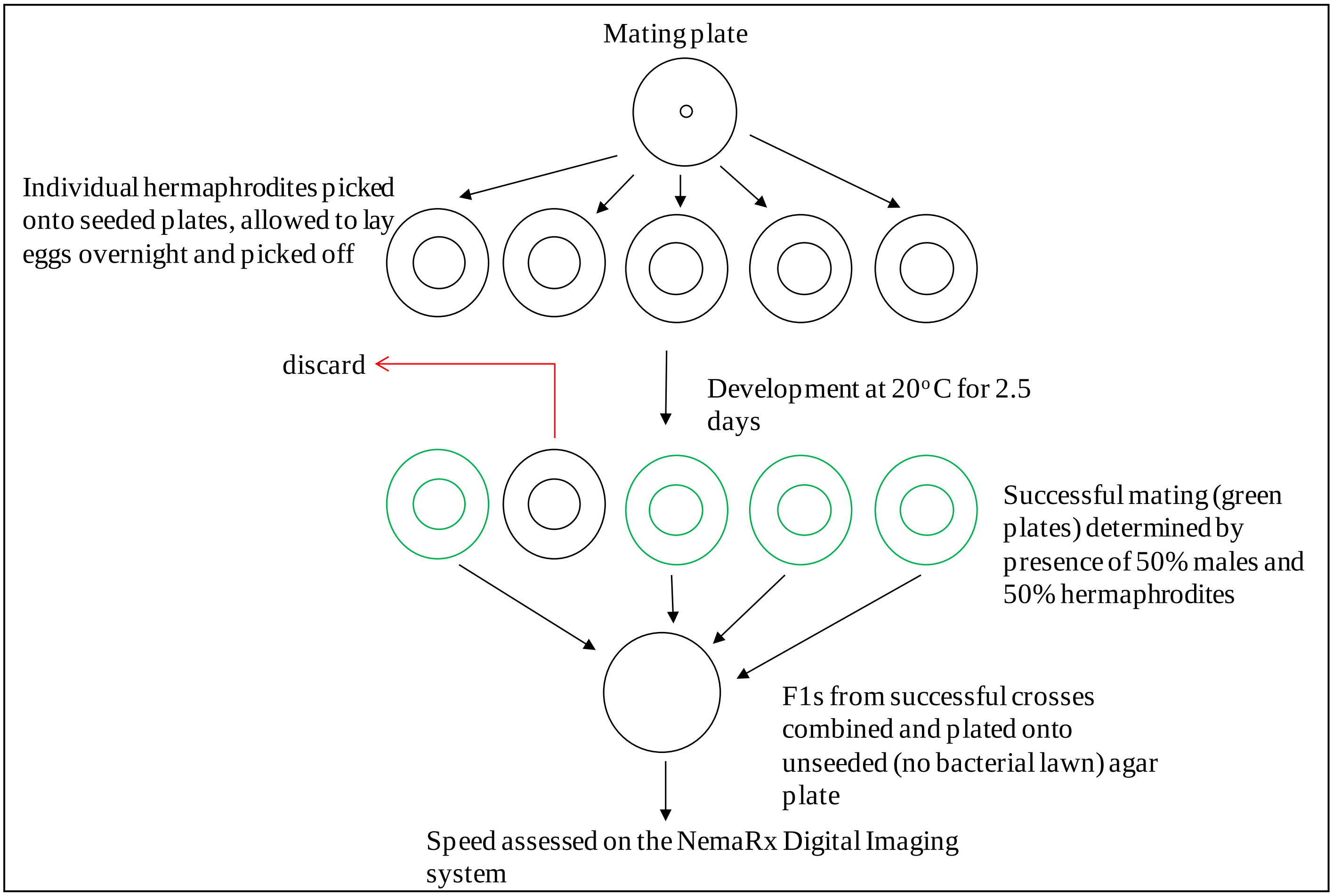


Figure 2.8 Overview of the genetic complementation experiments. Worms are allowed to mate overnight and 'mated' hermaphrodites picked individually onto *E.coli* OP50 seeded NGM agar drug plates and allowed to lay eggs overnight and picked off plates the next day. Eggs are allowed to develop and progeny from successful out crossing determined by the presence of 50% each of males and hermaphrodites (green plates) are used in subsequent phenotypic analysis.

Figure 2.9 Primer annealing sites in the *C. elegans ben-1* genomic locus. Screen shot from the Geneious program showing the location of primers (green annotations) and coding exons (yellow annotations) on the *ben-1* locus. oJG=oligo nucleotide John Gilleard (used to denote primers designed in by Gilleard lab members). Base numbers are marked on top. Primers oJG 216-223, oJG 240-247 were the first to be designed after which the *ben-1* locus was PCR amplified using these primers. When the PCR products were sequenced and assembled to the canonical N2 *ben-1* sequence there was lack of overlap between fragments in some regions. Thus, primers oJG 465-469 were designed to amplify and obtain sequence for these regions.

Table 2.3 Sequences of primers used to amplify the *C. elegans ben-1* locus

Primer	Sequence	Melting Temperatures/Tm (^{0}C)*
oJG211	ATGAGAGAAATTGTTCACGTTCAAGCC	66
oJG212	CTATTCAGCATCTCCATCAGTTCCATCA	66
oJG216	AATGAGAGAAATTGTTCACGTTCAAG	62
oJG217	GCGCAAGAGACACCACACGTTTAT	68
oJG218	CCGGCGTATACTAGTTTCTTGCACA	67
oJG219	GAAAGCTTGAGGGTTCTGAAGCAG	66
oJG220	GTCCACCAGCTCGTTGAAAATACC	66
oJG221	TTCGCCTCCAAATACCTAACGAGA	66
oJG222	AGTTGCTGGAAACGTGCTCCACC	71
oJG223	TCTATTCAGCATCTCCATCAGTTCC	64
oJG 240	AGGACGGAGCTCCCAATTTCC	68
oJG 241	TGCACTCCGGTTACAAAATATTC	61
oJG242	TTGGAAGTTAAAGAAAATTTCGG	57
oJG243	CGCTGCCACAAAAGTTTGAA	62
oJG244	TTGGCGAAACTAATTGAAACA	58
oJG245	AGGCGAAATTCGAAAAAGAATTTC	60
oJG246	ATGGCAGGAAGCAGGCGATAC	68
oJG247	TTCCGGAAAACCGAAAATTA	57
oJG465	ATCCGTGAAGAGTATCCAGATAG	61
oJG466	CTAATAATTAAAATTCAGGTCTCGG	57
oJG467	ACCGATGAGACTTTCTGCATTG	63
oJG468	GATCCGAGACACGGACGTTAC	65
oJG469	TGAAAAGTCGATAATTTTTTCGCG	60

* Melting temperatures were calculated using the New England Biolabs T_m calculator available at: https://www.neb.com/tools-and-resources/interactive-tools/tm-calculator

Table 2.4 Expected product sizes from PCR amplification of *ben-1* locus

Primer pair	Amplicon size (bp)*	Template
oJG 211/212	1335	cDNA
oJG 216/217	950	Genomic DNA
oJG 218/219	870	Genomic DNA
oJG 220/221	867	Genomic DNA
oJG 222/223	756	Genomic DNA
oJG 240/243	582	Genomic DNA
oJG 241/242	280	Genomic DNA
oJG 245/246	318	Genomic DNA
oJG 244/247	695	Genomic DNA

*Amplicon sizes are based on coding (NM_065327) and genomic (extracted from cosmid C54C6 (Z77131)) sequences of the *ben-1* locus of the N2 strain

Table 2.5 PCR component volumes per reaction

Component	Volume (µL)
Buffer (-Mg)	5
50mM $MgSO_4$	2
dNTP mix (25µM)	1
Forward primer (10mM)	1
Reverse primers (10mM)	1
Platinum®Taq DNA Polymerase High Fidelity	0.2
Template (cDNA/genomic DNA)	2
Water	37.8
Total	50

Chapter 3: Phenotypic characterization of wild *C. elegans* strains to assess their sensitivity to benzimidazole drugs

3.1 Introduction

The overall goal of this project was to identify naturally occurring novel mutations/mechanisms that confer benzimidazole resistance in nematodes using *C. elegans* as a model. As a first step to this goal, it was necessary to determine whether any wild *C. elegans* strains show phenotypic resistance to benzimidazoles and if so then go on to define the molecular basis of this resistance (Chapters 4 and 5).

To date over 200 wild *C. elegans* strains have been isolated from different geographic locations worldwide (Andersen, Gerke et al. 2012). I include the benzimidazole phenotyping results for a subset of eleven of these strains (AB1, CB3191, CB4853, CB4854, CB4856, CB4858, JU258, JU263, JU322, KR314 [*mab-23(e2518)*] and MY2) in this chapter. These strains were chosen because they have been previously shown to display a high degree of genetic diversity based on variable numbers of indels in their genomes relative to the canonical N2 strain (Maydan, Lorch et al. 2010). In the mean time, a recent phylogenetic study based on Restriction site Associated DNA (RAD) markers has demonstrated that they display a high degree of haplotype diversity (Figure 1.7) (Andersen, Gerke et al. 2012). Finally, the locations from which these strains were originally isolated (Table 1.2) are geographically spread out thus providing a reasonably representative sample of the global *C. elegans* population.

Two automated equipment were used for phenotyping wild strains: The NemaRx digital imaging system (Figure 2.1) which assesses the effects of drug based on speed of movement and size of worms was used in the preliminary screening cycle, while the IVT-MS100 (Figure 2.3) where phenotyping is based on total movement represented by Mean Squared Error (MSE) values was used for subsequent biological replicates (Section 2.3).

3.2 Determining the benzimidazole sensitivity of wild *C. elegans* strains using the NemaRx digital imaging system

To measure the effects of the benzimidazole drug albendazole on *C. elegans* strains, synchronized L1 stage worms were exposed to a series of concentrations of albendazole (0, 0.00075, 0.0015, 0.0030 and 0.0060mM) in liquid culture for 2.5 days at 20^{o}C during which time the L1s develop to adult stage (Section 2.2.1). At the end of the incubation period, adult worms were transferred to unseeded agar plates for measurement of speed of movement and size using the NemaRx digital imaging system which tracks individual worms and records behavioral (speed) and morphological (size) phenotypes in arbitrary units (a.u.). It was expected that this chronic drug exposure would have negative effects on the growth and motility of *C. elegans* (Spence, Malone et al. 1982). Therefore, sensitive worms were expected to appear shorter and show a greater reduction in movement than resistant worms. Hence, the degree to which the length and movement are affected by the drug in different worm strains should depend on their level of resistance to albendazole.

For all assays, the reference susceptible strain, N2 and reference resistant strain, CB3474 [*ben-1(e1880)*] were setup alongside the wild strains being tested. The latter strain had been previously generated by EMS mutagenesis of the N2 strain and shown to be resistant to benzimidazoles (Driscoll, Dean et al. 1989). Since screening on the NemaRx system can take a considerable amount of time (3 minutes per sample plate), in order to avoid prolonged experiment times within which worms could potentially recover from effects of the drug a maximum of three wild strains (alongside the two reference strains) were assayed in each experiment.

Once the screening on the NemaRx system was complete, the raw data was imported into excel and further analysis was conducted using GraphPad version 6.0. A box and whiskers plot of speed data for the reference susceptible N2 strain at increasing albendazole concentrations showed the presence of a small number of biologically implausible values in the data set (Figure 3.1A). Thus, the speed data was subject to an outlier test (ROUT method with Q set at 1%) to remove the extreme outliers (Figure 3.1B). Normality tests (D' Agostino-Pearson Omnibus) for both the speed and size data showed non-normal distributions in most cases (Figure 3.1B). Thus, the median speed and size was used as the phenotypic measure (rather than mean) and non parametric tests were used to compare data sets (Kruskal Wallis test followed by Dunn's multiple comparisons test/Mann Whitney test; if multiple comparisons were carried out the significance level is indicated as adjusted P values). Since the different strains may vary in their inherent behaviour and morphology, the phenotype at each drug concentration was normalized to the relevant strain's DMSO control to give a relative value (Section 2.3.3.1). In this way such strain differences independent of drug exposure were accounted for and any changes seen could be taken to be solely due to drug exposure.

The results of the screens performed on different days (shown as separate dose response curves) for 11 wild *C. elegans* strains are shown in the Summary Data Tables 3.1 to 3.4 and Figures 3.2 to 3.5 and are ordered according to the dates on which they were carried out. Recordings of visual observation of assay plates are included in Appendix C. As this was a preliminary screen each wild strain was only assayed in a single experiment with the objective of replicating the assays for only those strains that displayed a benzimidazole resistant phenotype.

3.2.1 Albendazole sensitivity of strains AB1, CB3191 and JU322:

The first screen on the NemaRx system included strains AB1, CB3191 and JU322 the results of which are summarized in Tables 3.1A and B and Figure 3.2. As expected the median speed of movement of the CB3474 [*ben-1(e1880)*] reference resistant control worms was not affected even at the highest concentration of 0.006mM albendazole compared to the median speed in DMSO (P=0.8773). Similarly, there was no effect on the median size of this strain at the highest albendazole concentration compared to the DMSO control (P=0.0912). In contrast and as expected, the speed of movement of the N2 reference sensitive control worms was dramatically affected at the highest concentration with an 84% reduction in median speed compared to the N2 DMSO control (P<0.0001). The size of N2 worms was also affected showing a 34% reduction in the median length compared to N2 worms in DMSO (P<0.0001).

Strains AB1, CB3191 and JU322 were all affected by albendazole in a manner similar to N2 worms (Figure 3.2A and B). The median speeds of these wild strains at the highest albendazole concentration were reduced by 77.4%, 79.3% and 79.3% respectively compared to DMSO controls of the same strains (P<0.0001) (Figure 3.2A). Further, the median speeds of these strains at the highest albendazole concentration were not significantly different to the N2 worms (P=0.5021 for AB1, P>0.9999 for CB3191 and P=0.2368 for JU322), but were significantly different to that of the CB3474 [*ben-1(e1880)*] reference resistant strain (P>0.0001).

Similarly, these wild strains showed 25.5%, 36.8%, 31.4% reductions in size respectively at the highest albendazole concentration compared to DMSO controls of the same strains (P<0.0001) (Figure 3.2B). The median sizes of these strains at the highest albendazole concentration were not significantly different to N2 worms (P=0.0154, P=0.2791 and P=0.0934), but were significantly different to that of the CB3474 [*ben-1(e1880)*] reference resistant strain (P>0.0001).

Overall, it can be concluded that the wild strains AB1, CB3191 and JU322 are susceptible to benzimidazoles and have a phenotype similar to the reference susceptible N2 strain. In support of this, it should be noted here that microsatellite and copy number based studies have previously shown that CB3191 is almost identical to the N2 strain and that it is possibly a laboratory contaminant or escape (Haber, Schüngel et al. 2005; Rockman and Kruglyak 2009; Maydan, Lorch et al. 2010). This further confirms the result obtained for this particular strain.

3.2.2 Albendazole sensitivity of strains CB4853, JU258 and JU263:

The second screen on the NemaRx system included strains CB4853, JU258 and JU263 the results of which are summarized in Tables 3.2A and B and Figure 3.3. As in the previous screen, the median speed of CB3474 [*ben-1(e1880)*] worms were not affected even at the highest concentration and they behaved the same way to those exposed to DMSO only (P=0.3709). Similar results were obtained through size analysis confirming that albendazole did not have an effect on the growth of this strain even at the highest concentration tested (P>0.9999). The median speed and size of N2 worms again showed a dramatic decrease of 76% and 41% at the highest albendazole concentration tested compared to the DMSO controls (P<0.0001) indicating that the drug affects both movement and growth.

Strains CB4853, JU258 and JU263 were all affected by albendazole in a manner similar to N2 worms (Figure 3.3A and B). The median speeds of these wild strains at the highest albendazole concentration were reduced by 80.1%, 80.1% and 80.6% respectively compared to DMSO controls of the same strains (P<0.0001) (Figure 3.3A). Further, the median speeds of these strains at the highest albendazole concentration were not significantly different to the N2 worms ($P>0.9999$ for all three strains), but were significantly different to that of the CB3474 [*ben-1(e1880)*] reference resistant strain ($P<0.0001$ for all three strains).

These wild strains showed 33.3%, 34.6%, 34.6% reductions in size respectively at the highest albendazole concentration compared to DMSO controls of the same strains ($P<0.0001$) (Figure 3.3B). The median sizes of these strains at the highest albendazole concentration were in this case significantly different to both N2 and CB3474 [*ben-1(e1880)*] strains ($P>0.0001$). However, looking at the graphical representation (Figure 3.3B), it is clear that the growth of these wild strains is much more affected than the CB3474 [*ben-1(e1880)*] strain which did not show any reduction in size even at the highest albendazole concentration. In summary, as in the previous screen the results suggest that the wild strains CB4853, JU258 and JU263 are susceptible to benzimidazoles.

3.2.3 Albendazole sensitivity of strains CB4854, CB4856 and CB4858:

The third screen on the NemaRx system included strains CB4854, CB4856 and CB4858 the results of which are summarized in Tables 3.3A and B and Figure 3.4. In this screen although it appeared as though the median speed of CB3474 [*ben-1(e1880)*] worms was being affected by the increasing albendazole concentration (Figure 3.4A) worms of this strain exposed the highest concentration of albendazole behaved similarly to those exposed to DMSO only ($P=0.7864$). The apparent discrepancy in the dose response curve may be due to technical rather than biological reasons. In fact the arbitrary speed values recorded for this day for this strain are lower than those recorded in the previous screens for the same concentrations (see median speed columns for this strain in Tables 3.1, 3.2 and 3.3). The dose-response curve for relative speed in turn is probably a reflection of these low recordings. Size analysis confirmed that albendazole did not have an effect on the growth of this strain even at the highest concentration tested (P=0.1043). The median speed and size of N2 worms again showed a dramatic decrease of 60% and 37% at the highest albendazole concentration tested compared to the DMSO controls (P<0.0001) indicating that the drug affects both its movement and growth.

Strains CB4854, CB4856 and CB4858 were all affected by albendazole in a manner similar to N2 worms (Figure 3.4A and B). The median speeds of these wild strains at the highest albendazole concentration were reduced by 80.4%, 78.2% and 82.6% respectively compared to DMSO controls of the same strains (P<0.0001 in all three cases) (Figure 3.4A). Although it appeared as though the wild strains were more sensitive than N2, the median speeds of these strains at the highest albendazole concentration were not significantly different to the N2 worms (P>0.9999 for all three strains), but were significantly different to that of the CB3474 [*ben-1(e1880)*] reference resistant strain (P>0.0001 for all three strains).

Similarly, these wild strains showed 38.2%, 37.8%, 35.4% reductions in size respectively at the highest albendazole concentration compared to DMSO controls of the same strains (P<0.0001) (Figure 3.4B). The median sizes of these strains at the highest albendazole concentration were not significantly different to N2 (P>0.9999) but, were significantly different to CB3474 [*ben-1(e1880)*] strain (P>0.0001). In summary, the results suggested that the wild strains CB4854, CB4856 and CB4858 are susceptible to benzimidazoles.

3.2.4 Albendazole sensitivity of strains KR314 [*mab-23(e2518)*] and MY2:

The fourth and final screen on the NemaRx system relevant for this chapter was for the KR314 [*mab-23(e2518)*] and MY2 strains the results for which are summarized in Tables 3.4A and B and Figure 3.5. This screen also yielded the most striking outcome in that the movement and growth of wild strains KR314 [*mab-23(e2518)*] and MY2 were not affected by the increasing concentration of albendazole (Figures 3.4A and B).

As in the previous occasions, the median speed of the CB3474 [*ben-1(e1880)*] reference resistant worms at the highest concentration of 0.006mM albendazole compared to the median speed of this strain in DMSO only ($P=0.7605$). However, this strain showed an 11% reduction in size at the highest concentration which was considered statistically significant ($p<0.0001$). Visual observation of plates however suggests otherwise, and so the effect may be due to factors other than drug effects (i.e. worms clumping etc.). The N2 strain showed an 80% reduction in speed and 41% reduction in size at the highest concentration both of which are both significantly different from the worms of this strain exposed to DMSO only ($P<0.0001$).

As previously mentioned the wild strains KR314 [*mab-23(e2518)*] and MY2 were unaffected in terms of speed and size with the increasing albendazole concentration (Figure 3.5A and B). The speed of movement of the KR314 [*mab-23(e2518)*] worms was not affected even at the highest concentration tested ($P>0.9999$) (Figure 3.5A). The same observation was made for the MY2 strain where the speed of worms at 0.006mM albendazole was not significantly different to those exposed to DMSO only ($P>0.9999$) (Figure 3.5A). Similarly, the growth of strains KR314 [*mab-23(e2518)*] and MY2 were not affected by the increasing albendazole concentration (Figure 3.5B) and the worms at the highest albendazole concentration did not significantly differ from the worms of the same strains exposed to DMSO only ($P>0.9999$). In summary, these results demonstrated that the KR314 [*mab-23(e2518)*] and MY2 strains are resistant to benzimidazoles at the dose range tested (0.00075mM to 0.006mM).

Overall, of the eleven wild strains screened on the NemaRx digital imaging system, two *C. elegans* strains KR314 [*mab-23(e2518)*] and MY2 strains displayed phenotypic resistance to benzimidazoles comparable to the reference resistant CB3474 [*ben-1(e1880)*] strain (Table 3.5).

3.3 Confirmation of the benzimidazole resistance phenotype of wild *C. elegans* strains KR314 [*mab-23(e2518)*] and MY2 using the IVT-MS100 system

Biological replicates were set up to verify and more accurately define the resistant phenotype observed in the KR314 [*mab-23(e2518)*] and MY2 strains. Independent assays were set up on different days (three for KR314 [*mab-23(e2518)*] and four for MY2) and worms were screened after albendazole exposure using the IVT-MS100 system. The reason for switching from the NemaRx system to the IVT-MS100 system was because this new system only became available after the initial round of screens and the shorter time taken for the assays (Section 3.4) made it more feasible to undertake multiple assays allowing the generation of replicate data.

It was also considered beneficial to use a different assay system to confirm the resistance phenotype. The output from the IVT-MS100 is in the form of Mean Squared Error (MSE) which reflects total movement within each well of the 96-well plate (Section 2.3.1.2). For the dose response curves 'Relative MSE' was used as the behavioral parameter/ phenotype. The reason for using relative values is the same as discussed for the NemaRx system i.e. to account for inherent differences between the strains. Since the output files are in excel format, preliminary analysis was done on the program itself and statistical analysis was done using GraphPad. Unlike the raw data from the NemaRx system, those from the IVT-100 were mostly normally distributed (Figure 3.6) and thus I could apply parametric tests for these data sets for statistical analysis.

3.3.1 Confirmation of benzimidazole phenotype of the KR314 [*mab-23(e2518)*] strain:

Combined results from three biological replicates for this strain confirmed its benzimidazole resistance phenotype (Figure 3.7). As expected, the movement of worms of the reference resistant CB3474 [*ben-1(e1880)*] strain was not affected by the increasing concentration of albendazole (p>0.05 at all concentrations compared to the DMSO control). The slight increase seen in the dose-response curve from control to first dose of albendazole is not statistically significant (P>0.05). In contrast, the worms of the reference sensitive N2 strain showed an 85% reduction in movement at the highest concentration.

The KR314 [*mab-23(e2518)*] strain followed a similar trend to the reference resistant CB3474 [*ben-1(e1880)*] strain, where the movement of worms at the second highest concentration (0.006mM) was the same as those exposed to DMSO only (P>0.05). The highest concentration of 0.012mM was used in only two of the biological replicates, and is only included in Figure 3.7 for reference but was not included in the comparisons. However, it is clear from the Figure 3.7 that the KR314 [*mab-23(e2518)*] strain is still not affected even at this concentration. A two-way ANOVA test (using strain and concentration as factors) for strains KR314 [*mab-23(e2518)*] and CB3474 [*ben-1(e1880)*] showed that they behave similarly at each concentration (P=0.0643). Taken together, results of the biological replicates confirm that the KR314 [*mab-23(e2518)*] strain is as resistant to benzimidazole drugs as the reference resistant CB3474 [*ben-1(e1880)*] strain.

3.3.2 Confirmation of benzimidazole phenotype of the MY2 strain

Results of four biological replicates confirmed that the MY2 strain is resistant to benzimidazole (Figure 3.8). Except for the one additional biological replicate, the rest were set up alongside the KR314 [*mab-23(e2518)*] strain as discussed above (with the same population of reference strains). Thus, the results for the reference strains are not discussed here to avoid repetition. Once again, similar to the reference resistant CB3474 [*ben-1(e1880)*] strain, the MY2 strain was not affected even at this second highest concentration tested (0.006mM) compared to worms only exposed to DMSO ($P>0.005$). As mentioned for the KR314 [*mab-23(e2518)*] strain 0.012mM was used in only two of the biological replicates as the highest concentration and therefore was not included in the comparisons. However, once again as for the KR314: *mab-23(e2518)* V strain, the MY2 strain was also not affected even at 0.012mM. In addition, a two way ANOVA between the CB3474 [*ben-1(e1880)*] strain and MY2 strains (up to 0.006mM) showed no significance in difference throughout the dose-range tested ($P>0.05$). Collectively, these results confirm that the MY2 strain is as resistant to benzimidazole as the reference resistant CB3474 [*ben-1(e1880)*] strain.

3.4 Discussion

The albendazole exposure assay allowed the phenotypic characterization of eleven wild *C. elegans* strains in terms of their sensitivity to this drug. Overall, two out of the eleven strains tested (KR314 [*mab-23(e2518)*] and MY2) were identified as benzimidazole resistant.

Two different technologies, NemaRx digital imaging system and IVT-MS100 have been employed to characterize the benzimidazole phenotype of wild *C. elegans* strains. The NemaRx system used in the initial round of screening helped to identify the two wild strains (KR314 [*mab-23(e2518)*] and MY2) that showed phenotypic resistance to the benzimidazole drug used (albendazole). The IVT-MS100 system helped to confirm the resistant phenotype of these strains when biological replicates were performed. In order to account for inherent differences between strain in terms of growth rate, food consumption and response to the various components in the assays set up the raw data obtained for the different concentrations for the different strains was normalized to its own control (no drug) conditions.

This work is the first time the NemaRx system has been used in this way and the first time the recently developed IVT-MS100 system has been applied. Each system has its own advantages and disadvantages over the other. The major drawback of the NemaRx system is the time taken to carry out assays. Sample plate preparation for screening on the NemaRx system is very time consuming including washing steps and drying of plates (Section 2.3.2.1). The average scan time for each sample plate is 3 minutes. A typical assay includes 5 strains (3 wild strains and the 2 reference strains), exposed to 6 different drug doses (inclusive of DMSO control, i.e. 0 drug dose). This amounts to 30 sample plates (5X6), which adds up to 90 minutes (3X30) of scan time (for a single experiment). Furthermore, the sample plates need to be switched manually by the operator at 3 minute intervals.

In contrast, the IVT-MS100 offers a less labor intensive higher throughput option with minimal time requirements. Screening can be done in 96-well plate format and there are no special preparatory steps required. Readings are taken from worms suspended in liquid so all that requires to be done is to transfer an aliquot (100 µL) of worms from the assay plate (6-well) into the 96-well plate (Section 2.3.2.2). Also each well (equivalent to a sample plate for the NemaRx system), is only scanned for 3 seconds (as opposed to 3 minutes) and so replicates of the same sample can be analyzed within a considerably lower time period. As an example, considering a typical assay which includes 5 strains, the total scan time here would be 90 seconds (3 technical replicates X6 concentrations X5 strains) as opposed to 90 minutes on the NemaRx. Done in triplicate this will still only be 4.5 minutes as opposed to 270 minutes on the NemaRx system.

However, the NemaRx system is able to generate many more data points (anywhere between 100 to 900 readings per sample) due to the higher scan time, compared to the IVT-MS100 which produces fewer data points (8 per sample). Although acquiring more data points is potentially more powerful in statistical terms, a balance needs to be made between high throughput and statistical accuracy. Thus, in statistical terms a possible strategy would be to do the initial high throughput screens on the IVT-MS100 system and move on to the NemaRx system to confirm positive results.

A valuable feature of the IVT-MS100 system is the auto focus function. This feature is not available in the NemaRx system and thus care needs to be taken to pour NGM agar plates accurately to contain identical depth of each plate to maintain focus. On occasions where the depth is reduced due to drying of agar or errors in pouring, fewer worms will be detected on the surface leading to a loss of data.

In terms of the data generated, the NemaRx system offers the options of acquiring both behavioral (speed of movement) and morphological data (worm length). However, from the results that I have obtained through the screens it appears that behavioral data is able to better distinguish between phenotypes (susceptible vs. resistant) compared to size measurements. This is probably also a manifestation of the effect of the drug itself, in that it may be affecting the motility to a greater extent compared to the growth. Thus, it would be worthwhile focusing just on the behavioral data for future experiments.

For screening on the NemaRx system, worms need to be transferred from a liquid culture onto solid agar plates after several washes. At higher drug concentrations, the sensitive worms tend to clump in the middle of the agar plate where the liquid is first dispensed (Figure 2.2; positions 1-5 marked on template). This limits the number of objects detected upon traversal of the agar plate by the NemaRx system. Such recordings may thus carry less weight and may not be representative of the whole population since only the worms that have moved away from the primary point will be detected as single worms since clumped ones would be considered objects larger than the maximum size set for worm identification and thus not detected. Recordings taken in liquid media do not have this clumping issue and so the IVT-MS100 is advantageous in this respect.

The biggest limitation of the accuracy of the IVT-MS100 is that the MSE values are heavily dependent on worm number. This is because the MSE values are dependent on total amount of pixel changes and not on single worms (Section 2.3.1.2). Therefore, it is extremely important to dispense the same number of worms into each well to ensure that the differences in MSE between wells are solely due to effects of the drug. One approach to mitigate this limitation is to have technical replicates for each dose series and using relative MSE values also helps to cancel out any effects on MSE due to changes in worm number as I have done in my work (Sections 2.3.2.2 and 3.3).

At this point, the IVT-MS100 system is still under development-however, assays carried out on it so far have provided valuable results. There are occasional issues with focusing which can potentially affect the MSE readings taken since the number of objects (worms) picked up will be affected. These issues are currently being addressed alongside efforts to improve optical aspects of the system. A potential consideration for improvement in terms of the software itself would be to incorporate codes that afford the normalization of MSE readings based on the number of worms identified. An alternative approach would be to use a worm sorter to dispense equal numbers of worms into each well. This will address the inevitable differences in worm number caused due to pipetting errors etc. Such an element will guarantee more accurate output which will clear even the minimal ambiguities in the phenotyping experiments. An additional recommendation would be to incorporate basic data analysis tools into the tracking program itself (rather than having to import them into excel or doing them manually) to output summary data for each sample. This feature is potentially useful for high throughput experiments which will help in the avoidance of manual errors (e.g. data mix ups). Considering the need for high throughput screening options in the present day, the IVT-MS100 is definitely preferable in terms of time involved and the ability to carry out multiple screens. Therefore, it will prove to be a very useful system in the future, especially if more strains are to be characterized and also if these assays are to be extended to parasitic worms.

3.5 Chapter 3: Figures and Tables

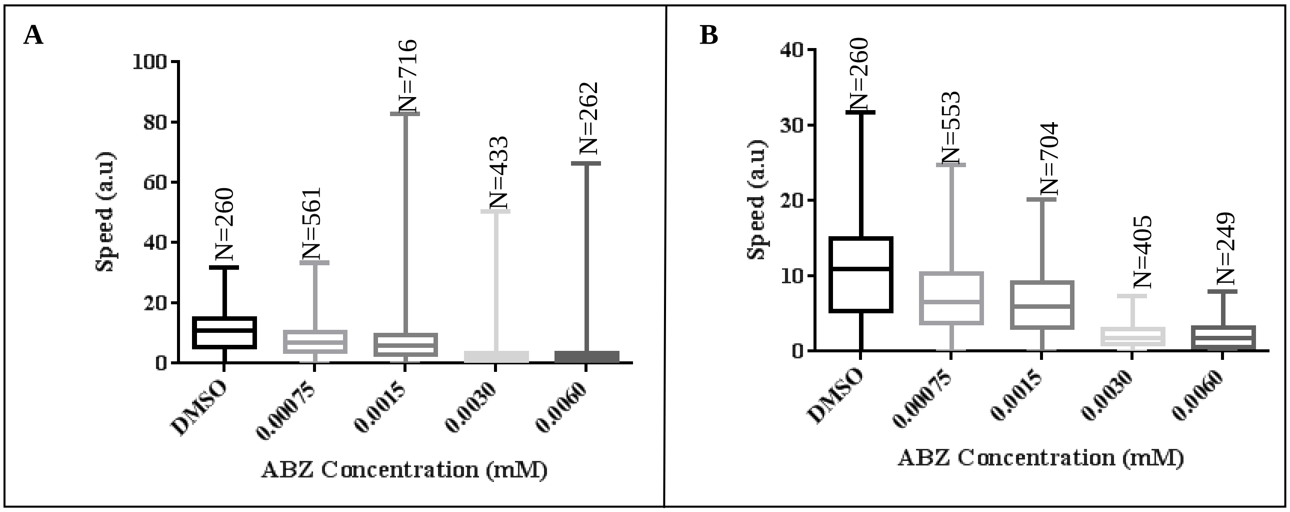


Figure 3.1 Outlier test for speed data acquired using the NemaRx system for the reference susceptible N2 strain. Data is taken from the very first screen carried out on the NemaRx system for this project (Section 3.2.1). Horizontal lines within boxes represent median speed at each concentration. N is the number of values in each data set. Whiskers begin and end at minimum and maximum speed values in the data sets at each albendazole (ABZ) concentration pre (A) and post (B) ROUT outlier test with Q set at 1%**.** It is apparent that the raw data set (A) has speed values that may be biologically irrelevant. The cleaned data set (B) has 61 outliers removed in total and has a more reasonable spread of data. It is also additionally apparent from the plots that the data sets are skewed (not normally distributed) since the whiskers are not of equal size on either side of the boxes.

Table 3.1A Summary of Speed Data from NemaRx system from screening of AB1, CB3191 and JU322 strains

ABZ Concentration (mM)	N2				CB3474				AB1				CB3191				JU322			
	Median Speed (a.u*)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N
0	11.0	100.0	6.9	260	7.8	100.0	5.4	522	8.9	100.0	5.5	88	8.2	100.0	5.2	517	10.2	100.0	5.2	610
0.00075	6.7	60.9	4.8	553	8.2	105.1	5.3	413	8.1	91.5	4.5	237	6.1	74.4	4.2	819	7.3	71.9	4.6	772
0.0015	6.1	55.5	4.1	704	9.3	119.2	5.4	327	6.3	71.2	4.4	285	3.9	47.6	3.3	912	5.8	57.1	4.2	915
0.0030	1.8	16.4	1.7	405	8.6	110.3	5.3	304	2.3	26.0	1.7	426	1.7	20.7	1.5	688	2.4	23.6	2.0	895
0.0060	1.8	16.4	1.8	249	7.5	95.5	4.8	402	2.0	22.6	1.8	474	1.7	20.7	1.6	445	2.1	20.7	1.8	865

*****a.u= arbitrary units
SD= Standard Deviation
N= the number of data points used to calculate median speed (this is not necessarily equal to the number of worms on a sample plate as the same worm may have been tracked more than once by the system).
Relative speed is the median speed at each concentration expressed as a percentage of the median speed under control conditions (i.e. in DMSO).

Table 3.1B Summary of Size Data from NemaRx system from screening of AB1, CB3191 and JU322 strains

ABZ Concentration (mM)	N2				CB3474				AB1				CB3191				JU322			
	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N
0.00000	58.0	100.0	10.4	260	56.0	100.0	9.3	527	55.0	100.0	11.5	88	57.0	100.0	9.8	520	51.0	100.0	8.2	621
0.00075	56.0	96.6	10.0	561	56.0	100.0	8.5	418	60.0	109.1	11.5	237	53.0	93.0	8.8	835	48.0	94.1	8.2	789
0.00150	49.5	85.3	8.6	716	55.0	98.2	8.6	327	54.0	98.2	11.1	289	48.0	84.2	10.4	942	45.0	88.2	9.5	939
0.00300	41.0	70.7	9.3	433	56.0	100.0	8.9	309	45.0	81.8	9.2	444	40.0	70.2	11.2	725	38.0	74.5	10.1	946
0.00600	37.0	63.8	10.7	262	57.0	101.8	8.5	410	41.0	74.5	10.5	489	36.0	63.2	10.0	461	35.0	68.6	10.4	922

*****a.u= arbitrary units
SD= Standard Deviation
N= the number of data points used to calculate median size (this is not necessarily equal to the number of worms on a sample plate as the same worm may have been tracked more than once by the system).
Relative size is median size at each concentration expressed as a percentage of the median size under control conditions (i.e. in DMSO).

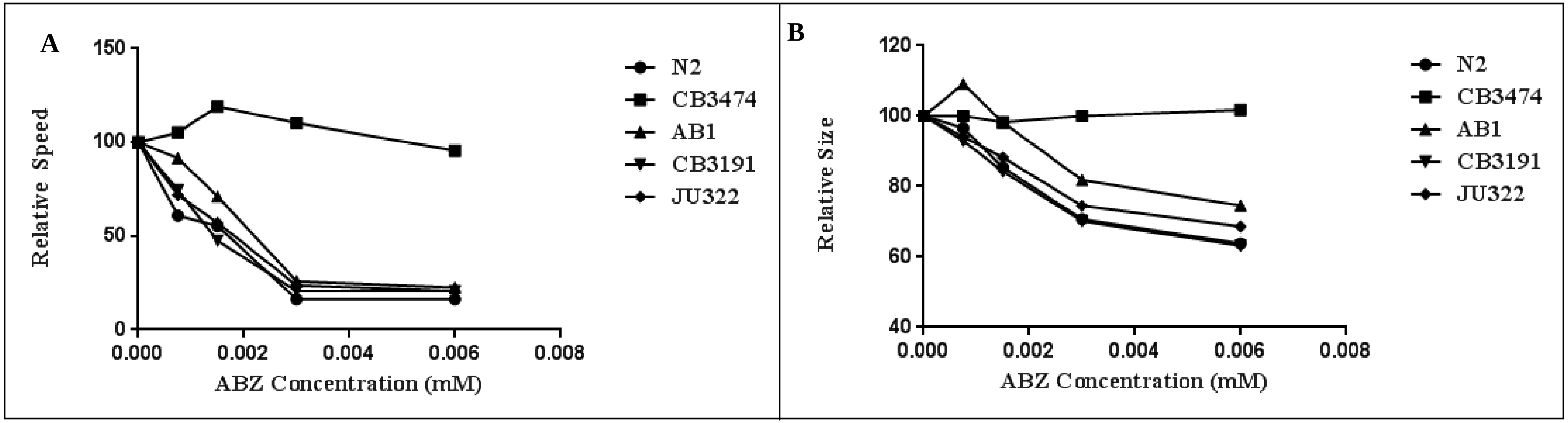


Figure 3.2 Effect of albendazole on movement (A) and size (B) of *C. elegans* strains AB1, CB3191 and JU322. Relative values are the median values at each concentration expressed as a percentage of the median value of the same strain when not exposed to drug. N2 is the reference susceptible strain and CB3474 [*ben-1(e1880)*] is the reference benzimidazole resistant strain. Error bars are not shown as results are from a single experiment.

Table 3.2A Summary of Speed Data from NemaRx system from screening of CB4853, JU258 and JU263 strains

ABZ Concentration (mM)	N2				CB3474				CB4853				JU258				JU263			
	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N
0.00000	8.1	100.0	4.9	333	6.8	100.0	4.8	491	9.05	100.0	5.3	504	11	100.0	6.5	687	10.3	100.0	7.0	687
0.00075	5.4	66.7	4.2	894	7.2	105.9	5.1	437	6.05	66.9	4.0	516	7.45	67.7	4.8	916	7.1	68.9	5.0	921
0.00150	3.1	38.3	2.7	885	7.7	113.2	5.2	814	4.6	50.8	3.7	623	4	36.4	3.3	908	4.6	44.7	4.1	824
0.00300	1.8	22.2	1.7	478	7.2	105.9	5.5	386	2.1	23.2	1.7	770	2.1	19.1	1.8	895	2.3	22.3	2.2	876
0.00600	1.9	23.5	1.8	74	6.5	95.6	4.9	776	1.8	19.9	1.8	839	2.1	19.1	1.9	877	2	19.4	2.0	849

*****a.u= arbitrary units
SD= Standard Deviation
N= the number of data points used to calculate median speed (this is not necessarily equal to the number of worms on a sample plate as the same worm may have been tracked more than once by the system).
Relative speed is the median speed at each concentration expressed as a percentage of the median speed under control conditions (i.e. in DMSO).

Table 3.2B Summary of Size Data from NemaRx system from screening of CB4853, JU258 and JU263 strains

ABZ Concentration (mM)	N2				CB3474				CB4853				JU258				JU263			
	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N
0	51	100.0	7.2	335	57	100	9.2	504	54	100.0	9.3	513	52	100.0	9.5	705	52	100.0	9.4	699
0.00075	46	90.2	10.1	933	57	100	8.9	448	49	90.7	8.0	527	47	90.4	9.7	937	49	94.2	9.2	940
0.0015	41	80.4	9.8	938	57	100	10.8	830	44.5	82.4	9.2	634	42	80.8	10.2	948	45	86.5	8.1	832
0.0030	35	68.6	11.5	506	57	100	10.6	395	39	72.2	8.6	802	36	69.2	10.7	943	38	73.1	9.9	928
0.0060	30	58.8	9.7	78	57	100	11.3	794	36	66.7	9.9	872	34	65.4	11.1	918	34	65.4	10.8	893

*****a.u= arbitrary units
SD= Standard Deviation
N= the number of data points used to calculate median size (this is not necessarily equal to the number of worms on a sample plate as the same worm may have been tracked more than once by the system).
Relative size is median size at each concentration expressed as a percentage of the median size under control conditions (i.e. in DMSO).

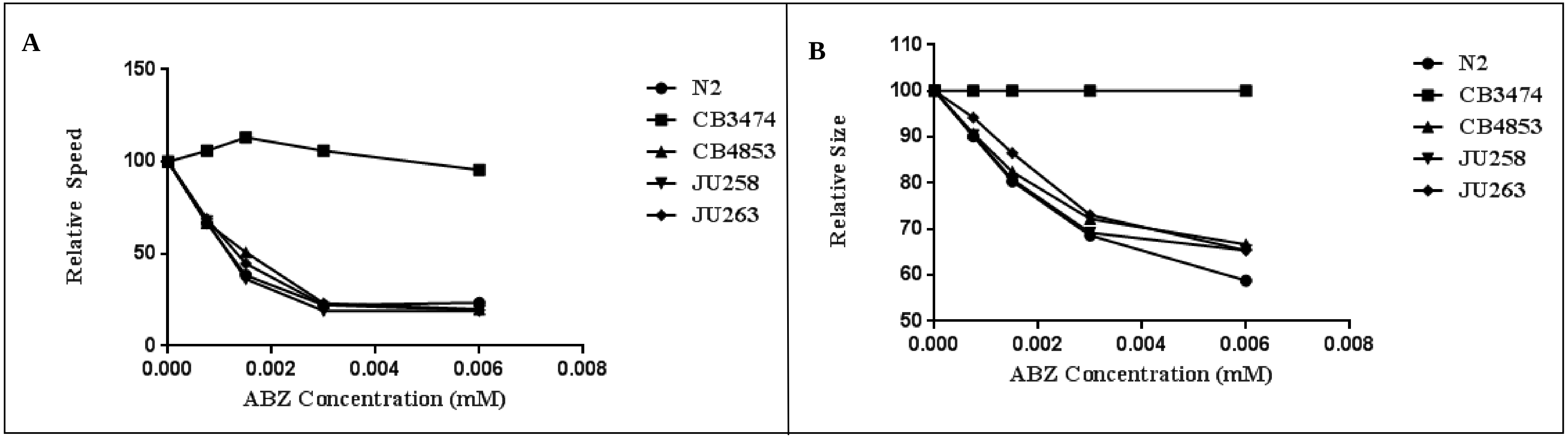


Figure 3.3 Effect of albendazole on movement (A) and size (B) of *C. elegans* strains CB4853, JU258 and JU263. Relative values are the median values at each concentration expressed as a percentage of the median value of the same strain when not exposed to drug. N2 is the reference susceptible strain and CB3474 [*ben-1(e1880)*] is the reference benzimidazole resistant strain. Error bars are not shown as results are from a single experiment.

Table 3.3A Summary of Speed Data from NemaRx system from screening of CB4854, CB4856 and CB4858 strains

ABZ Concentration (mM)	N2				CB3474				CB4854				CB4856				CB4858			
	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N
0	5.0	100.0	5.5	212	5.8	100.0	5.1	245	10.2	100.0	7.0	142	9.7	100.0	7.9	114	11.5	100.0	7.7	162
0.00075	4.2	84.8	3.6	378	7.2	123.3	5.6	190	7.4	72.5	5.6	207	6.0	62.2	4.8	159	7.1	61.7	4.7	102
0.0015	3.1	62.6	3.0	327	5.9	101.7	5.4	267	4.1	40.2	3.8	195	3.2	33.2	2.9	168	4.3	37.4	3.8	134
0.0030	1.8	36.4	1.6	296	4.8	82.8	5.5	176	2.4	23.5	1.7	147	2.0	20.7	1.7	168	2.5	21.7	2.2	133
0.0060	2.0	40.4	1.8	207	4.1	70.7	5.8	224	2.0	19.6	1.8	189	2.1	21.8	1.7	115	2.0	17.4	1.7	230

*a.u= arbitrary units
SD= Standard Deviation
N= the number of data points used to calculate median speed (this is not necessarily equal to the number of worms on a sample plate as the same worm may have been tracked more than once by the system).
Relative speed is the median speed and size at each concentration expressed as a percentage of the median speed under control conditions (i.e. in DMSO).

Table 3.3B Summary of Size Data from NemaRx system from screening of CB4854, CB4856 and CB4858 strains

ABZ Concentration (mM)	N2				CB3474				CB4854				CB4856				CB4858			
	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N
0.00000	65	100.0	18.2	219	70	100.0	18.1	249	68	100.0	15.9	143	67.5	100.0	15.4	114	65	100.0	15.5	162
0.00075	62	95.4	13.9	397	67	95.7	17.4	197	67	98.5	13.5	209	66	97.8	14.6	163	64	98.5	14.2	102
0.00150	54	83.1	10.7	345	72	102.9	17.1	271	54	79.4	11.6	197	52.5	77.8	10.0	174	57	87.7	10.3	134
0.00300	44	67.7	11.1	315	67	95.7	19.4	184	47	69.1	8.4	150	48	71.1	9.3	172	44	67.7	10.6	143
0.00600	41	63.1	11.5	217	66	94.3	18.2	233	42	61.8	7.9	193	42	62.2	9.6	121	42	64.6	8.0	234

*a.u= arbitrary units
SD= Standard Deviation
N= the number of data points used to calculate median size (this is not necessarily equal to the number of worms on a sample plate as the same worm may have been tracked more than once by the system).
Relative size is the median size at each concentration expressed as a percentage of the median size under control conditions (i.e. in DMSO).

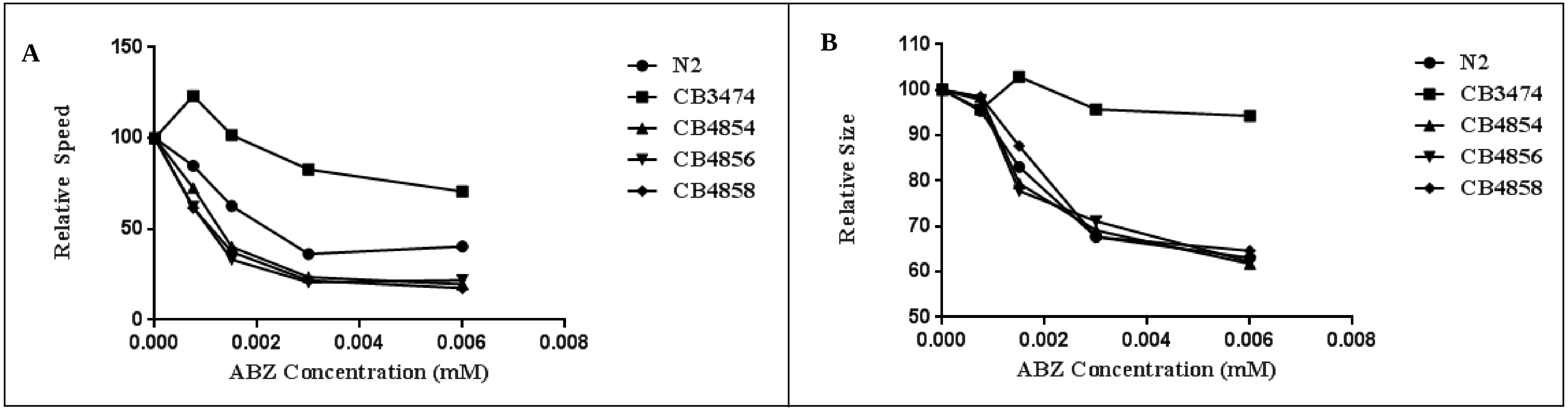


Figure 3.4 Effect of albendazole on movement (A) and size (B) of *C. elegans* strains CB4854, CB4856 and CB4858. Relative values are the median values at each concentration expressed as a percentage of the median value of the same strain when not exposed to drug. N2 is the reference susceptible strain and CB3474 [*ben-1(e1880)*] is the reference benzimidazole resistant strain. Error bars are not shown as results are from a single experiment.

Table 3.4A Summary of Speed Data from NemaRx system from screening of KR314: *mab-23 (e2518)* V and MY2strains

ABZ Concentration (mM)	N2				CB3474				KR314				MY2			
	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N	Median Speed (a.u)	Relative Speed	SD	N
0	10.8	100.0	7.7	468	7.6	100.0	6.8	859	10.4	100.0	7.5	672	12	100.0	7.3	875
0.00075	8.5	78.7	5.7	601	7.5	98.7	6.3	875	10.4	100.0	7.2	570	12.8	106.7	6.9	885
0.0015	4.6	42.6	3.8	765	8.6	113.2	6.5	877	9	86.5	7.7	521	11.5	95.8	7.1	827
0.0030	2	18.5	1.6	762	7.5	98.7	5.9	867	11.1	106.7	7.1	403	11.3	94.2	7.2	750
0.0060	2.2	20.4	1.9	146	7.7	101.3	6.5	851	10.7	102.9	7.1	590	11.7	97.5	7.7	907

*****a.u= arbitrary units
SD= Standard Deviation
N= the number of data points used to calculate median speed (this is not necessarily equal to the number of worms on a sample plate as the same worm may have been tracked more than once by the system).
Relative speed is the median speed at each concentration expressed as a percentage of the median speed under control conditions (i.e. in DMSO).

Table 3.4B Summary of Size Data from NemaRx system from screening of KR314 and MY2strains

ABZ Concentration (mM)	N2				CB3474				KR314				MY2			
	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N	Median Size (a.u)	Relative Size	SD	N
0.00000	64	100.0	13.1	472	57	100.0	12.3	887	51	100.0	11.0	689	55	100.0	11.7	902
0.00075	56	87.5	11.1	615	55	96.5	11.5	917	51	100.0	10.0	583	56	101.8	10.9	909
0.00150	48	75.0	9.4	789	55	96.5	11.1	899	49	96.1	10.7	529	56	101.8	11.2	839
0.00300	40	62.5	10.8	802	54	94.7	11.8	915	53	103.9	9.7	405	53	96.4	11.0	769
0.00600	38	59.4	13.2	161	51	89.5	11.8	877	53	103.9	11.9	603	55	100.0	11.8	920

*****a.u= arbitrary units
SD= Standard Deviation
N= the number of data points used to calculate median size (this is not necessarily equal to the number of worms on a sample plate as the same worm may have been tracked more than once by the system).
Relative size values are median size at each concentration expressed as a percentage of the median size under control conditions (i.e. in DMSO)

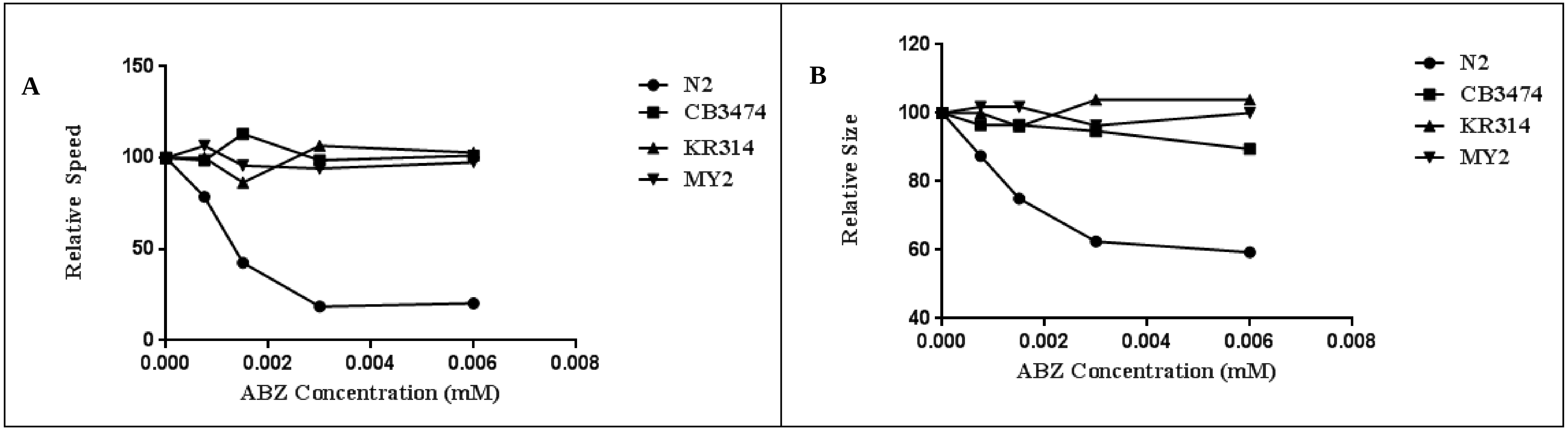


Figure 3.5 Effect of albendazole on movement (A) and size (B) of *C. elegans* strains KR314: *mab-23 (e2518)* V and MY2. Relative values are the median values expressed as a percentage of the median value of the same strain when not exposed to drug. N2 is the reference susceptible strain and CB3474 [*ben-1(e1880)*] is the reference benzimidazole resistant strain. Error bars are not shown as results are from a single experiment.

Table 3.5 Benzimidazole sensitivity of *C. elegans* strains

Strain	Phenotype
N2	**Susceptible**
CB3474: *ben-1 (e1880)*	**Resistant**
AB1	**Susceptible**
CB3191	**Susceptible**
CB4853	**Susceptible**
CB4854	**Susceptible**
CB4856	**Susceptible**
CB4858	**Susceptible**
JU258	**Susceptible**
JU263	**Susceptible**
JU322	**Susceptible**
KR314: *mab -23 (e2518)* V	**Resistant**
MY2	**Resistant**

N2 and CB3474 [*ben-1(e1880)*] strains were used as the reference controls for the benzimidazole exposure assays. Overall, two of the eleven strains tested (indicated in red) were resistant to albendazole (ABZ).

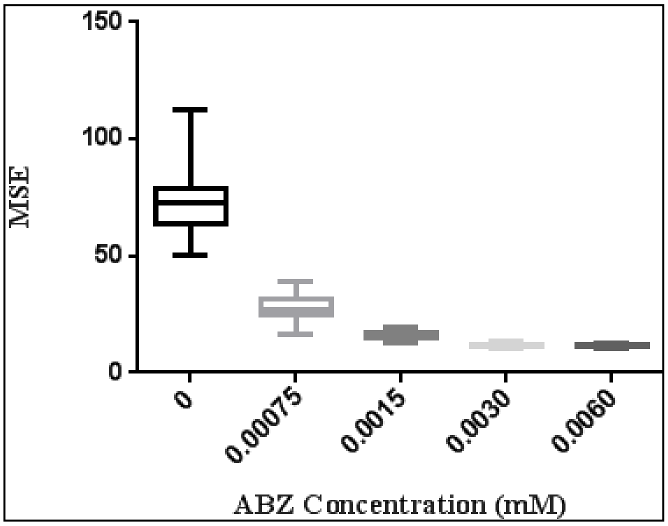


Figure 3.6 Distribution of raw data obtained for the N2 strain from the IVT-MS100 system. The mid-horizontal lines within the boxes indicate average Mean Squared Error (MSE) at each albendazole (ABZ) concentration. Whiskers represent complete range from minimum to maximum MSE values. It is apparent that the raw data is not skewed as in the case of data from the NemaRx system (Figure 3.1). Data is taken from the first three biological replicate assays carried out on this system for this thesis, and at each concentration includes 72 data points (Each assay includes three technical replicates. Each technical replicate gives 8 MSE readings and so 3x3x8=72).

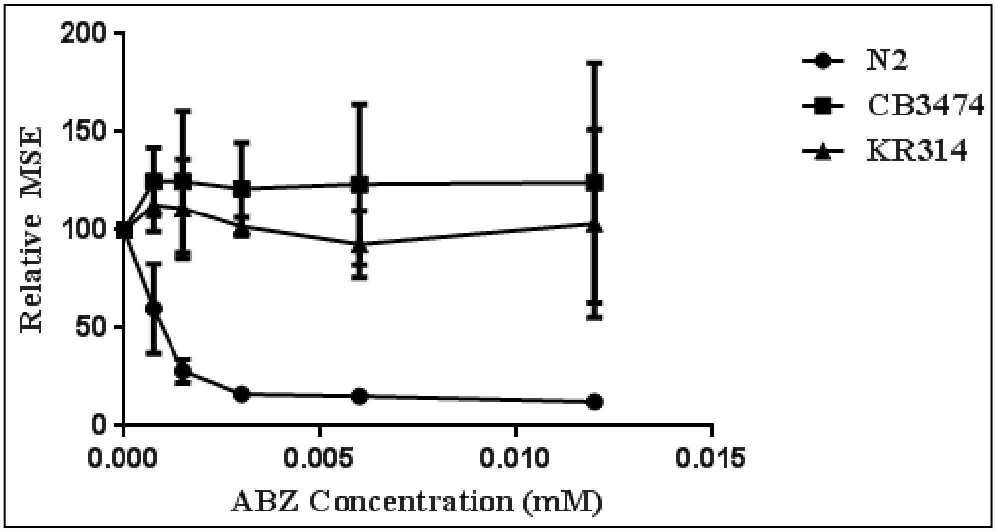


Figure 3.7 Effect of albendazole (ABZ) on the KR314 strain. Phenotyping of worm populations exposed to drug was carried out on the IVT-MS100. Relative MSE is the amount of movement at each concentration expressed as a percentage fraction of that under control conditions. Each data point is the mean of three biological replicates. Error bars represent standard deviation.

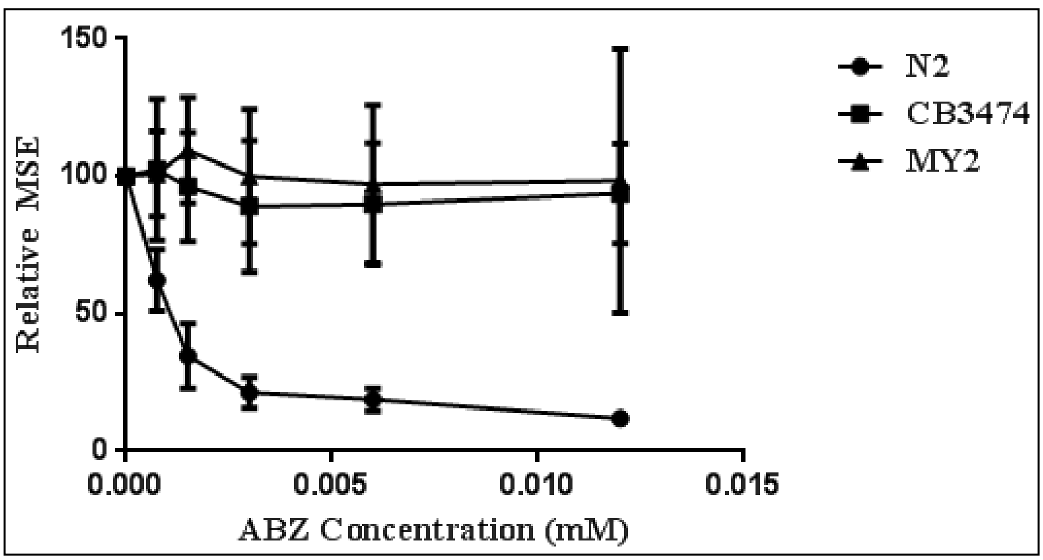


Figure 3.8 Effect of albendazole on the MY2 strain. Phenotyping of worm populations exposed to drug was carried out on the IVT-MS100. Relative MSE is the amount of movement at each concentration expressed as a percentage fraction of that under control conditions. Each data point is the mean of four biological replicates. Error bars represent standard deviation.

Chapter 4: Molecular characterisation of *ben-1* locus in albendazole resistant and susceptible wild *C. elegans* strains

4.1 Introduction

In the previous chapter two wild *C. elegans* strains that were phenotypically resistant to albendazole were identified: The KR314 [*mab-23(e2518)*] strain (isolated from Vancouver, Canada in 1984/85) and the MY2 strain (isolated from Munster, Northwest Germany in 2002). In this chapter, I present the results of experiments to investigate the molecular basis of benzimidazole resistance in these two strains.

A very common mechanism of drug resistance is mutations in the drug target that lead to loss of function. These could be point mutations leading to amino acid substitutions that interfere with drug binding or indels (insertions and deletions) that produce a frame shift that lead to loss of the drug target (Fojo 2007; James, Hudson et al. 2009).

Benzimidazole drugs act by binding to β-tubulin and inhibiting microtubule polymerisation (Figure 1.2) (Lacey 1990). Early studies showed that tubulins were altered in benzimidazole resistant *C. elegans* (Enos and Coles 1990) and later chemically induced mutations conferring benzimidazole resistance in *C. elegans* were mapped to the *ben-1* genomic locus, which codes for this β-tubulin (Driscoll, Dean et al. 1989). Furthermore, mutations in the β-tubulin polypeptide is a common mechanism of resistance both in nematode parasites (Kwa, Veenstra et al. 1994; Silvestre and Cabaret 2002; Rufener, Kaminsky et al. 2009) and fungi (Jung, Wilder et al. 1992; Koenraadt, Somerville et al. 1992; Albertini, Gredt et al. 1999; Ma, Yoshimura et al. 2003). Consequently, the first step in characterising the KR314 and MY2 strains was to sequence the *ben-1* locus to look for potential molecular lesions that could account for the benzimidazole resistance phenotype. Furthermore, I used data from the million mutations project to investigate the effect of additional polymorphisms in the *ben-1* locus of other wild *C. elegans* strains on benzimidazole resistance.

4.2 *C. elegans ben-1* coding sequence (CDS) from benzimidazole resistant strains identified in Chapter 3 (KR314 [*mab-23(e2518)*] and MY2)

The *ben-1* transcript was PCR amplified from cDNA prepared from total RNA extracted from strains N2, CB3474: *ben-1 (e1880)* III, KR314 [*mab-23(e2518)*] and MY2 using primers oJG 211 and oJG 212. The resulting amplicons are predicted to be 1335bp in length according to the *ben-1* coding sequence of the canonical N2 strain (NM_065327). Amplicons of the predicted size were produced (Figure 4.1) and these PCR products were purified and sequenced using primers oJG 211/212.

Firstly, following trimming of the poor quality sequence at the 5' and 3' ends forward (sequenced using oJG211) and reverse fragments (sequenced using oJG212) were assembled *de novo* and then aligned to the N2 reference sequence acquired from GenBank (accession number: NM_065327) (Figure 4.2). From this alignment, single nucleotide polymorphisms (SNPs) in the KR314 [*mab-23(e2518)*] and MY2 sequences with reference to the N2 sequence were identified (Figure 4.3). Since I sequenced the PCR products directly and not following cloning, the chromatograms at the 5' and 3' ends of the sequenced fragments were of poor quality and so I could not obtain the complete *ben-1* coding sequences of the KR314 [*mab-23(e2518)*] and MY2 strains. For the KR314: *mab-23(e2518)*V strain 115 and 300bp from the 5' and 3' ends was not available while for the MY2 strain 70 and 65bp from the 5' and 3' ends was not available (Figure 4.2).

According to the coding sequence that was available from sequencing the cDNA the KR314 [*mab-23(e2518)*] strain had an A/G change at the 206th base pair of its *ben-1* coding sequence (2nd base position of codon 69). Very close to that an A/- single bp deletion at the 210th base pair (3rd base position of codon 70) of the 1335bp long β-tubulin coding sequence exists (Figure 4.3A). Messenger RNA (mRNA) transcripts with a stop codon more than 50bp upstream of the last splice junction are known to undergo nonsense-mediated mRNA decay (Maquat 2002). Therefore this strain possibly lacks the β-tubulin drug target coded for by the *ben-1* gene. The MY2 strain has a G/C change at the 553rd base pair (1st base position of the 185th codon) of its *ben-1* coding sequence (Figure 4.3B). The changes in the KR314 [*mab-23(e2518)*] and MY2 strains are on the 2nd and 3rd exons on the β-tubulin coding sequence respectively and they were the only polymorphisms identified in these strains with respect to the N2 coding sequence.

Translation of the available KR314 [*mab-23(e2518)*] β-tubulin coding sequence showed that, the A/G change at codon 69 leads to a glutamic acid to glycine amino acid change (E69G), while the A/- deletion at codon 70 leads to a frame-shift and thus a premature stop codon at amino acid 119 (whereas the full length polypeptide should be 444 amino acids in length) (Figure 4.4A). This truncated polypeptide in KR314 is shown in Figure 4.8A. The G/C change at the 185th codon of the MY2 strain leads to an alanine to proline (A185P) change in the β-tubulin (Figure 4.4B). The rest of the polypeptide is not affected in this strain (Figure 4.8B).

4.3 *C. elegans ben-1* genomic sequence from benzimidazole resistant strains identified in Chapter 3 (KR314 [*mab-23(e2518)*] and MY2)

In order to confirm the molecular lesions identified in the cDNA of KR314 [*mab-23(e2518)*] and MY2 strains, and also to obtain the complete coding sequences of the *ben-1* locus of these strains, I proceeded to sequence the *ben-1* genomic locus of these strains. In order to obtain the complete coding sequences I designed 4 sets of primers that would include up to 200-300bp of flanking regions of the 5’ and 3’ ends of the *ben-1* locus (Figure 2.9). In addition to the wild strains, I also sequenced the *ben-1* genomic locus of the CB3474 [*ben-1(e1880)*] strain as I was interested in finding out the molecular lesion in this strain as it is the *ben-1* reference allele (this information is not available from WormBase yet as it is still pending curation).

Genomic DNA in crude lysates of the N2, CB3474: *ben-1 (e1880)* III, KR314: *mab-23(e2518)*V and MY2 strains was used as template and PCR amplification was performed using primers designed for the *ben-1* genomic locus (Figure 2.9 and Figure 4.5). The PCR reactions yielded amplicons of the predicted size (Table 4.1 and Figure 4.6). The PCR products were then purified and sequenced using the same primers used for PCR amplification.

Poor quality sequence at the 5' and 3' ends of each fragment were trimmed off and the fragments were directly assembled to the *ben-1* genomic sequence extracted from *C. elegans* cosmid C54C6 (GenBank accession number: Z77131). This was done instead of *de novo* assembling due to lack of overlap in intronic regions between some fragments (Figure 4.7).

Assembly of the genomic DNA sequence fragments of the KR314 [*mab-23(e2518)*] and MY2 strains to the N2 *ben-1* genomic sequence (Figure 4.7) confirmed the single nucleotide changes (SNPs) identified through coding sequence analysis (Figures 4.3 and 4.4). Furthermore, the complete *ben-1* coding sequences of the KR314 [*mab-23(e2518)*] and MY2 strains were obtained and translation of these sequences confirmed the amino acid changes identified through coding sequence analysis (Figure 4.8). In addition to this, the assembly of sequence fragments of the CB3474: *ben-1 (e1880)* strain to the N2 *ben-1* genomic sequence showed that this strain has a glycine (GGT) to aspartic acid (GAT) change at codon 104 (G104D).The complete coding sequences of all above strains are included in Appendix D.

4.4 Sequence variation in the *ben-1* locus for the benzimidazole susceptible strains identified in chapter 3 (AB1, CB3191, CB4853, CB4854, CB4856, CB4858, JU258, JU263 and JU322)

Having sequenced the *ben-1* genomic locus of the benzimidazole resistant wild strains KR314 [*mab-23(e2518)*] and MY2, I was also interested to investigate polymorphisms in those strains studied in Chapter 3 that had a benzimidazole susceptible phenotype. This was facilitated by the million mutations project data becoming available in which 40 wild strains recently had their entire genomes sequenced (Thompson, Edgley et al. 2013). The 40 strains included all 11 of the strains mentioned in the previous chapter. The variation data for all these strains is accessible through a dedicated website for the Million Mutations Project at http://genome.sfu.ca/mmp/ and also through WormBase.

Searching for sequence variation in the *ben-1* locus for the benzimidazole sensitive wild strains studied in this project (AB1, CB3191, CB4853, CB4854, CB4856, CB4858, JU258, JU263 and JU322) at http://genome.sfu.ca/mmp/search.html revealed that except for CB4856 (discussed below), none of these strains harboured non-synonymous polymorphisms at this locus relative to the N2 sequence Furthermore, the only strain where a synonymous change (P80P) was found was in CB4854.

4.5 Identification and phenotypic characterisation of additional wild *C. elegans* strains from the million mutations project that have non-synonymous changes in the *ben-1* gene

When I first became aware of the million mutations project, the dedicated website was not yet complete and so I obtained data directly from one of the lead researchers (Dr. D.G. Moerman, University of British Columbia). Of the data I received for 27 strains I found five strains that harboured non-synonymous polymorphisms in the β-tubulin polypeptide (Table 4.2).

Three of these five strains- JU1171, MY14 and MY16- carry the same A185P amino acid change seen in the MY2 strain. However, new amino acid substitutions were identified in two of the strains. In strain JU1088 an isoleucine was present at position 257 in place of a methionine in the N2 strain (M257I). In strain GXW1, an asparigine was present at position 404 in place of an aspartic acid in the N2 strain (D404N) (Table 4.2).

The benzimidazole resistance phenotype of strains JU1088 (M257I), JU1171 (A185P), MY14 (A185P) and MY16 (A185P) was determined using the NemaRx system (mutations in the β-tubulin drug target are indicated in parentheses). Strain GXW1 was not originally available and so was not tested during these experiments. However, its phenotype was examined in a separate experiment when it later became available (as described in further detail below). Strain MY16 was examined in one experiment along with the N2 and CB3474: *ben-1 (e1880)* controls (Figure 4.9) and the JU1088, JU1171 and MY14 strains were examined in a second independent experiment again alongside the N2 and CB3474: *ben-1 (e1880)* controls (Figure 4.10).

4.5.1 Characterisation of the benzimidazole phenotype of the MY16 strain

The median speed of movement of the CB3474: *ben-1 (e1880)* reference resistant strain was not affected even at the highest concentration tested (0.006mM) compared to the DMSO control ($P>0.9999$). The reference susceptible N2 worms showed a marked reduction in median speed (82%) at the highest concentration compared to worms exposed to DMSO only ($P<0.0001$). The MY16 strain behaved similar to the reference resistant CB3474: *ben-1 (e1880)* strain in that the median speed of movement was not affected by the increasing albendazole concentration (Figure 4.9A). In terms of median size the N2 strain showed the highest reduction (33%) followed by the CB3474: *ben-1 (e1880)* and MY16 strains (11% and 6% reductions respectively) at the highest concentration compared to worms from these strains exposed to

DMSO only (Figure 4.9B). The reduction in size for the MY16 strain was not statistically significant (P=0.3405), but those for the CB3474: *ben-1 (e1880)* and N2 strains were (P<0.0001). However, the effect seen for the CB3474: *ben-1 (e1880)* strain was probably an experimental artefact since the change in median size was very modest compared to N2 (33% vs. 11%). Overall, these observations suggested that the MY16 strain had a similar high level of benzimidazole resistance as the CB3474: *ben-1 (e1880)* strain.

4.5.2 Characterisation of the benzimidazole phenotype of strains JU1088, JU1171 and MY14

The results of the screen for strains JU1088, JU1171 and MY14 on the NemaRx system are shown in Figure 4.10. In this screen, the CB3474: *ben-1 (e1880)* reference resistant control strain showed a 19% drop in median speed at the highest concentration compared to the worms of the same strain exposed to DMSO. This drop however was not statistically significant (P=0.1300). The reference susceptible N2 control worms in contrast showed a 76% reduction in median speed which was statistically significant (P<0.0001). The median speeds of both the JU1171 and MY14 strains were not affected by the drug even at the highest concentration (P=0.6660 and P>0.9999 respectively) and they behaved similar to the CB3474: *ben-1 (e1880)* reference resistant strain (Figure 4.10A). The JU1088 strain showed a 44% reduction in median speed at the highest concentration which was statistically significant (P<0.0001) and appeared to have an intermediate level of sensitivity between the two reference strains (Figure 4.10). Therefore, I decided to confirm the phenotype of this strain through two additional biological replicates (see below).

In terms of median size, the reference resistant CB3474: *ben-1 (e1880)*, JU1171 and MY14 strains were similar to worms of these strains not exposed to drug (Figure 4.10B).

Although there was a lack of consistency in the dose-response curve for relative median size for the JU1171 strain, the strain still more closely resembled the reference resistant strain than the N2 strain. The N2 strain and JU1088 strain showed 23% and 17% reductions in median size respectively at the highest concentration. Inconsistencies were seen in the dose-response curve for the JU1088 strain where there was an initial dip then and increase in median size and then again a gradual decrease displaying almost a zigzag pattern (Figure 4.10B). This may have been due to a mix up in sample plates or other technical issue related to the screening procedure relevant to the NemaRx system. At the highest concentration, size-wise JU1088 worms were more similar to the reference susceptible N2 strain ($P=0.3194$), whereas at 0.003mM it was different to both the reference strains ($P<0.0001$), which again suggested that this strain may possess an intermediate level of resistance. In summary, the results of this screen demonstrated that the JU1171 and MY14 strains had a level of resistance similar to the reference resistant strain CB347: *ben-1 (e1880)* strain and that the JU1088 strain probably had an intermediate level of resistance.

The JU1171 and MY14 strains carry the same A185P mutation (Thompson, Edgley et al. 2013) found in the MY2 strain (Section 4.2) which was identified as having a benzimidazole resistance phenotype in the previous chapter (Section 3.2.4). From this section, it is apparent that they also have the same level of resistance as the MY2 strain, since all of these strains behave similar to the reference resistant CB347: *ben-1 (e1880)* III strain. A recent genome-wide restriction site associated DNA (RAD) marker based phylogenetic study had alluded to the fact that although the strains JU1171 (from Chile), MY14 and MY2 (from Germany) are from distant locations but belonged to the same isotype set (Table 1.2), there may be a case of strain confusion (Andersen, Gerke et al. 2012). To verify this I examined the polymorphisms in genes close to *ben-1* on chromosome III (*unc-45, par-2, dpy-1, smg-6, let-805, mec-12, unc-93, pal-1, lon-1, sma-3*) in these three strains (JU1171, MY2 and MY14). Information regarding the flanking genes were obtained from WormBase (Figure 4.11) and variation data for genes was obtained from the dedicated website for the Million Mutations Project at http://genome.sfu.ca/mmp/.

Interestingly these three strains (MY2, MY14 and JU1171) have the same molecular variations in the genomic loci flanking the *ben-1* gene (Table 4.3). This raises the possibility that they may indeed be the same strain, although broadening this search to include more genes on other chromosomes would be beneficial. Also, although there is a possibility that MY2 and MY14 may be the same strain, it is unlikely that the JU1171 strain is since they were isolated by different people at different times; 4-5 years apart in different locations (Caenorhabditis Genetics Center; Andersen et al 2012). Thus, these results confirm that the naturally occurring A185P mutation in β-tubulin can give benzimidazole resistance comparable to the reference resistant CB3474 [*ben-1 (e1880)*] strain.

4.5.3 Confirmation of benzimidazole phenotype of the JU1088 strain

Results from the first phenotyping experiment carried out for the JU1088 strain suggested that it may have an intermediate level of resistance in between the two reference strains N2 and CB3474 [*ben-1(e1880)*] (Figures 4.10 and 4.12A). Therefore, two additional biological replicates were set up for this strain alongside the reference strains and the results of the screens on the NemaRx system are shown in Figures 4.12B and C.

In the first of these experiments (Figure 4.12B), the median speed of the reference resistant CB3474 [*ben-1(e1880)*] strain, was not affected at the highest concentration compared to worms exposed to DMSO only ($P>0.9999$), but the reference susceptible N2 strain showed a significant 85% reduction in median speed at the highest concentration ($P<0.0001$). The JU1088 strain displayed a gradual decrease in median speed with the increasing albendazole concentration but, at the highest concentration there was a small increase. At 0.0075mM there is an increase in the median speed of movement for this strain which was significantly different to worms just exposed to DMSO ($P = 0.003$). This effect was also seen with the reference resistant strain but in that case the difference was not significant ($p=0.4746$). This may possiblybe due to a residual effect of DMSO and it is also possible that the effect of DMSO on the strains varies. Since it was an increase and not decrease in speed it is likely that the JU1088 strain was not affected at 0.00075mM. Following that, up to 0.003mM, the worms of the JU1088 strain behaved similarly to worms of this strain not exposed to drug ($P=0.8771$ and $P>0.9999$ for 0.0015mM and 0.0030mM respectively). However, at 0.0045mM and 0.0060mM the median speed was decreased by 30% and 13% respectively which was significantly different to worms exposed to DMSO only ($P<0.0001$ and $P=0.0403$ respectively). It seemed that the worms at the highest concentration (0.006mM) were affected to a lesser extent than those at the 2nd highest (0.003mM) concentration; however, at the highest concentration the JU1088 worms were significantly different to the CB3474: *ben-1 (e1880)* strain ($P=0.0003$) which suggested that the JU1088 strain had a lower level of resistance compared to the reference strain.

In terms of median size the N2, CB3474: *ben-1 (e1880)* and JU1088 strains showed 35%, 0% and 18% reductions at the highest concentration compared to the DMSO controls. Looking at the dose-response curves for relative median size (Figure 4.12B) it was apparent that the JU1088 strain was moderately affected since its curve clearly fell in between the two reference strains. The reduction in median size for the N2 and JU1088 strains were considered statistically significant compared to worms of these strains exposed to DMSO only ($P<0.0001$) but they were not significantly different to each other at the highest concentration (P>0.9999). Even at lower concentrations the JU1088 strain was affected the same way as N2 (P>0.9999) in terms of size. Hence it is possible that in terms of growth, the magnitude of effect on the drug was same on the N2 and JU1088 strains.

For the second experiment in addition to the two reference strains, I also included the MY2 strain identified as benzimidazole resistant in the previous chapter for comparison. From the dose response curves (Figure 4.12C) it was apparent that the MY2 strain behaved in a similar fashion to the reference resistant CB3474: *ben-1 (e1880)* strain while the drug had slightly more effect on the movement of the JU1088 strain indicative of its lower level of resistance. The worms of the JU1088 strain exposed to DMSO and up to a concentration of 0.0015mM were not significantly different to each other (P=0.5405 at 0.00075mM; P>0.9999 at 0.0015mM), however beyond 0.0015mM the worms were significantly affected and showed a 32% reduction in median speed at the highest concentration (P<0.0001). Furthermore, the JU1088 strain behaved significantly different to both the reference strains at every single concentration tested (P<0.0001). Combined with the dose-response curves for speed these statistical inferences confirmed the intermediate level of resistance of the JU1088 strain.

Dose-response curves for relative median size data provided a similar suggestion as to the intermediate level of resistance of the JU1088 strain (Figure 4.12C). Up to 0.0015mM the JU1088 strain was not affected by the drug (P>0.9999) but beyond that there was a significant effect with a 12% reduction at the highest concentration. The CB3474: *ben-1 (e1880)* and MY2 strains were not affected by the drug in terms of median size even at the highest concentration compared to DMSO exposed worms (P>0.9999) while the N2 strain was (P<0.0001). Furthermore the JU1088 strain significantly differed from the N2, CB3474: *ben-1 (e1880)* and

MY2 strain at the highest drug concentration ($P<0.0001$, $P=0.0145$ and $P<0.0001$ respectively). Thus, the results of this second screen also validated the primary observation regarding the intermediate level of resistance of the JU1088 strain.

Hence, in this section I verified the intermediate level of resistance of the JU1088 strain and demonstrated that naturally occurring mutations in the β-tubulin drug target, A185P and M257I give varying levels of benzimidazole resistance-the former higher than the latter.

4.5.4 Albendazole sensitivity of strain GXW1

Pilot experiments carried out for the GXW1 strain on the NemaRx system indicated that it may be susceptible to benzimidazole (Figure 4.13). However, phenotypic data from a separate experiment carried out on the IVT-MS100 system suggested that whilst the GXW1 (D404N) strain was not highly resistant to the drug, it may not be as susceptible as N2 (Figure 4.14). To investigate this further, the dose-response curves for GXW1 strain were repeated using three independent biological replicates on the NemaRx system alongside the reference susceptible N2 strain and this time the JU1088 was used as the resistant control (Figure 4.15). The JU1088 strain was used as the resistant control in this case because it shows an intermediate level of resistance between the CB3474 [*ben-1(e1880)*] and N2 references and was considered likely to help define the level of resistance of the GXW1 strain (Figure 4.14).

In the first experiment of the series of three biological replicates (Figure 4.15A), all three strains (N2, GXW1 and JU1088) showed a decrease in median speed of movement with increasing albendazole concentration with significant 84%, 66% and 36% reductions in speed respectively at the highest concentration (0.006mM) compared to worms of the respective strains exposed to DMSO only ($P<0.0001$ for all three strains). The dose-response curve for the GXW1 strain fell intermediate to both the N2 and JU1088 strains. Statistical analysis supported this observation in that the GXW1 strain was not significantly different to the JU1088 strain at 0.00075mM but differed significantly to both N2 (including at 0.00075mM) and JU1088 strains at every concentration thereafter (except at 0.003mM where $P=0.7287$ compared to JU1088 where there was a dip in the JU1088 curve at this point). Hence, this experiment supported the preliminary observation of the intermediate level of resistance for the GXW1 strain between the N2 and JU1088 strains (Figure 4.14).

In the second experiment (Figure 4.15B) all three strains showed a decrease in median speed of movement with increasing albendazole concentration. The N2, GXW1 and JU1088 strains showed 86%, 56% and 48% decrease at the highest concentration compared to worms of the respective strains exposed to DMSO only ($P<0.0001$). The dose-response curve for the GXW1 strain once again fell in between those of the N2 and JU1088 strains and was significantly different to the N2 strain at all concentrations tested ($P<0.0001$). Comparison of GXW1 and JU1088 strains at 0.0045mM and 0.006mM did not yield significance ($P=0.4419$ and $P>0.9999$ respectively) possibly due to lack of differentiation of the GXW1 strain beyond 0.003mM (curve had reached a plateau). Since the dose-response curves of these strains did not overlap (Figure 4.15B) these results could be taken to support the hypothesis that the GXW1 strain had a lower level of resistance compared to the JU1088 strain.

In the third and final experiment (Figure 4.15C), the speed of movement of strains N2, GXW1 and JU1088 decreased with increasing albendazole concentration by 74%, 58% and 35%, all significantly different from worms of these strains exposed to DMSO only ($P<0.0001$). The dose-response curve of the GXW1 strain once again fell in between those of the N2 and JU1088 strains. The GXW1 strain was significantly different to the N2 strain at every concentration tested ($P<0.0001$). This strain was similar to the JU1088 strain up to a concentration of

0.0045mM albendazole. At 0.006mM the GXW1 strain was affected somewhat more than the JU1088 strain (P=0.0054). Thus, this experiment suggested that the GXW1 strain had the same level of resistance as the JU1088 strain up to a concentration of 0.0045mM albendazole but beyond this it is possibly more susceptible.

Dose-response curves for size from the three biological replicates showed that on average the N2, GXW1 and JU1088 strain showed a 34%, 17% and 14% respective reductions in size at the highest concentration (0.006mM). Since the difference for GXW1 and JU1088 strains was narrow in terms of size, I did not used this data here to distinguish the level of resistance between these strains.

Taking the results of the three experiments together (Figure 4.15A, B and C), it was apparent that the GXW1 strain most likely had a level of benzimidazole resistance below the JU1088 strain. The fact that the mutation in the β-tubulin of the GXW1 strain is at the C-terminal (D404N) which has no direct involvement in drug binding could be the reason for this apparent lower level of resistance. Phenotyping at a lower concentration range may prove fruitful in obtaining clearer results.

Compiling of phenotypic data for all resistant wild *C. elegans* strains identified in this study (Figure 4.16) showed that strains KR314: *mab-23(e2518)* V, MY2, JU1171, MY14 and MY16 had levels of resistance similar to that of the reference resistant CB3474 [*ben-1(e1880)*] strain while the JU1088 strain has an intermediate level of resistance between the two reference strains (N2 and CB3474: *ben-1 (e1880)*). The GXW1 strain had an even lower level of resistance, falling in between the reference susceptible N2 strain and the JU1088 strain.

4.6 Effect of non-synonymous mutations in the *ben-1* gene on the secondary structure of β-tubulin

A preliminary analysis was undertaken to assess the possible structural consequences of the amino acid substitutions identified above using the secondary structure predication function of the Geneious software (Figure 4.17). The A185P mutation (in strains MY2, MY14, MY16 and JU1171) causes an α-helix in this region to disappear and is replaced by a coil-turn-β strand-coil motif (Figure 4.17A). Furthermore, the coil preceding this motif is also pushed towards the left due to the introduction of this motif in place of the α-helix. The M257I mutation (in the JU1088 strain) increases the length of the β strand and introduces an extra coil in this region (Figure 4.17B). Furthermore it also causes a very short α-helix in this region to disappear. Therefore, these alterations in the presence and positions of the secondary structural elements in these strains relative to the N2 stain are likely to also affect the specific folding of the β-tubulin protein (also see Chapter 6). The differences in the levels of benzimidazole resistance seen in the MY2 and JU1088 strain may be explained by the relative importance of the specific regions which are altered in these strains in drug binding.

Since I did not sequence the *ben-1* locus of the GXW1 strain, the complete amino acid sequence for the β-tubulin protein of this strain was obtained by replacing the 404th residue of the N2 sequence as per data obtained from the Million Mutations Project website (http://genome.sfu.ca/mmp/). The D404N substitution (in the GXW1 strain) does not seem to affect the structure of the protein at a secondary level as much as the previous two cases. The only notable difference in this sequence relative to N2 is that the α-helix in the region is reduced by one amino acid (Figure 4.17C). Furthermore, this substitution occurs in the C-terminal region of the protein (which is also highly variable) which appears to have no role in benzimidazole binding (Robinson, McFerran et al. 2004). This may well explain the much lower level of resistance observed for this strain compared to the strains carrying the A185P or M257I mutations (Section 4.4.1.3 and Figure 4.15). Similar predictions were also done for the reference resistant CB3474: *ben-1 (e1880)*III strain and the result is included in Appendix E. Secondary structure predictions were also done for the *H. contortus* isotype-1 β-tubulin sequence into which the substitutions most encountered in the field (F200Y, E198A and F167Y) were introduced by residue replacement. These results are also included in Appendix E.

4.7 Identification of wild *C. elegans* strains with deletion polymorphisms in the *ben-1* gene from the Million Mutations Project

Examination of the sequence data of the 40 wild type strains from the Million Mutations Project identified four strains (CB4856, ED3021 MY6 and JU360) that carry deletions in the *ben-1* coding sequence (Table 4.4). The CB4856 strain was phenotyped previously in this project (Section 3.2.3) but the ED3021, MY6 and JU360 strain were not.

Even though the CB4856 strain has an in frame deletion in the ben-1 locus it has a benzimidazole susceptible phenotype (Figure 3.4 and Section 3.2.3). However, large deletions and nonsense alleles in the *ben-1* locus have been previously shown to give benzimidazole resistance (KR314 [*mab-23(e2518)*] strain identified in this project and Driscoll et al. 1989 in chemically mutagenised strains). To confirm that there was no cross contamination of strains, an independent sample of this strain was obtained from Dr. Paul Mains, University of Calgary and its phenotype assessed by the IVT-MS100 system after exposure to albendazole (0.0015mM and 0.0060mM) alongside the reference susceptible N2 strain.

Results of this experiment confirmed the susceptibility of the CB4856 strain (Figure 4.18). The CB4856 strain had a lower MSE value measurement (probably due to low worm number or objects identified by the IVT-MS100) under control conditions (worms exposed to DMSO). This is reflected in the higher relative values for the two albendazole concentrations compared to the N2 strain. This difference however, is not statistically significant ($p>0.05$). In addition to this, upon direct observations, the CB4856 strain displayed similar behaviour to the N2 strain in the DMSO control as well at the two drug concentrations used. Thus, it was confirmed that the CB4856 strain is susceptible to benzimidazoles.

The phenomenon of the presence of a small in-frame *ben-1* deletion with no benzimidazole resistance can be explained by the fact that the deletion in the CB4856 strain falls in the C-terminus of the β-tubulin drug target; deletion of amino acids 440-442 (total size of peptide is 444 amino acids). Since the C-terminal is highly variable certain substitutions may not have a large impact on the protein structure and function. In addition, as mentioned previously, nonsense mediated RNA decay only occurs when the deletion is >50bp upstream of the last splice junction which is not the case for this strain. This observation is further supported by the fact that in yeast, deletion of amino acid residues downstream of position 431 leads to benzimidazole super-sensitivity as opposed to resistance (Matsuzaki, Matsumoto et al. 1988).

4.8 Discussion

The probable causal mutations of benzimidazole resistance in the two wild *C. elegans* strains KR314 [*mab-23(e2518)*] and MY2 were identified in this chapter by sequencing the *ben-1* locus of these strains. The *ben-1* locus of the KR314 [*mab-23(e2518)*] strain harbours a null allele which results in a truncated transcript, which is most likely degraded by the RNA decay machinery of the cell. Therefore, this strain may be lacking the *ben-1* β-tubulin drug target. This result is in agreement with a previous study that utilised chemically mutagenised *C. elegans* strains and identified that deletion alleles at the *ben-1* locus conferred benzimidazole resistance to these strains (Driscoll, Dean et al. 1989). The MY2 strain harbours a G/C SNP at the *ben-1* locus which leads to an alanine to proline change at amino acid 185 of the β-tubulin polypeptide (A185P). Both these lesions are also confirmed by million mutations project data and the *ben-1* alleles in these strains have been denoted *cewivar00796699* (for the deletion in KR314) and *cewivar00420700* (for MY2).

Also from million mutations project data, three additional strains (JU1171, MY14 and MY16) were identified which have the same *ben-1* allele as the MY2 strain (*cewivar00420700*) and so the same A185P amino acid substitution. These strains also have the same level of phenotypic resistance the MY2 strain (as well as the KR314: *mab-23(e2518)* V).

Additionally, two other amino acid substitutions were identified which can give medium to low levels of benzimidazole resistance to those mentioned previously. They are a M257I change in the JU1088 strain and a D404N change in the GXW1 strain. The benzimidazole phenotypes of all these strains are summarised in Figure 4.16. These findings show that mutations in different parts of the β-tubulin drug target can give varying levels of phenotypic resistance possibly reflecting the differences in importance of these regions for drug binding.

Secondary structure predictions revealed that the A185P, M257I and D404N substitutions cause changes in the structural elements of the β-tubulin protein to varying levels (Figure 4.17). Homology modelling and docking studies will prove fruitful in further understanding the three dimensional structural consequences of the changes and the potential effects on drug binding (Robinson, McFerran et al. 2004). Further discussion on possible structural consequences of mutations in the β-tubulin drug target is included in Chapter 6. The functional significance of these changes is investigated in the next chapter.

4.9 Chapter 4: Figures and Tables

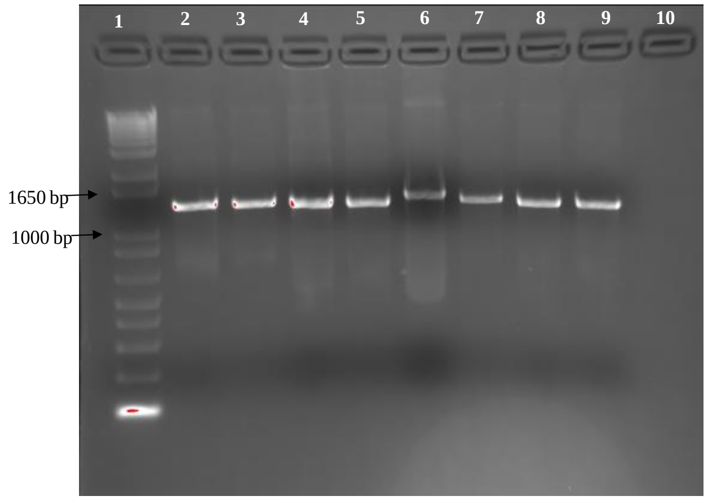


Figure 4.1 PCR amplification of *ben-1* cDNA from *C. elegans* strains. Primers oJG 211 and oJG 212 were used for amplification of cDNA. Expected product size is 1335bp. Lane 1: Invitrogen™ 1kb plus ladder. Lanes 2-9: amplification products (cDNA synthesis reactions were carried out in duplicate). Lane 2 &3: N2; lane 4&5: CB3474: *ben-1 (e1880)* III; lanes 6&7: KR314: *mab-23 (e2518)* V and lanes 8&9: MY2. Negative and positive controls were run on a separate gel (image not taken)**.**

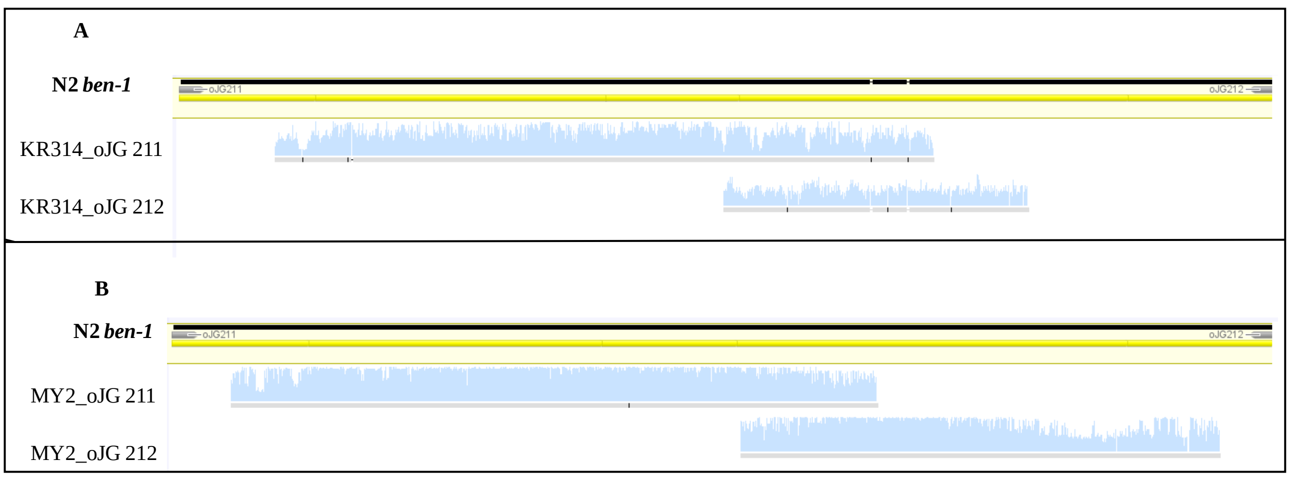


Figure 4.2 *ben-1* β-tubulin coding sequence assembly for *C. elegans* wild strains KR314 (A) and MY2 (B) and subsequent alignment to the canonical N2 sequence (NM_065327). Fragments are named (left) according to the primer used to sequence the amplicons. Annealing sites of the primers oJG 211 and oJG 212 are indicated by grey annotations (between yellow and black lines). After trimming poor quality ends, the oJG 211 and 212 fragments were *de novo* assembled and then aligned to the N2 sequence. Parts of the 5' and 3' regions of the KR314 and MY2 sequences are missing as the poor quality chromatograms were trimmed off. The complete sequences are included in the Appendix D. The snapshots are taken from Geneious software where the sequence analyses were performed.

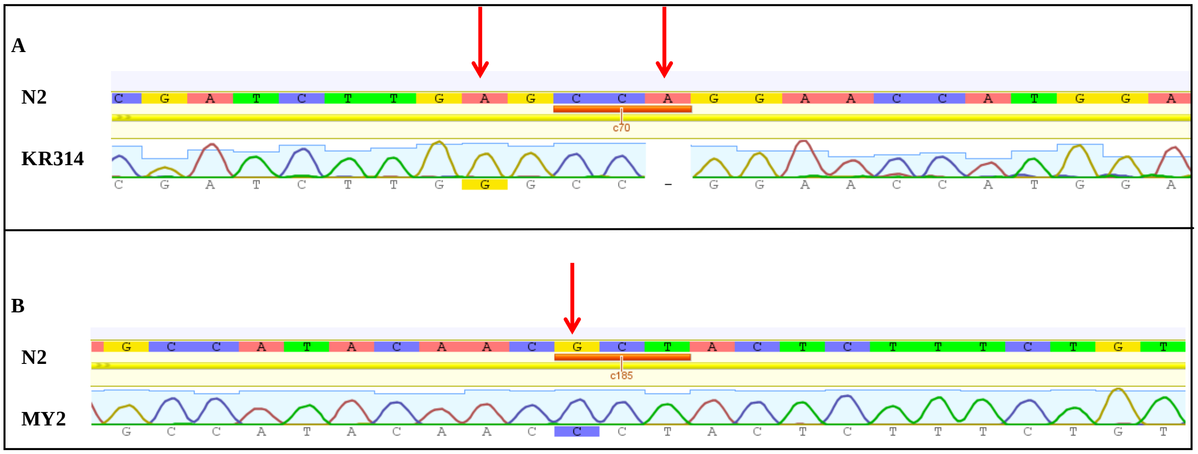


Figure 4.3 Non-synonymous polymorphisms in the *ben-1* coding sequence of wild *C. elegans* strains resistant to benzimidazole relative to the canonical N2 sequence. Only a region of the coding sequence (exon 2 for KR314 and exon 3 for MY2) where the non-synonymous changes occur are shown (indicated by red arrow). (A) KR314: single nucleotide change (A/G) at codon 69 and one base pair deletion (A/-) at codon 70 (c70) in exon 2 of the *ben-1* coding sequence. The deletion at codon 70 in the KR314 strain leads to a premature stop at codon 119 (not shown here; see Figure 4.4) (B) MY2: single nucleotide change (G/C) at codon 185 (c185) in exon 3 of the *ben-1* coding sequence. The complete *ben-1* coding sequences are included in Appendix D.

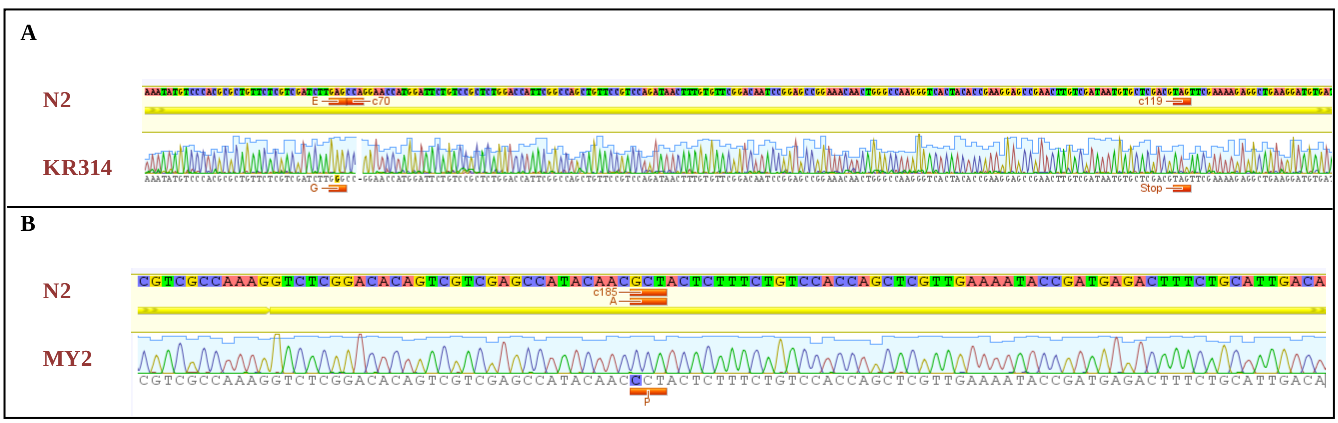


Figure 4.4 Amino acid changes in the β-tubulin drug target of wild *C. elegans* strains KR314 (A) and MY2 (B) resistant to benzimidazole. The specific changes and codons (c) are indicated by orange annotations. The KR314 strain has a single nucleotide deletion (A/-) at codon 70 (c70) which leads to a frame-shift resulting in a premature stop at codon 119 (c119). In addition, it also has a glutamic acid (E) to glycine (G) change at codon 69. The MY2 strain has a single nucleotide change (G/C) that leads to an alanine to proline change (A/P) at codon 185 (c185). The snapshots are taken from Geneious software where the sequence analyses were performed.

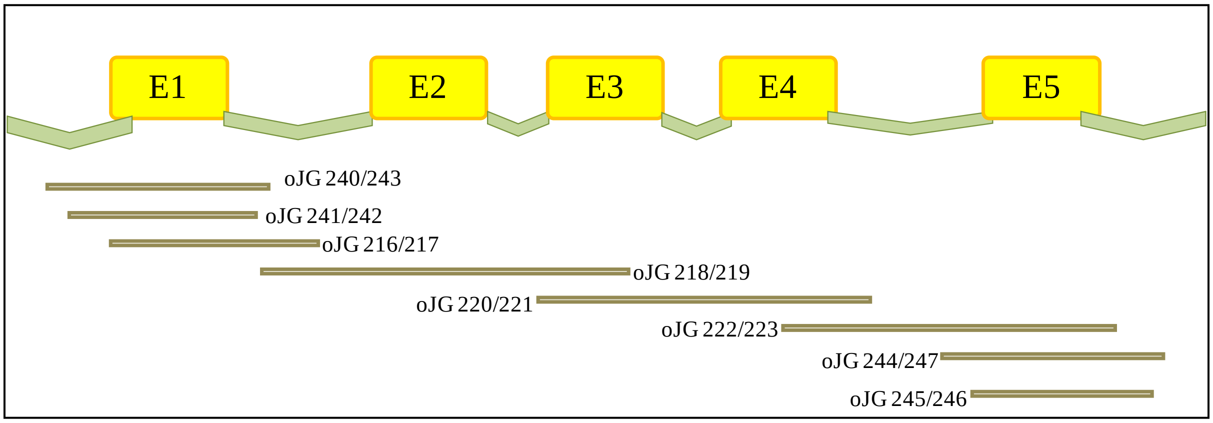


Figure 4.5 Schematic representation of fragments resulting from PCR amplification of the *ben-1* genomic locus of *C. elegans*. Yellow boxes E1-E5 represent the exonic regions and green regions connecting the exons represent introns. The primer pairs (prefixed 'oJG') used to amplify each region are indicated next to the amplicons. Primers were designed such that there will be at least a 60-70bp overlap between the amplicons. Primers oJG 216-223 were the very first set to be designed while oJG 240-243 and oJG 244-247 were designed later on to amplify the 5' and 3' flanking regions to obtain the complete sequence of the *ben-1* locus.

Table 4.1 Expected product sizes from PCR amplification of *C. elegans* genomic DNA using primer pairs designed for the *ben-1* genomic locus

Primer Pair	Expected PCR product size (bp)
oJG 240/243	582
oJG 241/242	280
oJG 216/217	950
oJG 218/219	870
oJG 220/221	867
oJG 222/223	756
oJG 244/247	695
oJG 245/246	318

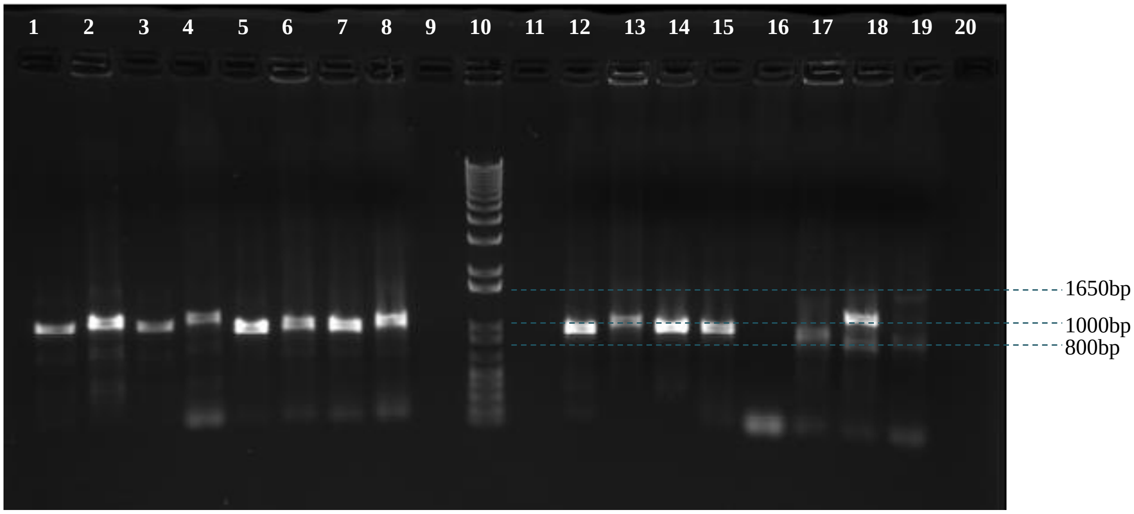
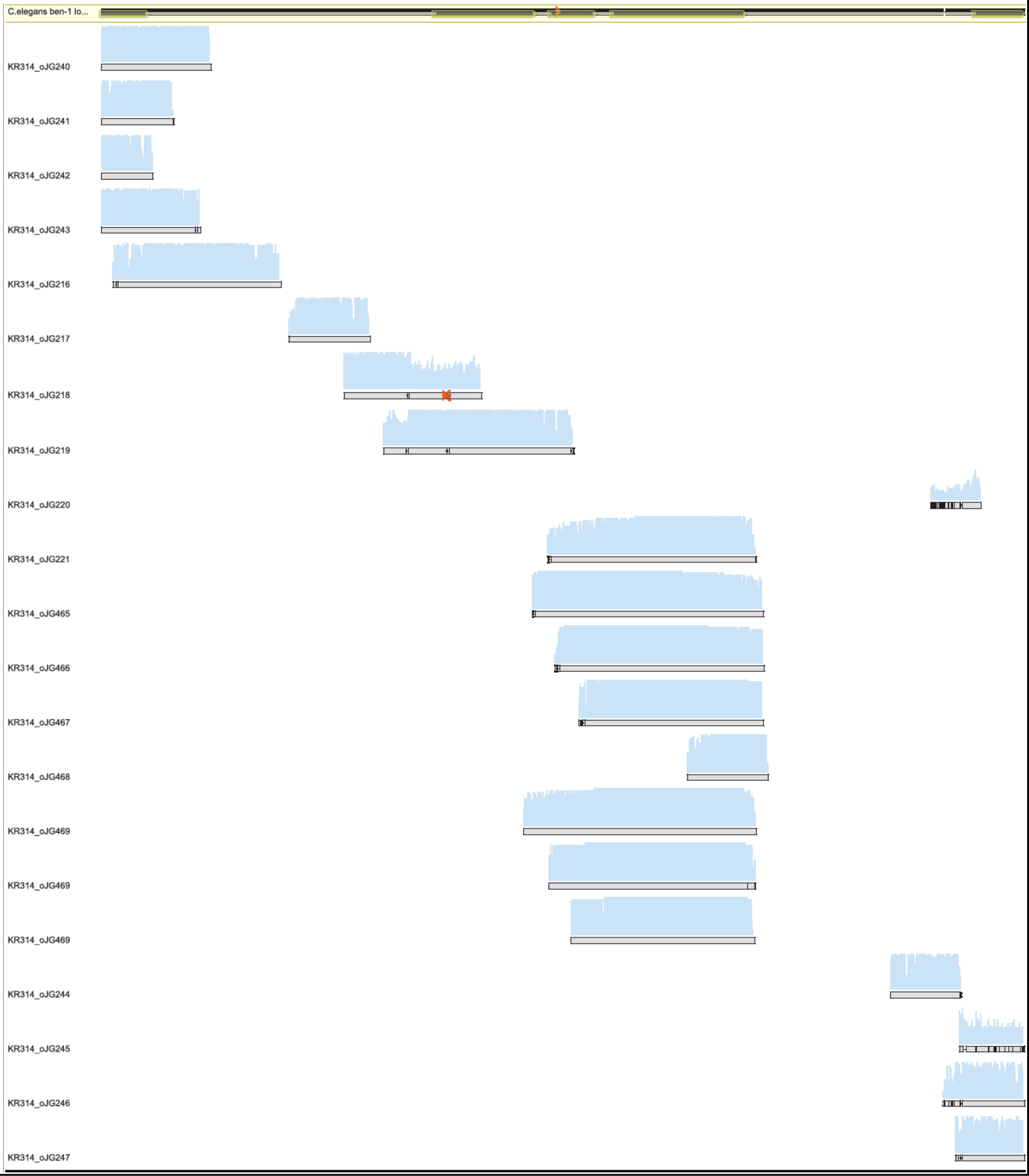


Figure 4.6 PCR amplification of the *ben-1* genomic locus of *C. elegans* strains. Genomic DNA lysate amplified using primer sets- oJG 216/217: Lanes 1-4 (1:N2, 2:CB3474, 3:KR314 and 4:MY2), oJG 218/219: Lanes 5-8 (5:N2,6:CB3474, 7:KR314 and 8: MY2), oJG 220/221: Lanes 12-15 (12:N2, 13:CB3474, 14:KR314 and 15: MY2) and oJG 222/223: Lanes 16-19 (16:N2, 17:CB3474, 18:KR314 and 19: MY2). Lane 10: Invitrogen 1kb plus ladder. Lanes 9, 11 and 20 are empty. Negative controls for each primer set were run on a separate gel (image not available).

A


C.elegans ben-1 lo...
KR314_oJG240
KR314_oJG241
KR314_oJG242
KR314_oJG243
KR314_oJG216
KR314_oJG217
KR314_oJG218
KR314_oJG219
KR314_oJG220
KR314_oJG221
KR314_oJG465
KR314_oJG466
KR314_oJG467
KR314_oJG468
KR314_oJG469
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KR314_oJG244
KR314_oJG245
KR314_oJG246
KR314_oJG247

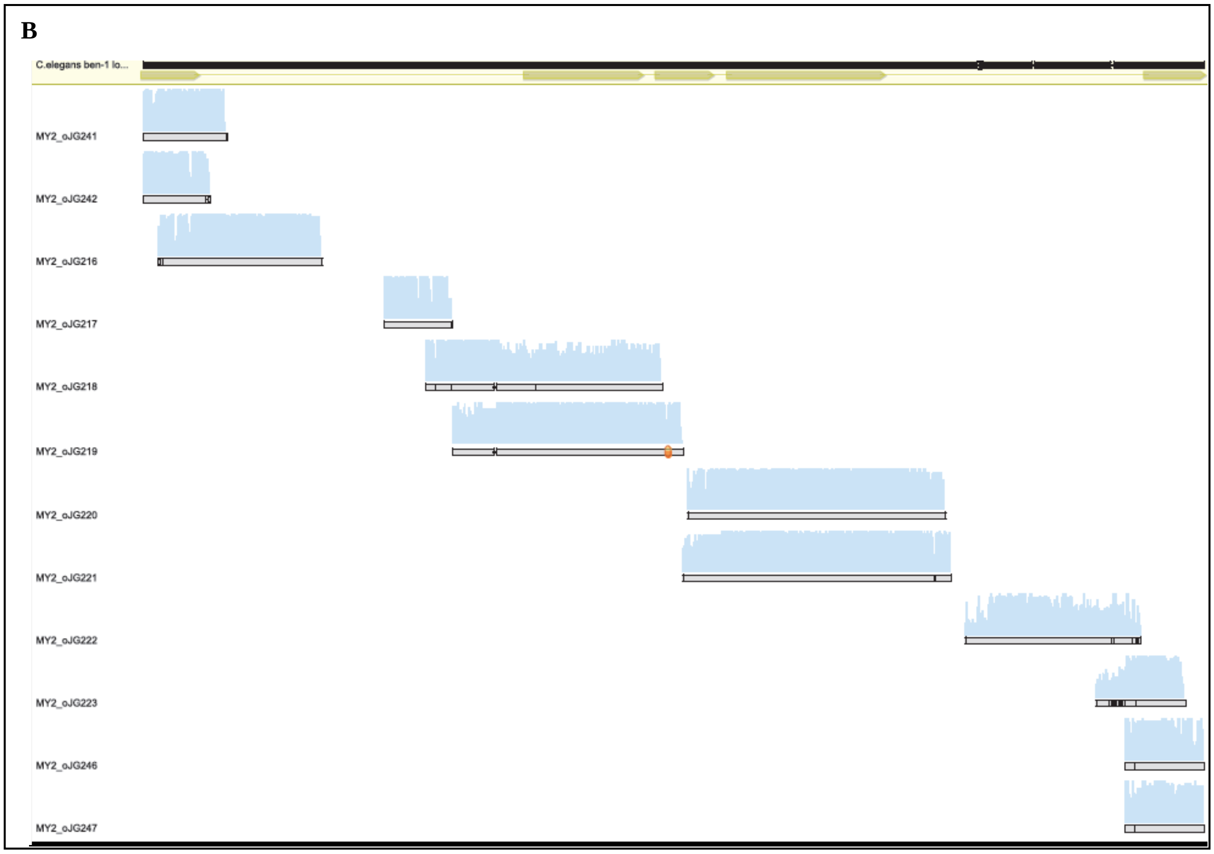


Figure 4.7 *ben-1* β-tubulin genomic locus assembly for *C. elegans* wild strains KR314: *mab-23 (e2518)* V (A) and MY2 (B) to the canonical N2 sequence. Fragments are named (left) according to the primer used to sequence the PCR product. After trimming off ends consisting of high noise, the fragments were assembled to the *ben-1* genomic sequence of the canonical N2 strain (due to lack of overlap between fragments for *de novo* assembling). The N2 *ben-1* genomic sequence was extracted from *C. elegans* cosmid C54C6 sequence (Z77131). Yellow arrow annotations indicate the five exonic regions in the sequence. The orange annotation on fragments (oJG218 for KR314 and oJG219 for MY2) indicates the point of conflict. The complete sequences are included in Appendix D.

Figure 4.8A Complete amino acid sequence of the *ben-1* β-tubulin drug target of *C. elegans* wild strain KR314: *mab-23 (e2518)* V (Vancouver, Canada) showing changes relative to the canonical N2 sequence (CAB00853). Point of frame-shift starting from codon 71 onwards (orange annotation) and the premature stop (white asterix within black box) at codon 119 are circled.

Figure 4.8B Complete amino acid sequence of the *ben-1* β-tubulin drug target *C. elegans* wild strain MY2 (Munster, NW Germany) showing changes relative to the canonical N2 sequence (CAB00853). Amino acid change at codon 185 from alanine to proline (A185P) is marked by the orange annotation (circled region). The rest of the protein is unaffected in the MY2 strain.

Table 4.2 Additional wild *C. elegans* carrying non-synonymous amino acid changes in the β-tubulin drug target

Strain	SNP *	Base position	Amino acid Change	Country of Isolation
JU1088	C/T	3539686	M257I	Japan
JU1171	C/G	3539961	A185P	Chile
MY14	C/G	3539961	A185P	Germany
MY16	C/G	3539961	A185P	Germany
GXW1	C/T	3538438	D404N	China

*SNP= Single Nucleotide Polymorphism. Data from million mutations project (2011-2013).

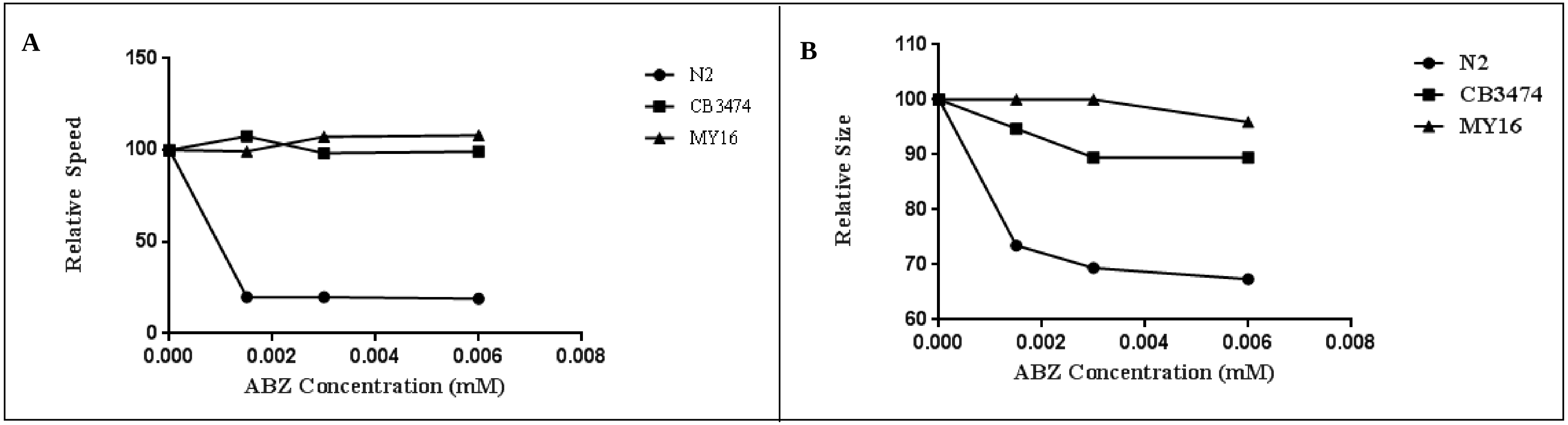


Figure 4.9 Effect of albendazole on movement (A) and size (B) of wild *C. elegans* strain MY16 as assessed by the NemaRx system. Relative values are the median values at each concentration expressed as a percentage of the median value of the same strain when not exposed to drug. N2 is the reference susceptible strain and CB3474 [*ben-1(e1880)*] is the reference benzimidazole resistant strain. Error bars are not shown as results are from a single experiment.

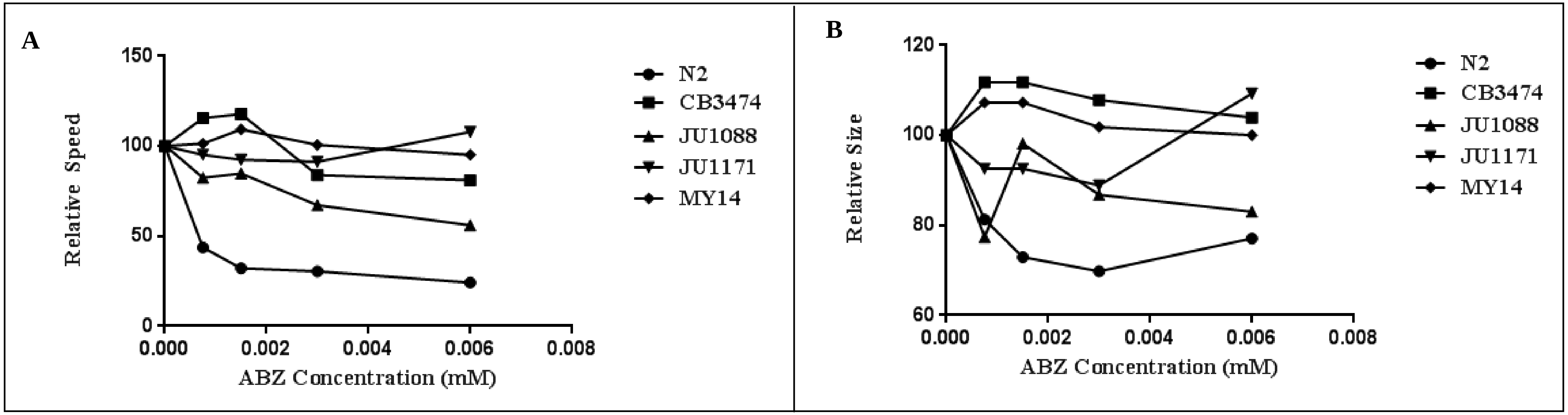


Figure 4.10 Effect of albendazole on movement (A) and size (B) of *C. elegans* strains JU1088 (M257I), JU1171(A185P) and MY14 (A185P) as assessed by the NemaRx system. Relative values are the median values at each concentration expressed as a percentage of the median value of the same strain when not exposed to drug. N2 is the reference susceptible strain and CB3474 [*ben-1(e1880)*] is the reference benzimidazole resistant strain. Error bars are not shown as results are from a single experiment.

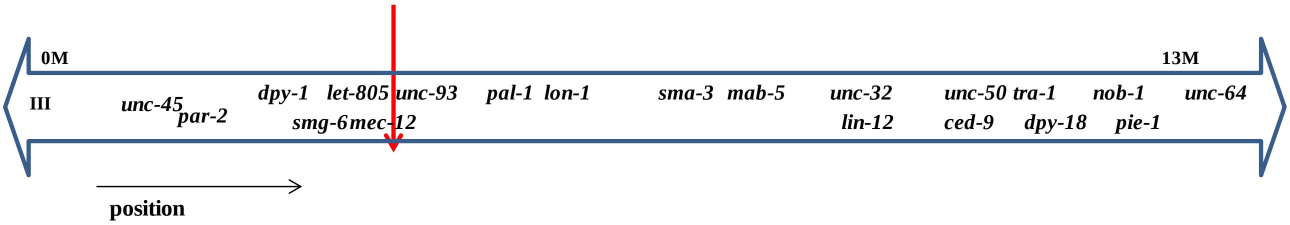


Figure 4.11: Schematic map of *C. elegans* chromosome III. Red arrow indicates region within which the *ben-1* locus is located. Figure adapted from WormBase map of chromosome III available at www.wormbase.org. The genes close to *ben-1* for which variations are included in Table 4.3 were chosen from this map.

Table 4.3 Variation in genes close to *ben-1* on chromosome III for the JU1171, MY14 and MY2 strains

Gene	Position	Mutation	Effect	Domain affected	Type
unc-93	3647409	G->T	M502I*	transmembrane_domain	missense
ben-1	3539974	C->G	A185P*	Tubulin	missense
par-2	1088162	G->A	R257Q		missense
par-2	1088207	T->C	V272A		missense
dpy-1	2042973	A->G	I416T		missense
dpy-1	2042897	A+15N->A	TEAPPT441T		coding exon, deletion
smg-6	2478203	C->G	P502A*		missense
smg-6	2483936	T->C	Y945H		missense
smg-6	2483978	A->G	S959G		missense
smg-6	2484103	G->C	R1000S		missense
smg-6	2484195	G->A	G1031E*		missense
smg-6	2476476	G->GAAGCTCAGA	E295EAQK	coiled_coil_region	coding exon, insertion

Data downloaded from http://genome.sfu.ca/mmp/. /.
Strains JU1171, MY2 and MY14 display these same variations with '*' indicating mutations also found in the MY16 strain which shares the A185P change in *ben-1* β-tubulin. Where a cell is blank, data was not available for that particular instance on the website.

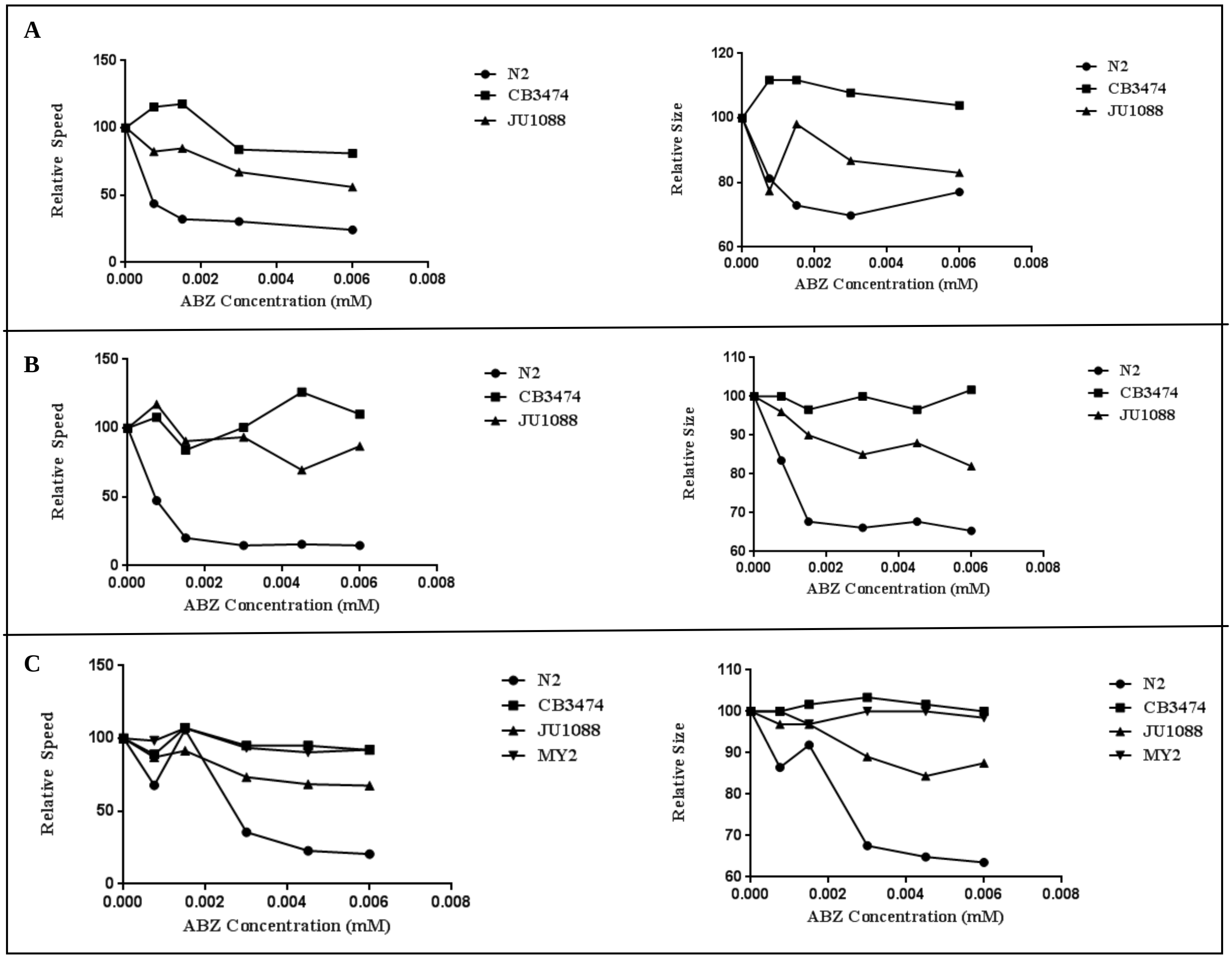


Figure 4.12 Biological replicates for the JU1088 strain to confirm its benzimidazole phenotype. The effect of albendazole on the speed and size of worms was assessed in three independent experiments (A, B and C). Data in panel A is same the same as that in Figure 4.10 for the JU1088 strain. Note that the Y axis in dose-response curves for size starts at 60%. In the third experiment (C) the MY2 strain was included to compare the level of resistance to JU1088 strain as a ‘reference wild isolate’ with a benzimidazole resistant phenotype.

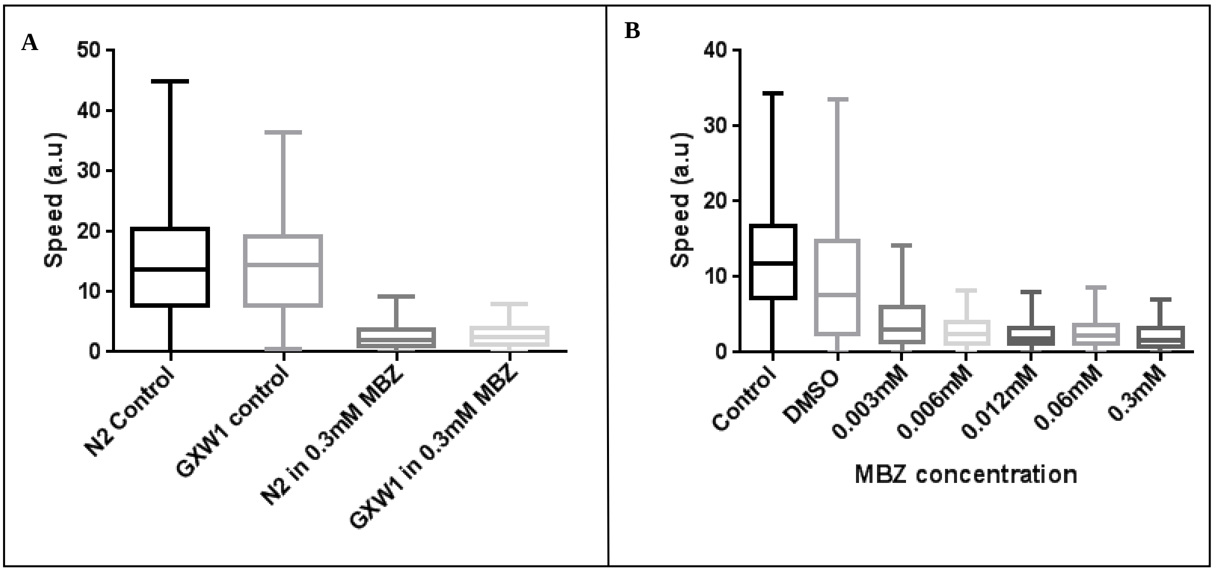


Figure 4.13 Preliminary phenotyping of wild *C. elegans* strain GXW1 on the NemaRx system. (A) Comparison of movement of GXW1 strain to the reference susceptible N2 strain at 0.3mM mebendazole (MBZ). (B) Effect of increasing concentration of mebendazole on the movement of GXW1 strain. Box plots show the speed distribution of populations of worms exposed to the different concentrations of mebendazole (after removal of outliers).

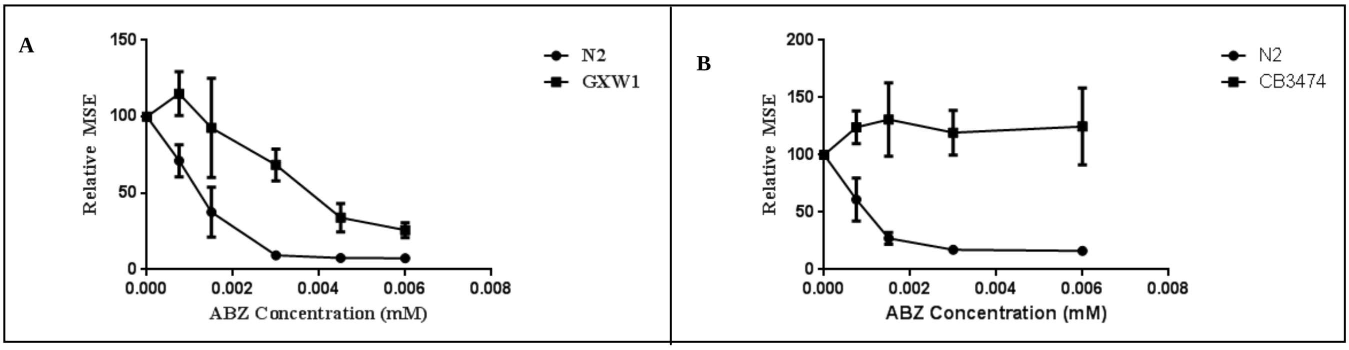


Figure 4.14 Preliminary phenotyping of GXW1 strain on the IVT-MS100 system. (A) Comparison of GXW1 strain against N2. Error bars are standard deviation, and each data point is the average of readings from three technical replicates. (B) Comparison of CB3474 [*ben-1(e1880)*] and N2 strains on IVT-MS100. This curve (computed from previous data) was included to show that the GXW1 strain is not as resistant as the reference resistant CB3474: *ben-1 (e1880)* strain. Each point in this curve is the average of three biological replicates and error bars are standard deviation.

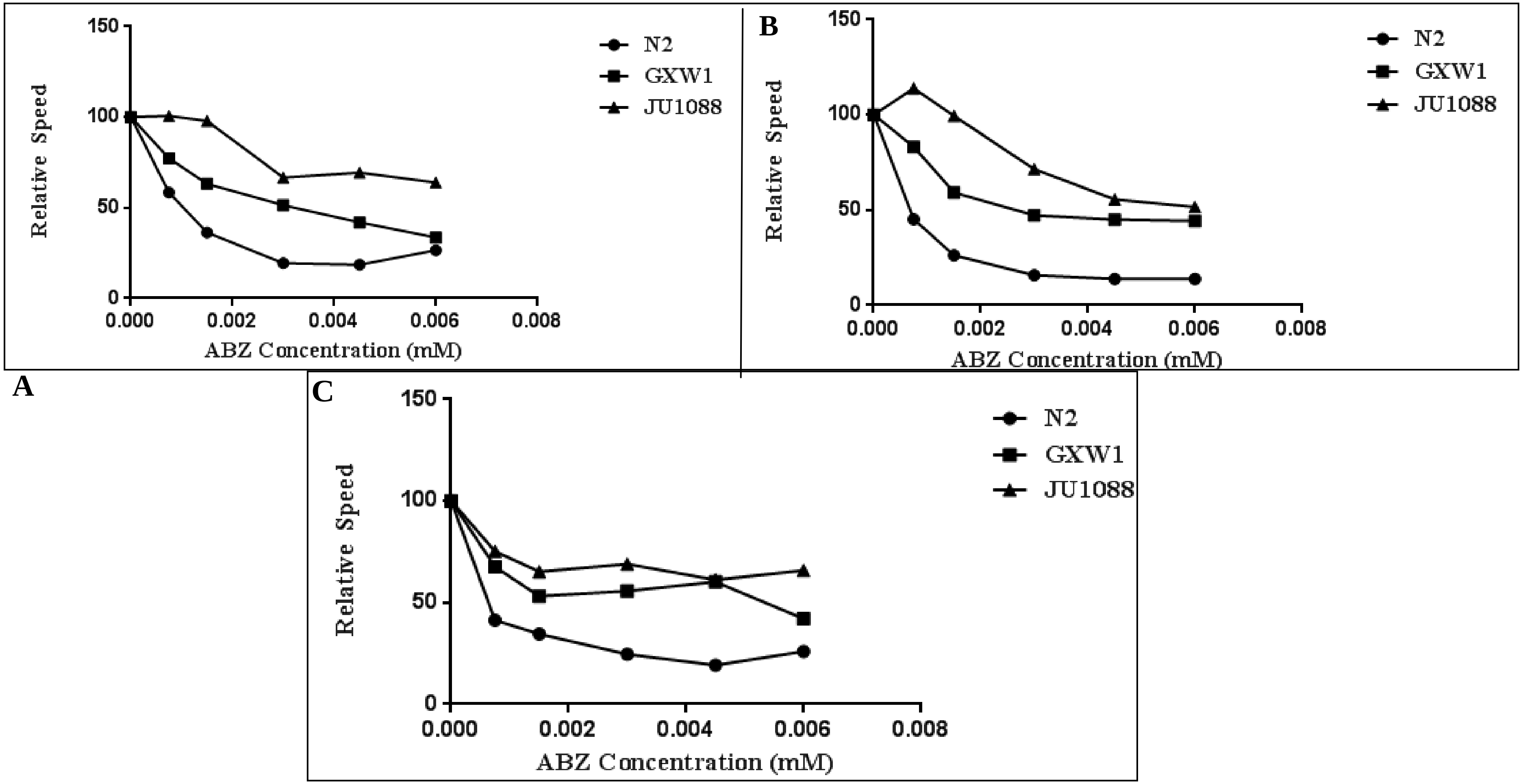


Figure 4.15 Effect of albendazole on the speed of *C. elegans* wild strain GXW1 as assessed by the NemaRx system. Three independent biological replicates (A, B and C) were carried out on different days. JU1088 is used as the reference resistant control and N2 is the reference susceptible strain on which the highest effect is seen with increasing albendazole concentration. The level of sensitivity of the GXW1 strain appears to be in between those of the JU1088 and N2 strains.

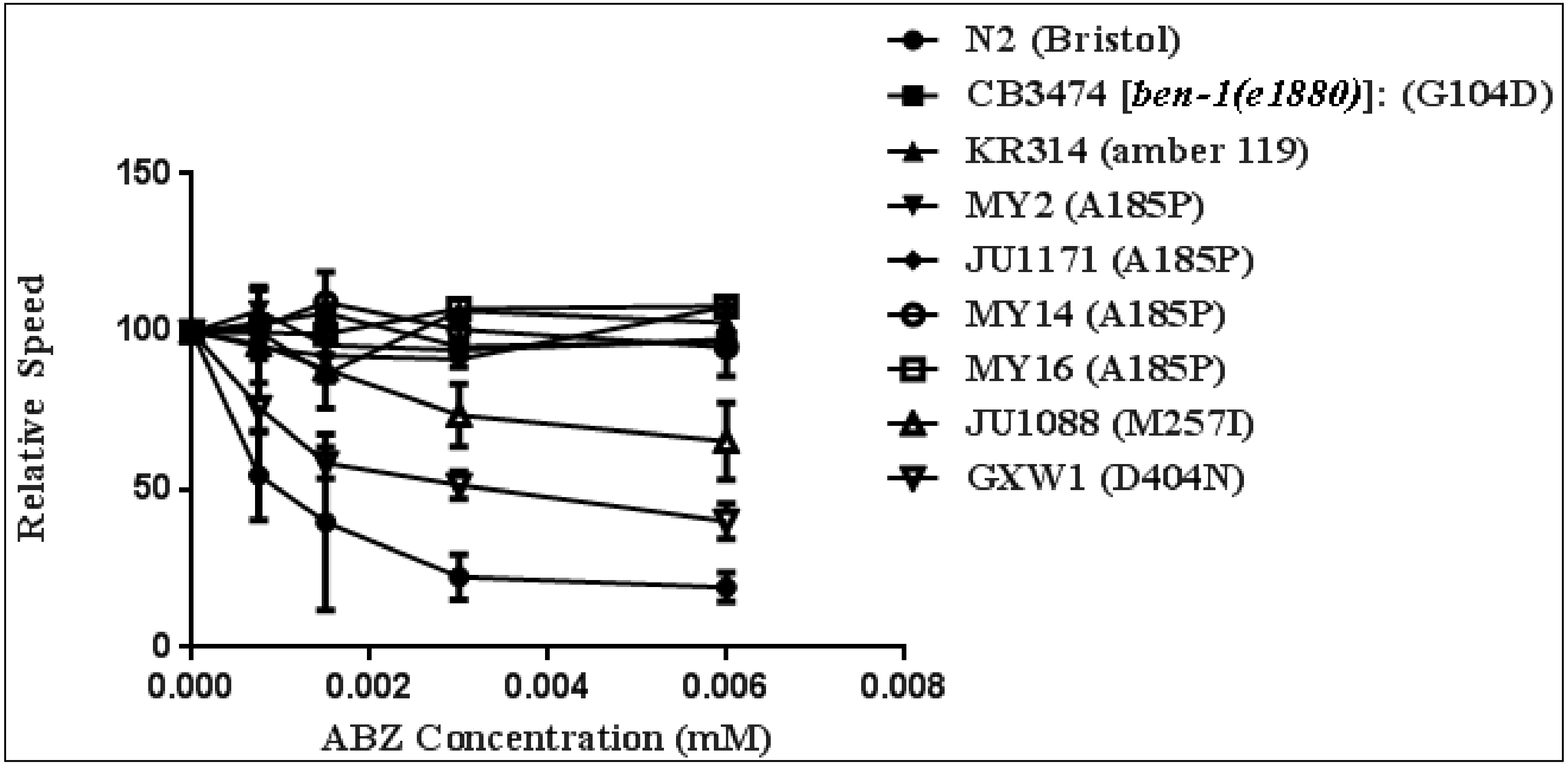


Figure 4.16 Comparison of benzimidazole resistance levels in wild *C. elegans* strains. N2 and CB3474 [*ben-1(e1880)*] are reference susceptible and resistant strains respectively. KR314: *mab-23 (e2518)* V, MY2, JU1088, JU1171, MY14, MY16 and GXW1 are wild *C. elegans* strains that showed varying levels of phenotypic resistance to albendazole (ABZ). The graph is based on data from the NemaRx digital imaging system. Error bars are standard deviation. For the N2 strain each data point is the average of values from 8 experiments, for CB3474 it's 5 experiments and for JU1088 and GXW1 three experiments each. Where error bars are not shown, the data points are based on the single experiment carried out for these strains on the NemaRx system (no replicate data available). The KR314, MY2, MY14, MY16 and JU1171 strains display the same level of resistance as the reference resistant strain. The resistance level of the JU1088 strain is lower than that of the reference resistant strain while the GXW1 strain displays a level of resistance even lower than the JU1088 strain. The changes in the amino acid sequences of the *ben-1* β-tubulin of these strains relative to the canonical N2 strain are indicated in parentheses.

A

B

C

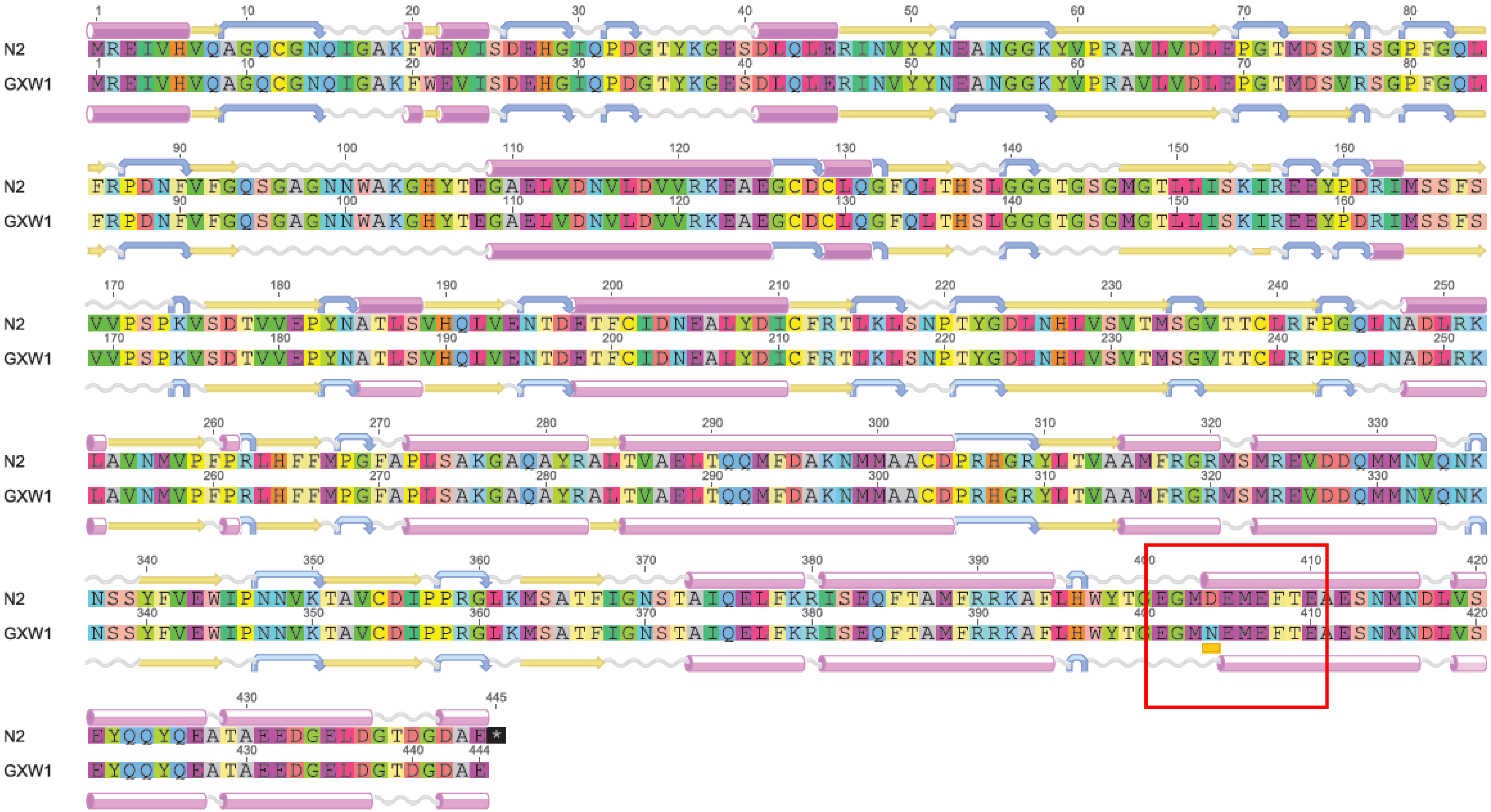


Figure 4.17 Secondary structure predictions for *ben-1* β-tubulin in wild *C. elegans* strains. The complete amino acid sequences of the MY2 (A), JU1088 (B) and GXW1 (C) were aligned to that of the N2 sequence and regions of α-helices (purple cylinder), β-strands (yellow arrow), coils (grey wave) and turns (blue arrow) were predicted using Geneious software. The positions where amino acid substitutions (and therefore changes in secondary structure) occur in the wild strains relative to the N2 strain are indicated by a red box (A185P for MY2 strain, M257I for JU1088 and D404N for the GXW1 strain).

Table 4.4 Wild *C. elegans* carrying deletions in the *ben-1* locus

Strain	Nucleotide Deletion	Base position	Amino acid Change	Country of Isolation
CB4856	GCATCTCCAT/G	3538333	DGDA443A	Hawaii
ED3021	TTG/T	3540406	68 stop*	United Kingdom
MY6	TTG/T	3540406	68 stop*	Germany
JU360	ACATACGTC/A	3539506	322 frame shift**	France

*Frame shift begins at amino acid position 58 leading to a premature stop codon at position 68
** Frame shift begins at amino acid 322 leading to a 441 amino acid long polypeptide. Data obtained from million mutations project (2011-2013).

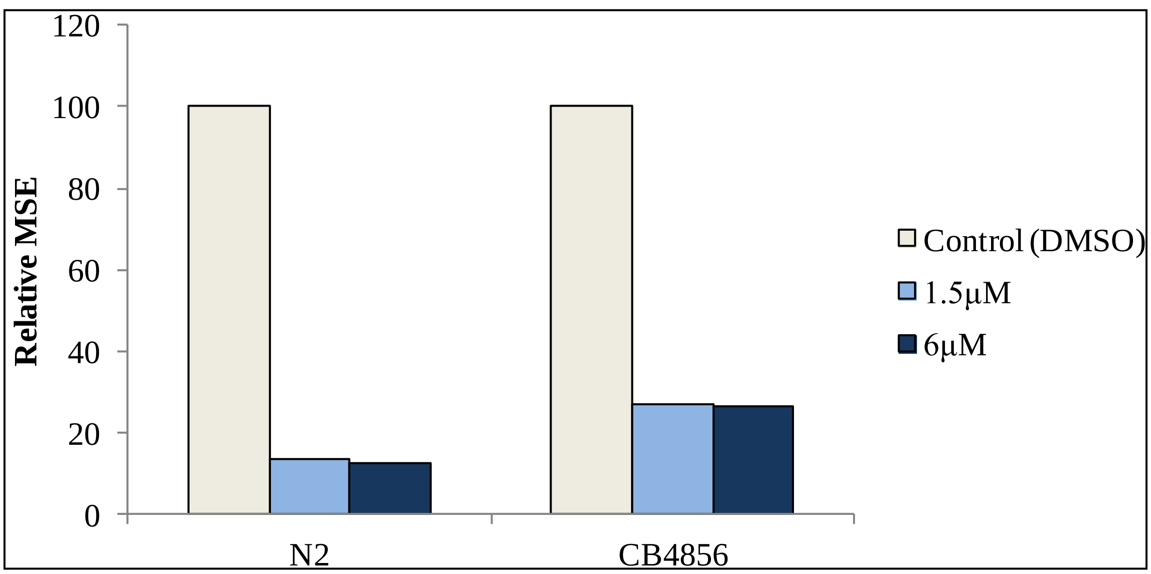


Figure 4.18 Assessment of benzimidazole sensitivity of an independent CB4856 strain on the IVT-MS100 system. MSE value at each concentration is expressed as a percentage of the control for the particular strain. Error bars not shown as results are from a single experiment. At each drug concentration tested the CB4856 strain behaved similar to the N2 strain (p>0.05).

Chapter 5: Genetic complementation to confirm the functional significance of polymorphisms in the *ben-1* locus of wild *C. elegans* strains resistant to albendazole

5.1 Introduction

In the previous chapter, I identified novel polymorphisms in the *ben-1* β-tubulin gene of wild albendazole-resistant *C. elegans* strains that are potentially responsible for the benzimidazole resistant phenotype. These were an alanine to proline substitution at amino acid position 185 (A185P) and a methionine to isoleucine substitution at position 257 (M257I). An aspartic acid to asparigine substitution at position 404 (D404N) was also identified in the GXW1 strain which gives much lower levels of resistance. In addition to these, a naturally occurring null mutation in the *ben-1* gene was identified in the KR314 [*mab-23(e2518)*] strain. In this chapter, I present the results of genetic complementation experiments carried out to determine the functional significance of these polymorphisms. Since the GXW1 strain which carries the D404N substitution only became available during the period during which this thesis was being written, genetic complementation was not performed for this strain.

Genetic complementation can be used to determine if a particular trait is due to mutations in a particular genetic locus (Figure 5.1). If two strains are crossed with different homozygous recessive mutations conferring the same phenotype and wild-type progeny are produced, complementation is said to have occurred. This indicates causal mutations are in different genes. Conversely, if the progeny have the mutant phenotype, complementation is said not to have occurred and the causal mutations must therefore be in the same gene.

In a previous study, all 28 mutations generated by EMS mutagenesis of *C. elegans* that give rise to benzimidazole resistance were mapped to the *ben-1* locus (Driscoll, Dean et al. 1989). The reference allele for this locus is *e1880* and so the CB3474 [*ben-1(e1880)*] strain has been used as the reference resistant strain throughout this project. Although the previous study determined that the *e1880* allele genetically mapped to the *ben-1* locus, the precise mutation had not been defined (Driscoll, Dean et al. 1989). Sequencing results from the previous chapter in this thesis identified the specific molecular lesion in this strain to be a single nucleotide change (G/A) at codon 104 leading to a glycine to aspartic acid change (G104D). Hence, it follows that if the F1 progeny from the cross between CB3474: *ben-1 (e1880)*III males and hermaphrodites of the albendazole resistant wild *C. elegans* strains are also resistant to benzimidazole drugs, the *ben-1* alleles from the two strains do not complement each other and therefore are in the same genetic locus. Consequently, it is possible to use genetic complementation to confirm that the non-synonymous polymorphisms (relative to N2) identified in the *ben-1* locus of the wild strains (KR314: *mab-23(e2518)* V, MY2 and JU1088) are the major cause of benzimidazole resistance in these strains. Alternatively, if the F1 cross progeny are sensitive to the drug then the alleles are said to complement each other and therefore are not of the same gene. If this is the case, then it suggests that the benzimidazole resistance in the wild strains is due to polymorphisms in other loci and may identify novel resistance loci.

One of the pre-requisites of genetic complementation experiments is that the trait in question be recessive under the conditions in which the genetic complementation experiments are to be carried out. To determine this, the wild strains identified as benzimidazole resistant in the previous chapter were first crossed with the reference benzimidazole susceptible N2 strain. If the F1 progeny from this cross are susceptible at a particular concentration, then we can conclude that the trait is recessive under the conditions tested allowing us to proceed with the genetic complementation cross (Figure 5.1).

5.2 Determining the lowest dose at which the N2 and CB3474 [*ben-1(e1880)*] reference strains can be unambiguously distinguished

The original phenotyping of wild *C. elegans* strains described in Section 3.2 was carried out using a dose-range of albendazole (Chapter 3). However, using this assay to test the phenotype of F1 progeny resulting from a genetic cross would be extremely labour intensive due to the following reasons: (1) huge number of crosses need to be set up to provide the required number of L1s for the assay (16,000+ per strain) (2) it is important to make sure that the progeny are derived from a successful mating (contains 50% each of males and hermaphrodites) and therefore progeny of each cross set up in (1) need to be examined at the L1 stage which is not possible since at this stage males and hermaphrodites are identical (Sulston and Horvitz 1977). Therefore, I decided to take an alternative approach for phenotyping the F1 progeny from crosses by testing them at a single benzimidazole concentration on agar plates. The first step was to determine the dose of benzimidazole drug that can unambiguously distinguish between the reference sensitive (N2) and the reference resistant CB3474 [*ben-1(e1880)*] strains. For this purpose a dose-response experiment was set up. The dose-range used here was much higher (0.06mM to 1.2mM) than that used in the initial bio assays (0.00075mM to 0.006mM) for phenotyping wild *C. elegans* strains. The higher dose range was opted for based on a previous study where the lowest paralysing dose for the *ben-1 (e1880)* allele was shown to be >0.28mM benomyl, another drug of the benzimidazole class (Driscoll, Dean et al. 1989). Thus, I was interested to know if the naturally occurring polymorphisms can confer resistance at similar or higher concentrations.

A single hermaphrodite from each of the reference strains was allowed to lay eggs on NGM agar plates in which relevant drug doses were incorporated into the agar and the eggs were allowed to develop for two days. Following the incubation period, the plates were visually examined and the number of L4 and adult stage worms in each plate was counted (Section 2.2.2).

The first dose-response assay was carried out using albendazole. However, the results of this experiment were not clear cut, in that the L4/adult counts did not accurately reflect the distinction between the two reference strains as sensitive or resistant (i.e. albendazole was not as discriminatory as in Chapter 3). The reason for this failure is unclear. Therefore, this experiment was repeated also including two other drugs of the benzimidazole class (mebendazole and thiabendazole) in case the discrepancy was due to the particular drug used (Table 5.1).

From scoring adults and observation of the movement of worms on plates it was apparent that 0.06mM mebendazole is the lowest concentration (in the dose range tested) that is able to unambiguously distinguish between the reference sensitive (N2) and reference resistant [CB3474: *ben-1 (e1880)* III] phenotypes. At this concentration, N2 worms were clearly paralysed and looked unhealthy and most had not reached L4/adult stage, whereas the worms of the CB3474 [*ben-1(e1880)*] strain had similar number of healthy adult worms as in a control (DMSO) plate and displayed unaffected sinusoidal movement. Above 0.3mM mebendazole the reference resistant strain was also beginning to be affected by the drug. Results for thiabendazole were similar at 0.06mM but above this concentration the worms from the reference resistant strain were also starting to get affected. With albendazole, a clear distinction was not made even at the lowest drug dose tested (Table 5.1). Hence, I decided to choose mebendazole at 0.06mM for all subsequent experiments in this section.

In addition to scoring plates of N2 and CB3474 [*ben-1(e1880)*] worm populations for L4/adults by visual observation, they were also phenotyped for movement velocity using the NemaRx digital imaging system (Figure 5.2). The speed of movement of N2 sensitive control worms was clearly affected at 0.06mM and 0.3mM mebendazole ($p<0.0001$ compared to the N2 worms in DMSO) while for the CB3474 [*ben-1(e1880)*] reference resistant worms the movement was not affected and not significantly different to those just exposed to DMSO ($p>0.05$).

The next dose-response assay was performed on the wild *C. elegans* strains (KR314 [*mab-23(e2518)*] and MY2) and compared to the N2 and CB3474 [*ben-1(e1880)*] strains (Table 5.2). Three technical replicates of this assay were performed (i.e. in each case three individual adult worms on separate drug plates were used for egg laying). This data again confirmed that at 0.06mM mebendazole, it is possible to distinguish between a susceptible and resistant phenotype. In each trial, no eggs of the N2 strain developed to the adult stage starting at 0.06mM whereas all the eggs of the resistant strains (KR314: *mab-23(e2518)* V, MY2 and CB3474: *ben-1 (e1880)* III) did. Overall, the key conclusion was that any individual worm that can develop to L4/Adult stage in 2 days at 0.06mM mebendazole had a resistant phenotype relative to N2 individuals. The speed of the worm populations from this assay was not assessed on the NemaRx system as the primary purpose was to check that the KR314 [*mab-23(e2518)*] and MY2 strains behaved in a similar fashion to the CB3474 [*ben-1(e1880)*] strain when exposed to 0.06mM mebendazole.

5.3 Establishing the mebendazole dose at which benzimidazole resistance phenotype is recessive for those *C. elegans* strains to be used in the genetic complementation assays

It has been previously shown that the dominance of benzimidazole resistance phenotype depends on both the concentration of drug and temperature (Driscoll, Dean et al. 1989). Hence, the next step was to determine a dose at which the benzimidazole resistance phenotype was sufficiently recessive in the reference resistant CB3474 [*ben-1(e1880)*] and wild *C. elegans* strains (KR314: *mab-23(e2518)* V, MY2 and JU1088) in order to undertake genetic complementation analysis. This was done by crossing hermaphrodites of each of these strains with N2 males and testing the benzimidazole phenotype of F1 progeny on the NemaRx system. Recessivity testing was not carried out for the JU1171, MY14 and MY16 strains as they carry the same amino acid change (A185P) in the *ben-1* β-tubulin drug target as MY2.

5.3.1 Determining the mebendazole concentration at benzimidazole resistance of CB3474 [*ben-1(e1880)*] strain is recessive

Visual examination of the F1 progeny resulting from the cross between N2 and CB3474 [*ben-1(e1880)*] (denoted N2/CB3474) exposed to 0.06mM mebendazole revealed them to be partially affected by the drug suggesting a partially dominant phenotype at this concentration (data not shown). Therefore the next highest concentration in the dose-range tested in Section 5.1 (0.3mM) was examined in more detail.

F1 progeny resulting from crosses between N2 males and CB3474 [*ben-1(e1880)*] hermaphrodites had a phenotype that was much more similar to N2 than CB3474 [*ben-1(e1880)*] although the F1 progeny did have slightly lower sensitivity to the drug than N2 (Figure 5.3). The median speed of the N2 population was 2 arbitrary units (a.u.), the N2/CB3474 cross progeny population was 2.7 a.u. and that of the CB3474 [*ben-1(e1880)*] population was 5.65 a.u., which indicates that the cross progeny are more similar to the N2 strain. Therefore, the benzimidazole resistance phenotype of the *ben-1 (e1880)* allele is largely, but not completely recessive at 0.3mM mebendazole (Figure 5.3). Hence, I reasoned that the recessivity was sufficient to carry out genetic complementation experiments at this same concentration.

5.3.2 Determining the mebendazole concentration at which the benzimidazole resistance phenotype of strains KR314 [*mab-23(e2518)*] and MY2 is recessive

Both visual examination of plates containing populations of worms exposed to drug and phenotyping on the NemaRx digital imaging system was utilised to confirm that the benzimidazole resistance phenotype of *C. elegans* wild strains KR314: *mab-23(e2518)*V and MY2 is recessive at 0.3mM mebendazole-the concentration at which I planned to carry out subsequent genetic complementation experiments.

5.3.2.1 Recessivity of the benzimidazole resistance phenotype of the KR314 [*mab-23(e2518)*] strain

Visual examination of F1 progeny from the crosses between N2 and KR314 [*mab-23(e2518)*] (denoted N2/KR314) exposed to 0.3mM mebendazole revealed a phenotype similar to the susceptible N2 parent in terms of size and shape of the worms (Figure 5.4; panels A-D). The motility of parental and F1 cross progeny was also tested using the NemaRx system (Figure 5.5). The N2/KR314 population had a motility phenotype much more similar to N2 than to KR314 [*mab-23(e2518)*] although the F1 progeny did have a slightly lower sensitivity to the drug than N2 (Figure 5.5 and Table 5.3). The median speed of the N2/KR314 population was 3.7a.u. and those of the N2, KR314: *mab-23(e2518)*V and CB3474: *ben-1 (e1880)*III populations were 1.7, 7.6 and 10.2 a.u respectively. This indicated that the N2/KR314 strain was more similar to the reference susceptible N2 strain than the KR314 [*mab-23(e2518)*] or CB3474 [*ben-*

1(e1880)] strains (also supported by visual examinations), although it presented a statistically significant difference to all three of these parental strains ($p<0.0001$) (Table 5.3). Hence, the benzimidazole resistance phenotype of the *ben-1 (cewivar00796699)* allele of the KR314 [*mab-23(e2518)*] strain was largely, but not completely recessive at 0.3mM mebendazole.

5.3.2.2 Recessivity of the benzimidazole resistance phenotype of the MY2 strain

Visual examination of F1 progeny from the crosses between N2 and MY2 (denoted N2/MY2) exposed to 0.3mM mebendazole revealed a phenotype similar to the susceptible N2 parent in terms of size and shape of the worms (Figure 5.6; panels A-D). The motility of parental and F1 cross progeny was also tested using the NemaRx system (Figure 5.7). The N2/MY2 population had a motility phenotype much more similar to N2 than to MY2 although the F1 progeny did have a slightly lower sensitivity to the drug than N2 (Figure 5.7 and Table 5.4). The median speed of the N2/MY2 population was 3.4a.u, and those of the N2, MY2 and CB3474 [*ben-1(e1880)*] populations were 1.7, 9.7 and 9.3 a.u. respectively. This indicated that the N2/MY2 strain was more similar to the reference susceptible N2 strain than the MY2 or CB3474 [*ben-1(e1880)*] strains (also supported by visual examinations), although it presented a statistically significant difference to all three of these parental strains ($p<0.0001$) (Table 5.4). Hence, the benzimidazole resistance phenotype of the *ben-1(cewivar00420700)* allele of the MY2 strain was largely, but not completely recessive at 0.3mM mebendazole. Therefore, for both wild strains KR314 [*mab-23(e2518)*] and MY2, as in the case of the CB3474 [*ben-1(e1880)*] strain, I reasoned that sufficient recessivity is displayed at 0.3mM mebendazole for subsequent genetic complementation experiments to be carried out.

5.3.3 Determining the drug concentration at which the benzimidazole resistance phenotype of the JU1088 strain is recessive

Although the KR314 [*mab-23(e2518)*] and MY2 strain were still resistant at 0.3mM mebendazole, the JU1088 strain was not. This is expected based on the original phenotyping of JU1088, where it showed only intermediate levels of resistance at even the lower dose range used in those experiments (Figure 4.10). Therefore, phenotyping of F1 progeny from crosses involving this strain was carried out at the lower concentration of 0.0045mM mebendazole. This concentration was chosen because it is mid-range between 0.003mM and 0.006mM and the JU1088 strain starts to become significantly different to the CB3474 [*ben-1(e1880)*] strain at 0.006mM in the dose response curve in Figure 4.10).

According to visual observations of the F1 progeny on the cross plates, in 0.0045mM mebendazole, the F1 progeny from the cross between JU1088 hermaphrodites and N2 males (denoted N2/JU1088) were similar to the reference sensitive N2 parent (Figure 5.8 panels A- D). Results from the NemaRx system confirmed the above observation (Figure 5.9). The N2/JU1088 population had a median speed of 2.9 a.u. and was not significantly different to the N2 strain (p=0.1337). Thus, it is possible to conclude that the benzimidazole resistance phenotype of the *ben-1 (cewivar00407429)* allele of the JU1088 strain is recessive at 0.0045mM and genetic complementation experiments can be carried out at this concentration.

5.4 Genetic Complementation Experiments

To confirm the functional significance of the benzimidazole resistance conferring mutations identified in the wild strains (KR314: *mab-23(e2518)* V, MY2 and JU1088), genetic complementation experiments were carried out by crossing these strains to the reference benzimidazole resistant CB3474 [*ben-1(e1880)*] strain. F1 progeny from the crosses were phenotyped at the concentrations determined in Section 5.3, both visually and using the NemaRx system.

5.4.1 Genetic complementation crosses between KR314 [*mab-23(e2518)*] and CB3474 [*ben-1(e1880)*] strains

F1 progeny resulting from crosses between CB3474 [*ben-1(e1880)*] males and KR314 [*mab-23(e2518)*] hermaphrodites (denoted CB3474/ KR314) were visually examined on drug plates containing 0.3mM mebendazole. They appeared much more similar to the resistant parental CB3474 [*ben-1(e1880)*] and KR314 [*mab-23(e2518)*] than N2 worms (Figure 5.4 panels A, B, C and E). The motility of parental and F1 progeny was also tested using the NemaRx system and results confirmed this observation as the F1 progeny were not significantly different to the reference resistant CB3474: *ben-1 (e1880)*III strain (P=0.2249) (Figure 5.5 and Table 5.3). The F1 population had a higher median speed (9.15 a.u.) compared to the KR314 [*mab-23(e2518)*] parental strain (7.6 a.u.). This difference is however statistically marginal (P=0.0476).

In contrast, the F1 progeny were significantly different (more resistant) to the N2/KR314 population (P<0.0001), F1 progeny produced by crossing N2 males and KR314 [*mab-23(e2518)*] hermaphrodites (Section 5.3.2.1). Collectively these observations and data demonstrate that the *ben-1 (e1880)* and *ben-1 (cewivar00796699)* alleles do not complement each other, providing evidence that the null mutation in the *ben-1* locus of the KR314 [*mab-23(e2518)*] strain is the major contributing factor to benzimidazole resistance in this strain.

5.4.2 Genetic complementation crosses between MY2 and CB3474 [*ben-1(e1880)*] strains

F1 progeny resulting from crosses between CB3474 [*ben-1(e1880)*] males and MY2 hermaphrodites (denoted CB3474/ MY2) were visually examined on drug plates containing 0.3mM mebendazole. They appeared much more similar to the resistant parental CB3474 [*ben-1(e1880)*] and MY2 strains than N2 worms (Figure 5.6 panels A, B, C and E). The motility of parental and F1 progeny was also tested using the NemaRx system and results confirmed the visual observations. The F1 progeny were not significantly different to either resistant parent CB3474 [*ben-1(e1880)*] or MY2 (P>0.9999) (Figure 5.7 and Table 5.4).

In contrast, the F1 progeny were significantly different (more resistant) to the N2/MY2 population (P<0.0001), the F1 progeny produced by crossing N2 males to MY2 hermaphrodites (Section 5.3.2.2). Taken together, these observations demonstrate that the *ben-1 (e1880)* and *ben-1 (cewivar00420700)* alleles do not complement each other, which is evidence that the A185P change in the *ben-1* β-tubulin of the MY2 strain is the major contributing factor to benzimidazole resistance in this strain.

5.4.3 Genetic complementation crosses between JU1088 and CB3474 [*ben-1(e1880)*] strains

F1 progeny resulting from crosses between CB3474 [*ben-1(e1880)*] males and JU1088 hermaphrodites (denoted CB3474/ JU1088) were visually examined on drug plates containing 0.0045mM mebendazole. They appeared much more similar to the resistant parental CB3474 [*ben-1(e1880)*] and JU1088 strains than N2 worms at this dose (Figure 5.8 panels A, B, C and

E). The motility of parental and F1 progeny was tested using the NemaRx system and results did not completely support this observation. The F1 progeny (CB3474/JU1088) were significantly different to both the resistant parents (CB3474 [*ben-1(e1880)*] and JU1088) as well as the N2/JU1088 population resulting from crosses between N2 males and JU1088 hermaphrodites (P<0.0001) (Figure 5.7 and Table 5.4). However, this observation is not due to the fact that the CB347/JU1088 population is more sensitive compared to the resistant parents as may be expected from the fact JU1088 only displays an intermediate level of resistance compared to the reference CB3474 [*ben-1(e1880)*] strain or wild strains KR314 [*mab-23(e2518)*] and MY2 (Section 4.4.1 and Figure 4.16), but quite the contrary. This observation is due to the fact that the median speeds recorded for the resistant parents CB3474 [*ben-1(e1880)*] (5.7a.u) and JU1088 (6.9a.u) is lower than expected on this day (see table on Figure 5.9). Some recorded median speeds for the CB3474 [*ben-1(e1880)*] strain on previous occasions includes values such as 10.2 a.u. and 9.3 a.u. (see tables in Figures 5.5 and 5.7). Thus it is possible that this difference is may not be due to biological phenomena directly relevant to this experiment (see discussion in section 5.6).

Considering that the CB3474/JU1088 strain is not similar to the reference sensitive N2 parent either (p<0.0001) and the explanations above, it is still fair to conclude that the *ben-1 (e1880)* and *ben-1 (cewivar00407429)* alleles do not complement each other and that the M257I change in the *ben-1* β-tubulin of the JU1088 strain is the major contributing factor to the benzimidazole resistance (albeit moderate) in this strain.

5.5 Additional Experiments carried out as a supplement to the genetic complementation experiments

The following experiments were carried out to address additional issues concerning the genetic complementation experiments described above.

5.5.1 Investigation of the potential phenotypic differences between male and hermaphrodite populations with regard to movement speed

In each of the three complementation experiments above (Figures 5.4 to 5.9) the parental strains were selfing hermaphrodite cultures whereas the F1 progeny of the genetic crosses consisted of 50% each of males and hermaphrodites. Hence, it is possible this could result in phenotypic differences for the NemaRx motility data between the F1 progeny and the parental strains due to simple differences in movement of male and female worms in the absence of drug. To investigate this possibility an independent experiment was carried out to show that there were no differences in the overall movement detected in a population as measured by the NemaRx assay whether it is made up solely of hermaphrodites or a 50% each mix of males and hermaphrodites (i.e. outcross progeny). The speed of worms in such populations (containing self vs. outcross progeny) was assessed (without exposure to drug using the NemaRx system (Figure 5.10). Results from this experiment confirmed the hypothesis that the two populations (self vs. outcross) were not significantly different to each other ($p=0.00891$) which validates the approach taken.

5.5.2 Confirmation that effects on speed of movement observed in the different parental and cross progeny populations are solely due to effects of mebendazole

I also investigated whether there was any fundamental differences in the movement, as measured by the NemaRx assay, of the F1 cross progeny compared to their respective parental strains that were independent of the effect of mebendazole (since cross progeny had not been previously tested after exposure to DMSO). Parental strains and cross progeny were allowed to develop on standard (no drug) NGM agar plates seeded with *E.coli* OP50 and their speed assessed using the NemaRx system (Figure 5.11). Results of this experiment showed that all F1 populations, except the CB3474/ KR314 population were not significantly different to the N2 strain ($p>0.05$). The median speed of the CB3474/KR314 population recorded on this occasion was 17.4 a.u. and from Section 5.4.1 after exposure to drug the recorded median speed for this population was 9.15a.u. This may suggest that the population is actually being affected by the drug. However visual examination of plates did not support this inference as the population appeared similar whether exposed to drug or not. Therefore, this result is possibly due to an experimental artefact.

Thus, the results from this section confirm that any effect seen on the speed of cross progeny is due to exposure to drug and where they are not affected, they are truly resistant.

5.6 Discussion

The functional significance of two naturally occurring amino acid substitutions (A185P and M257I) in the β-tubulin drug target rendering wild *C. elegans* strains resistant to benzimidazole anthelmintics, was confirmed by the work presented in this chapter. The A185P substitution occurs in strains MY2, MY14 and MY16 (all three isolated from Germany in 2002) and JU1171 (isolated in Chile in 2007). The M257I substitution occurs only in the JU1088 strain (isolated in Japan in 2007) of the total of 40 wild isolates whose genomes have been fully sequenced to date (Thompson, Edgley et al. 2013).

Furthermore, the functional significance of a naturally occurring deletion in the *ben-1* gene was also confirmed in conferring benzimidazole resistance. This molecular lesion occurs in the KR314 [*mab-23(e2518)*] strain (isolated from Vancouver, in the late 1980s). Previous studies had demonstrated that *C. elegans* was completely viable and displayed no detectable phenotype under laboratory conditions despite harbouring deletions in the *ben-1* locus which led to the inference that this gene is functionally redundant (Driscoll, Dean et al. 1989). Now, the finding from this thesis work and whole genome sequencing efforts (Thompson, Edgley et al. 2013) have demonstrated that deletions also occur in the *ben-1* gene of wild *C. elegans* strains (Table 4.4). These findings not only support the notion of redundancy of this gene under natural conditions but also demonstrate that that there should be little to no fitness cost to losing this gene in the natural environment.

In Sections 5.3.1, 5.3.2 and 5.3.3 it was noted that the F1 progeny resulting from crosses between N2 males and hermaphrodites of the CB3474 [*ben-1(e1880)*] or wild *C. elegans* strains KR314 [*mab-23(e2518)*] and MY2 were slightly less sensitive than the parental N2 strain (Figures 5.3, 5.5 and 5.7). This phenomenon may be explained by the fact that a previous study by Driscoll, Dean et al. (1989) that used mutagenised *C. elegans* strains demonstrated that the benzimidazole sensitivity is both temperature and concentration dependent. Almost all the *ben-1* alleles generated in that study were recessive at 15 ^{0}C and dominant at 25 ^{0}C (heterozygotes were not resistant at 15 ^{0}C) and resistance was not observed at benomyl concentrations greater than 0.012mM. From these findings, it can be inferred that the lower levels of sensitivity seen with N2/CB3474, N2/KR314 and N2/MY2 populations relative to the parental N2 strain may be due to the fact that experiments were carried out at an intermediate temperature of 20 ^{0}C which rendered the heterozygotes only partially sensitive (due to partially recessive alleles). However, the concentration at which these heterozygotes were tested in this project was much higher (0.3mM) than used in the previous study (0.012mM). It is possible that a combination of lower temperature but higher concentration is leading to this partially recessive phenotype. The reason why I decided to perform the genetic complementation experiments at 20 ^{0}C was to maintain a consistency between previous phenotyping assays (Chapter 3) and these experiments. In hindsight, the assays used for phenotyping wild *C. elegans* strains and F1 progeny from crosses are still fundamentally different since for preliminary phenotyping of wild *C. elegans* strains the worms were exposed to drug in liquid culture, whereas in the case of the genetic complementation experiments the drug was infused in solid media. Therefore, these observations of reduced sensitivity seen for heterozygotes may be further clarified by carrying out these experiments at a lower temperature of 15 ^{0}C where recessivity is expected at least for alleles arising from chemical mutagenesis. However, since the heterozygotes were more similar to the sensitive N2 strain than the resistant parents the benzimidazole resistance trait was accepted as being sufficiently recessive to carry out genetic complementation at this concentration.

In Section 5.5.1 it was shown that there was no significant difference in the median speeds of populations consisting of 100% hermaphrodites and 50% each of males and hermaphrodites. This is interesting since males and hermaphrodites are known to possess distinct locomotory features since males are smaller, thinner and more agile (Hodgkin 1974). In fact, a recent quantitative behavioural analysis supports this in that males were found to move at a higher velocity (0.17mm/s) compared to hermaphrodites (0.15mm/s) (Mowrey, Bennett et al. 2014). In the experiment carried out in Section 5.5.1 of this thesis however, one of the populations had only 50% males. Adding this to the fact that the velocities only differ by 0.02 mm/s it is not entirely surprising that a difference was not apparent between the two populations in Section 5.5.1 (Figure 5.10) possibly also owing to the limited sensitivity of the equipment. Thus, it is fair to conclude that the use of progeny resulting from self-fertilisation of the parental strains does not affect the outcome of the genetic complementation experiments.

5.7 Chapter 5: Figures and Tables

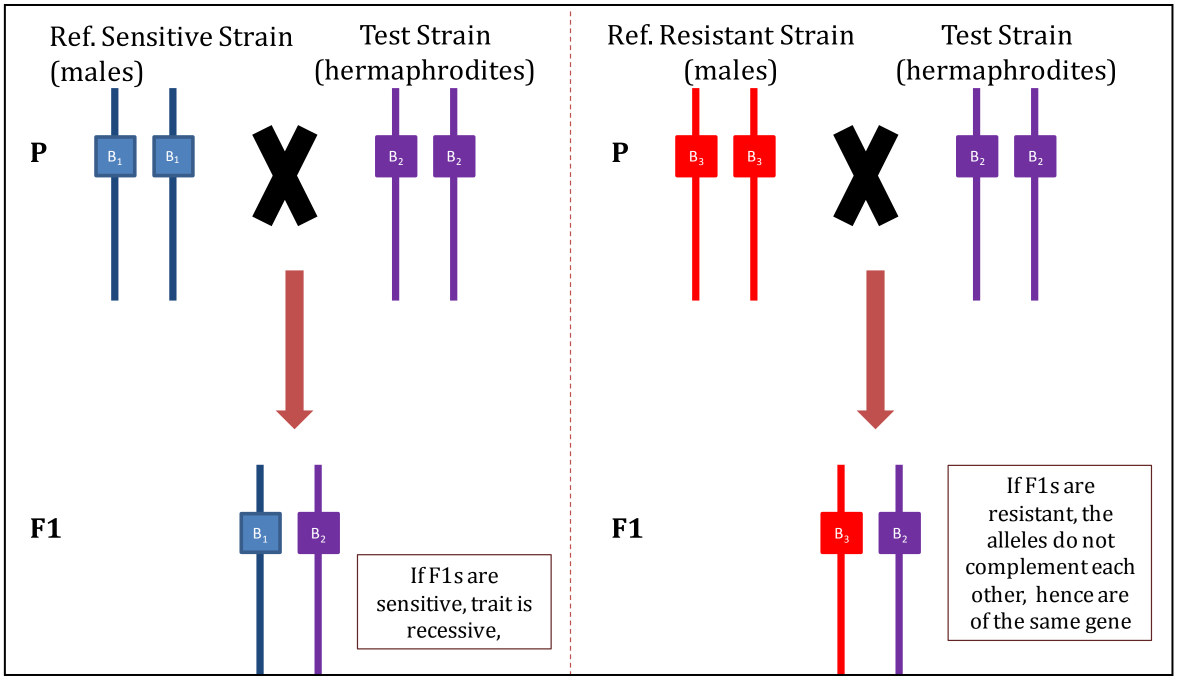
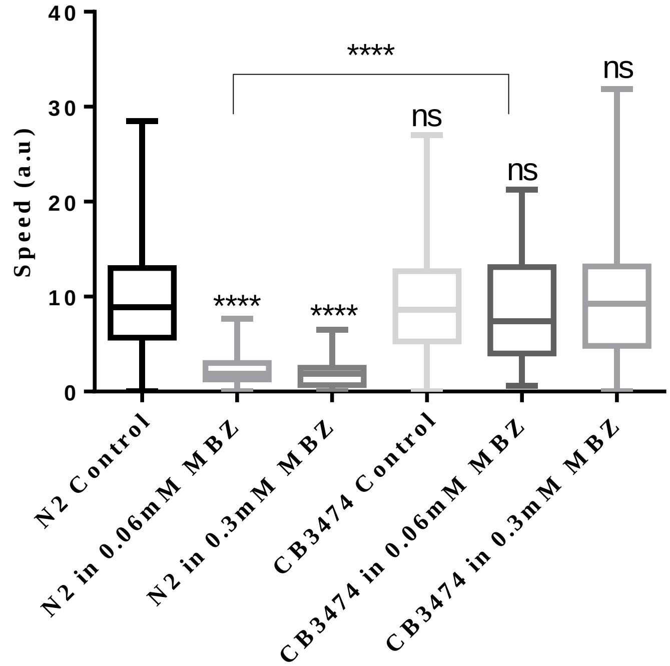


Figure 5.1 Outline of genetic complementation crosses. P= Parental generation; F1: First generation; B1, B2 and B3 are used to denote the *ben-1* alleles in the different strains. These crosses are carried out to confirm the functional significance of polymorphisms identified in the *ben-1* locus of *C. elegans* wild strains (KR314: *mab-23 (e2518)* V, MY2 and JU1088) referred to as 'Test Strain'. To assess the recessivity of the benzimidazole resistance trait in the wild strains, they are first crossed to the reference susceptible N2 strain. Once the recessivity is confirmed by examination of the F1 progeny they are crossed to the reference resistant CB3474 [*ben-1(e1880)*] strain and the benzimidazole sensitivity of the F1 progeny of this cross examined.

Table 5.1 Number of L4s or Adults after 2 days of incubation in benzimidazole drugs

Drug and concentration (mM)	No of L4 or A* stage worms	
	N2	CB3474
Albendazole		
0	22	>25
0.06	5	12
0.3	0	16
0.6	0	10
0.9	2	12
1.2	0	10
Mebendazole		
0	14	>25
0.06	0	>25
0.3	0	>25
0.6	0	12
0.9	0	6
1.2	0	8
Thiabendazole		
0	16	>25
0.06	13	>25
0.3	0	17
0.6	0	13
0.9	0	3
1.2	0	5

*A=adult stage; L4= fourth larval stage.
An adult worm was allowed to lay eggs overnight on NGM agar plates containing the different drug concentrations. The eggs were then allowed to develop and the number of L4s or adults on each plate was counted (up to 25 individuals) manually. The presence of 25 or more individuals at the adult/L4 stage/ mixture of the two stages was used as the cut off to accept the strain as resistant at a particular concentration.



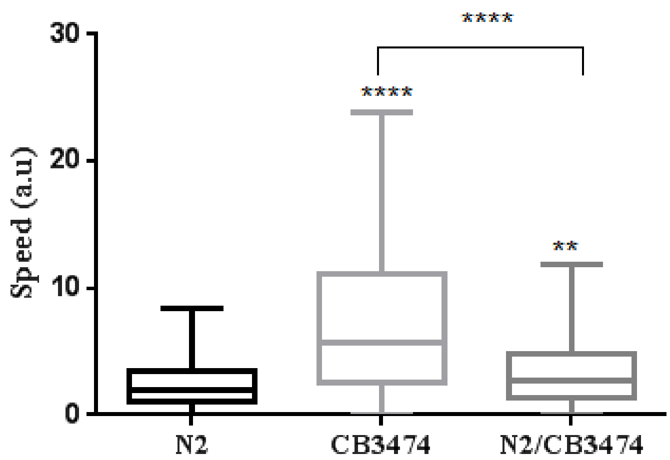
Population	Median Speed (a.u.)	N
N2 Control	8.9	299
N2 in 0.006mM MBZ	1.9	94
N2 in 0.3mM MBZ	1.9	71
CB3474 control	8.65	390
CB374 in 0.006mM MBZ	7.4	63
CB3474 in 0.3mM MBZ	9.25	202

Figure 5.2 Sensitive-Resistant phenotype distinctions in mebendazole (MBZ). Box and whisker plots show the minimum to maximum speed range in the populations of worms tested on the NemaRx digital imaging system. Horizontal line within the box indicates the median speed for which the corresponding values for each population are given in the table on the right of the box plots (N=the number of data points used to compute the median speed, this value is not necessarily equal to the number of worms in the population since the same worm may have been tracked more than once). Each population is compared to the N2 reference population (N2 control). ns= non significant; **** indicates P<0.0001; a.u= arbitrary units. The difference in speed between the CB3474 [*ben-1(e1880)*] populations at the different concentrations is not significant (p>0.05).

Table 5.2 Number of L4s or Adults in drug plates after 2 days incubation in mebendazole

Trial	Strain	MBZ concentration (mM)					
		0	0.06	0.3	0.6	1.2	1.8
	N2	>25	0	0	0	0	0
	CB3474	>25	>25	>25	>25	>25	10
	KR314	>25	>25	>25	0	>25	13
1	MY2*	0	>25	14	6	15	0
	N2	>25	0	0	0	0	0
	CB3474	>25	>25	>25	>25	>25	12
	KR314	>25	>25	>25	>25	>25	>25
2	MY2*	5	23	23	>25	>25	18
	N2	>25	0	0	0	0	0
	CB3474	>25	>25	>25	>25	18	>25
	KR314	>25	23	0	>25	5	21
3	MY2*	0	3	18	2	>25	>25

≥25 was used as the cut-off for scoring number of L4s and adults in order to save time with the counting process.
* In some instances the parental worm allowed to lay eggs may have crawled out of the plate or may have not laid enough number of eggs giving low counts. For the MY2 strain all the first generation worms on the plate (although low counts) were of L4 or adult stage supporting the fact that this strain is resistant to the drug.



Population	Median Speed (a.u.)	N
N2	2	559
CB3474	5.65	926
N2/CB3474	2.7	339

Figure 5.3 Recessivity of benzimidazole resistance in the CB3474: *ben-1 (e1880)* reference resistant strain assessed by speed distribution in F1 populations after exposure to 0.3mM mebendazole. Box and whisker plots show the minimum to maximum speed range. Horizontal lines within boxes indicate median speed and the corresponding values for each population are given in the table on the right of the box plots (N is the number of data points on which the median speed is based on). N2/CB3474 is the F1 progeny from a cross between N2 males and CB3474 [*ben-1(e1880)*] hermaphrodites. The CB3474 [*ben-1(e1880)*] and N2/ CB3474 populations were compared to N2 and also between themselves. **** or ** indicates a significant difference where, $p<0.0001$.

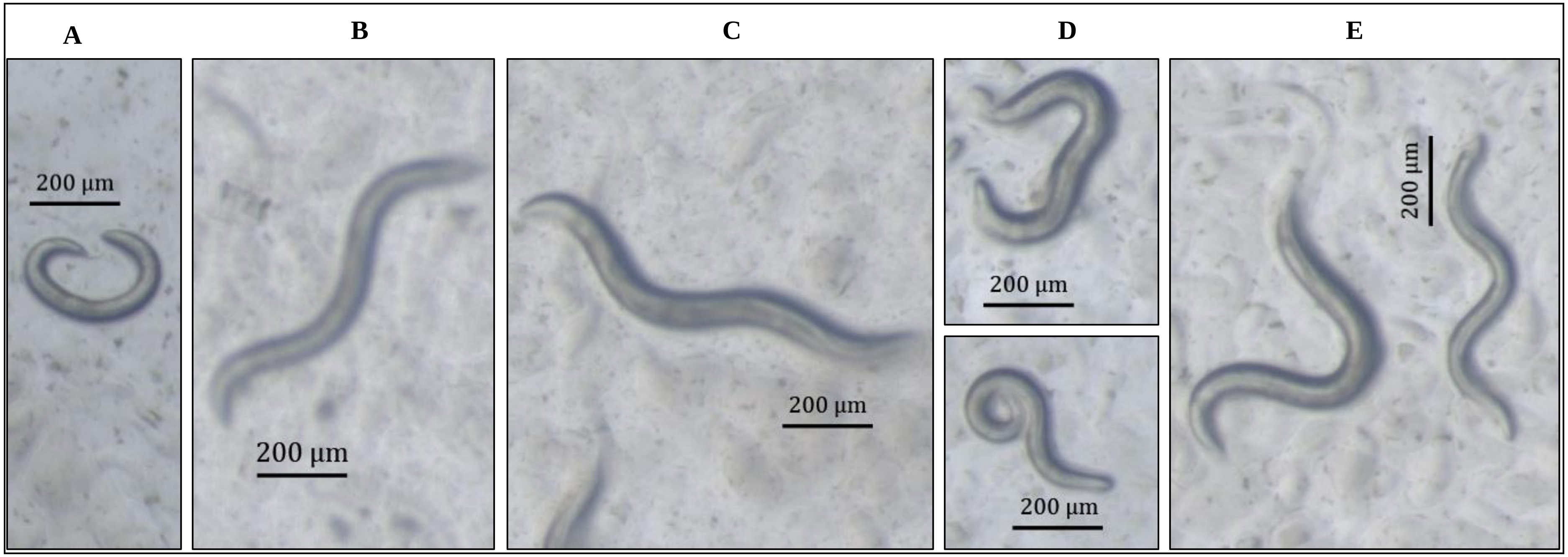
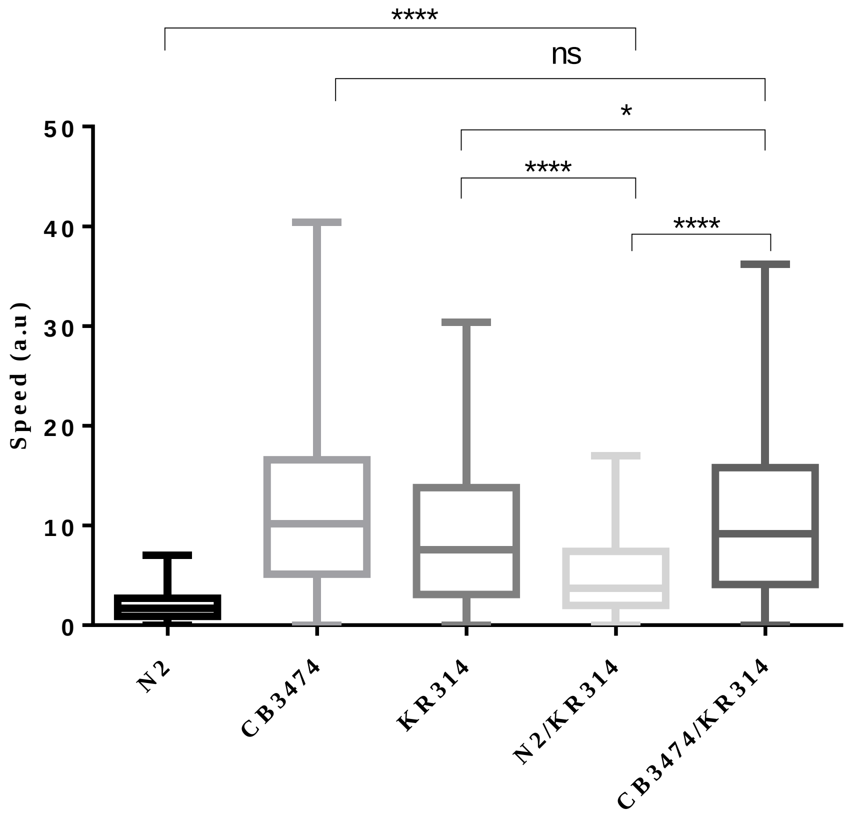


Figure 5.4 Appearance of worms grown on 0.3mM MBZ containing plates for genetic complementation experiments for the KR314: *mab-23 (e2518)* V strain. A: N2; B: CB3474: *ben-1 (e1880)* III; C: KR314: *mab-23 (e2518)* V; D: N2/KR314; E: CB3474/KR314. N2/KR314 indicates F1 progeny from a cross between N2 males and KR314: *mab-23 (e2518)* V hermaphrodites and CB3474/KR314 indicates F1 progeny from a cross between CB3474 [*ben-1(e1880)*] males and KR314: *mab-23 (e2518)* V hermaphrodites. Panels D and E contain the images of a hermaphrodite and male from the respective populations and where they were not on the same plate they are shown as separate sub panels (in the case of D). The N2 reference susceptible control worms (A) and N2/KR314 worms (D) were curled up and did not have sigmoidal movement, while the CB3474 [*ben-1(e1880)*] reference resistant control worms (B) KR314: *mab-23 (e2518)* V worms (C) and CB3474/KR314 worms (E) displayed normal morphology and sigmoidal movement.



Population	Median Speed (a.u.)	N
N2	1.7	315
CB3474	10.20	608
KR314	7.6	556
N2/KR314	3.7	557
CB374/KR314	9.15	562

Figure 5.5 Speed distributions of worm populations used in the genetic complementation experiments for the KR314: *mab-23 (e2518)* V strain after exposure to 0.3mM mebendazole. Box and whisker plots represent complete range of speed values recorded for each population from minimum to maximum. Horizontal lines within boxes indicate the median speed of each population and the corresponding values are indicated in the table to the right of the box plots (N is the number of data points on which these values are based on). Each population was compared to the N2 population as well to each other as is relevant such as indicated by the bars above box plots. ns= non significant and * , **** indicates P<0.05

Table 5.3 Comparison of populations from the genetic complementation experiment for the KR314: *mab-23 (e2518)* V strain

Dunn's multiple comparisons test	Significant?	Summary	Adjusted P Value
N2 vs. CB3474	Yes	****	< 0.0001
N2 vs. KR314	Yes	****	< 0.0001
N2 vs. N2/KR314	Yes	****	< 0.0001
N2 vs. CB3474/KR314	Yes	****	< 0.0001
CB3474 vs. KR314	Yes	****	< 0.0001
CB3474 vs. N2/KR314	Yes	****	< 0.0001
CB3474 vs. CB3474/KR314	No	ns	0.2249
KR314 vs. N2/KR314	Yes	****	< 0.0001
KR314 vs. CB3474/KR314	Yes	*	0.0476
N2/KR314 vs. CB3474/KR314	Yes	****	< 0.0001

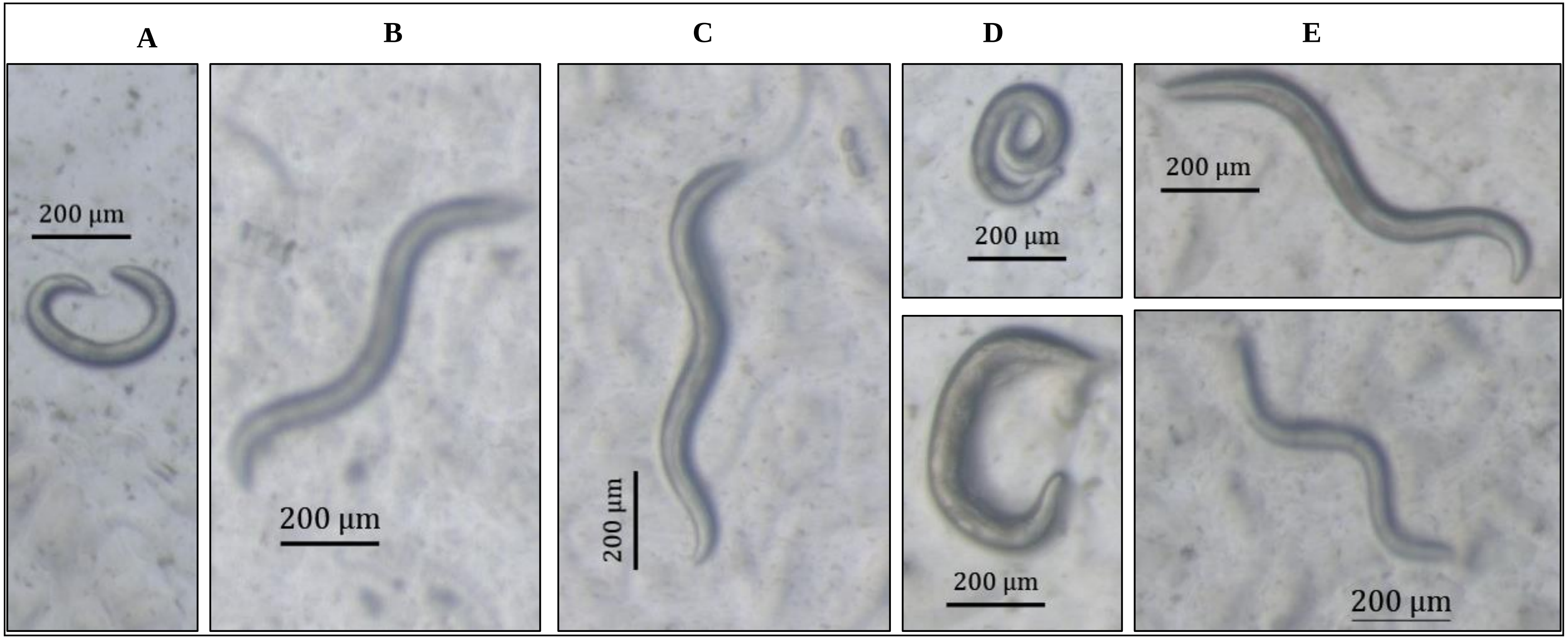
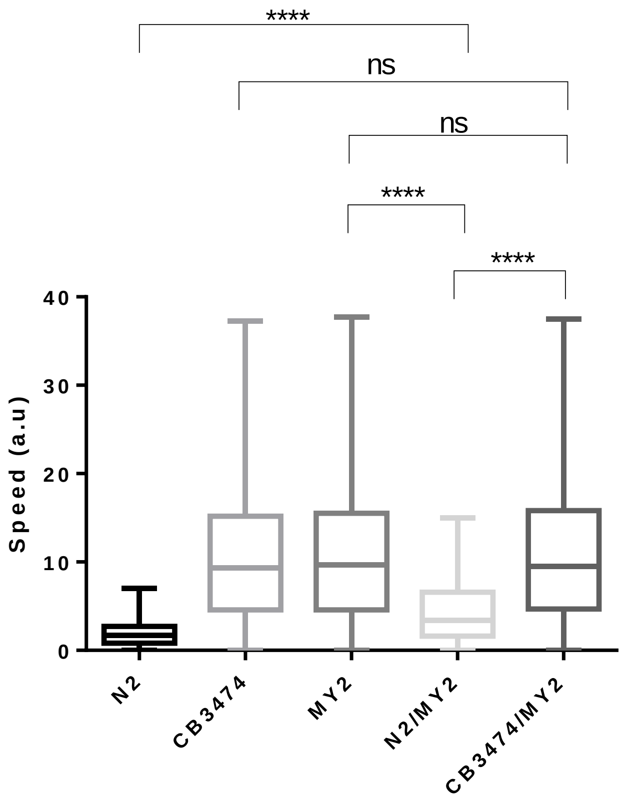


Figure 5.6 Appearance of worms grown on 0.3mM MBZ containing plates for genetic complementation experiments for the MY2 strain. A: N2; B: CB3474: *ben-1 (e1880)* III; C: MY2; D: N2/MY2; E: CB3474/MY2. N2/MY2 indicates F1 progeny from cross between N2 males and MY2 hermaphrodites and CB3474/MY2 indicates F1 progeny from cross between CB3474 [*ben-1(e1880)*] males and MY2 hermaphrodites. Panels D and E contain the images of a hermaphrodite and male from the respective populations and where they were not on the same plate they are shown as separate sub panels (in the case of D). The N2 reference susceptible control worms (A) and N2/MY2 worms (D) were curled up and did not have sigmoidal movement, while the CB3474 [*ben-1(e1880)*] reference resistant control worms (B) MY2 worms (C) and CB3474/MY2 worms (E) displayed normal morphology and sigmoidal movement. * Genetic complementation crosses for the KR314 and MY2 strains were set up at the same time and therefore the same images have been used for the controls in panels A and B in Figures 5.4 and 5.6.



Population	Median Speed (a.u.)	N
N2	1.7	488
CB3474	9.3	919
MY2	9.7	506
N2/MY2	3.4	624
CB374/MY2	9.5	900

Figure 5.7 Speed distributions of worm populations used for genetic complementation experiments of the MY2 strain after exposure to 0.3mM mebendazole. Box and whisker plots represent complete range of speed values recorded for each population from minimum to maximum. Horizontal lines within boxes indicate the median speeds of each population and the corresponding values are indicated in the table to the right of the box plots (N is the number of data points on which these values are based on). Each population was compared to the N2 populations as well to each other as is relevant such as indicated by the bars above box plots. ns= non significant and **** indicates P<0.0001

Table 5.4 Comparison of populations from the genetic complementation experiment for the MY2 strain

Dunn's multiple comparisons test	Significant?	Summary	Adjusted P Value
N2 vs. CB3474	Yes	****	< 0.0001
N2 vs. MY2	Yes	****	< 0.0001
N2 vs. N2/MY2	Yes	****	< 0.0001
N2 vs. CB3474/MY2	Yes	****	< 0.0001
CB3474 vs. MY2	No	ns	> 0.9999
CB3474 vs. N2/MY2	Yes	****	< 0.0001
CB3474 vs. CB3474/MY2	No	ns	> 0.9999
MY2 vs. N2/MY2	Yes	****	< 0.0001
MY2 vs. CB3474/MY2	No	ns	> 0.9999
N2/MY2 vs. CB3474/MY2	Yes	****	< 0.0001

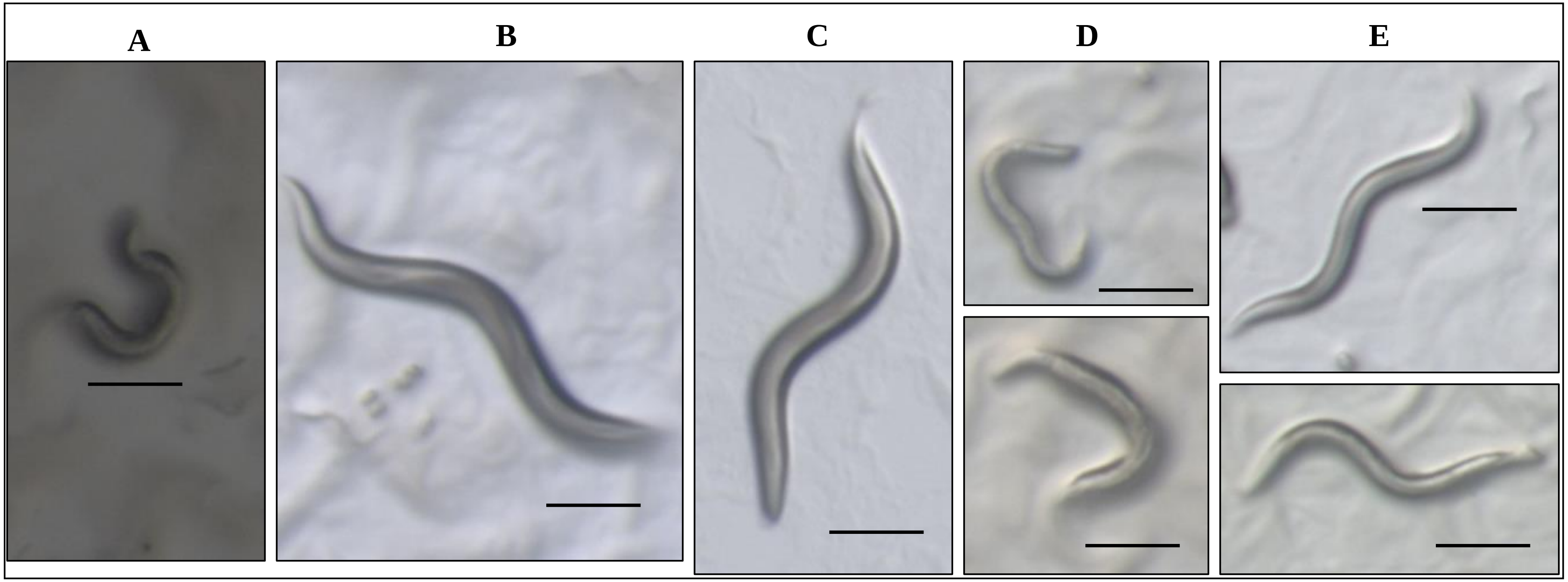
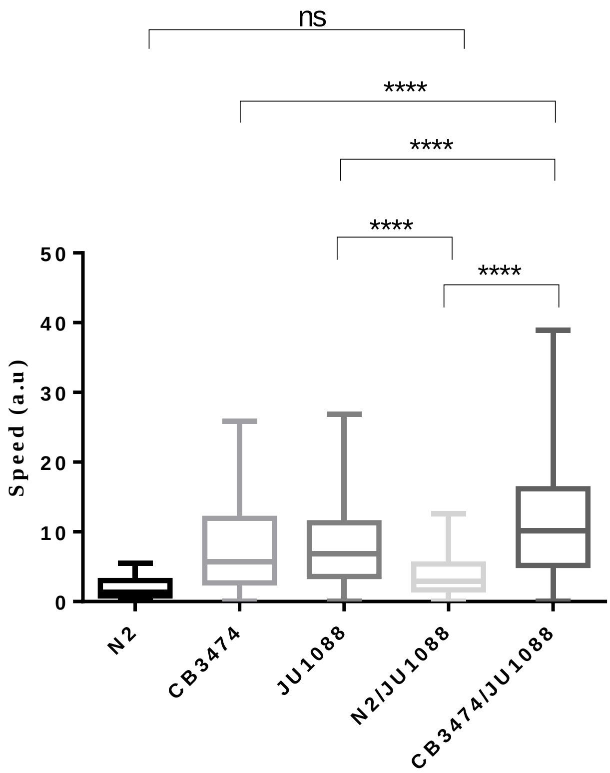


Figure 5.8 Appearance of worms grown on 0.0045mM MBZ containing plates for genetic complementation experiments for the JU1088 strain. Scale bar = 200µm. A: N2; B: CB3474: *ben-1 (e1880)* III; C: JU1088; D: N2/JU1088; E: CB3474/JU1088. N2/JU1088 indicates F1 progeny from cross between N2 males and JU1088 hermaphrodites and CB3474/JU1088 indicates F1 progeny from cross between CB3474 [*ben-1(e1880)*] males and JU1088 hermaphrodites. Panels D and E contain the images of a hermaphrodite and male from the respective populations and where they were not on the same plate they are shown as separate sub panels (in the case of D). The N2 reference susceptible control worms (A) and N2/JU1088 worms (D) were curled up and did not have sigmoidal movement, while the CB3474 [*ben-1(e1880)*] reference resistant control worms (B) JU1088 worms (C) and CB3474/JU1088 worms (E) displayed normal morphology and sigmoidal movement.

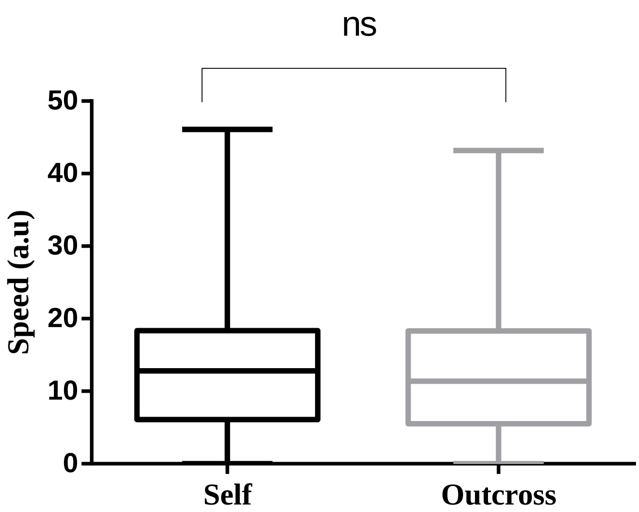


Population	Median Speed (a.u.)	N
N2	1.35	28
CB3474	5.7	221
JU1088	6.9	327
N2/JU1088	2.9	284
CB374/JU1088	10.2	624

Figure 5.9 Speed distribution of worm populations used in the genetic complementation experiments for the JU1088 strain after exposure to 0.0045mM mebendazole. Box and whisker plots represent complete range of speed values recorded for each population from minimum to maximum. Horizontal lines within boxes indicate the median speed of each population and the corresponding values are indicated in the table to the right of the box plots. Each population was compared to the N2 populations as well to each other as is relevant such as indicated by the bars above box plots. ns= non significant and **** indicates P<0.0001

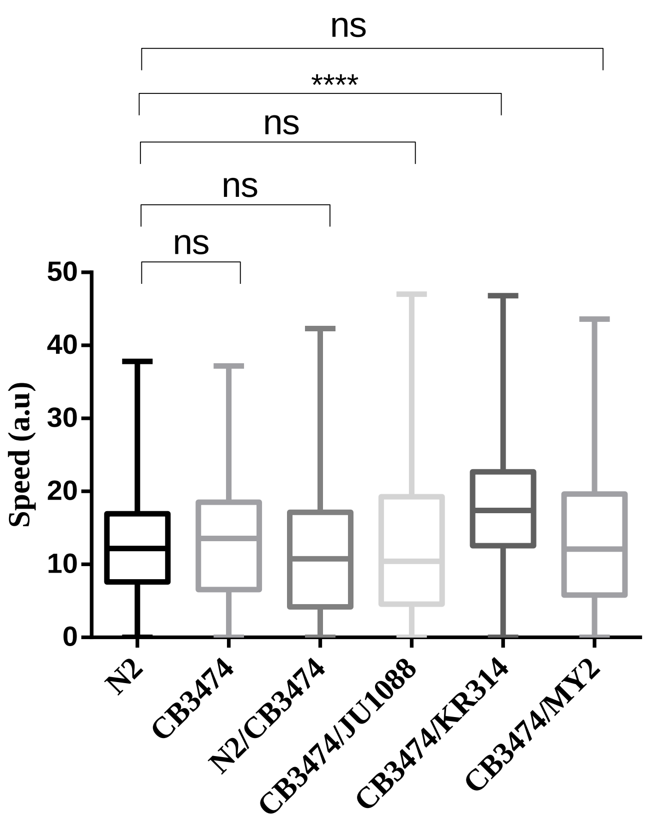
Table 5.5 Comparison of populations from the genetic complementation experiment for the JU1088 strain

Dunn's multiple comparisons test	Significant?	Summary	Adjusted P Value
N2 vs. CB3474	Yes	****	< 0.0001
N2 vs. JU1088	Yes	****	< 0.0001
N2 vs. N2/JU1088	No	ns	0.1337
N2 vs. CB3474/JU1088	Yes	****	< 0.0001
CB3474 vs. JU1088	No	ns	> 0.9999
CB3474 vs. N2/JU1088	Yes	****	< 0.0001
CB3474 vs. CB3474/JU1088	Yes	****	< 0.0001
JU1088 vs. N2/JU1088	Yes	****	< 0.0001
JU1088 vs. CB3474/JU1088	Yes	****	< 0.0001
N2/JU1088 vs. CB3474/JU1088	Yes	****	< 0.0001



Population	Median Speed (a.u.)	N
Self	12.8	1077
Outcross	11.4	696

Figure 5.10 Comparison of F1 progeny resulting from self fertilisation and out crossing in N2 worms not exposed to drug. Box and whisker plots represent the minimum to maximum speed values in each population. Horizontal lines within boxes indicate median speed and the corresponding values are indicated in the table to the right of the plots. Mann-Whitney test showed that the two populations are not different to one another (p=0.0891).



Population	Median Speed (a.u.)	N
N2	12.2	521
CB3474	13.55	282
N2/CB3474	10.75	248
CB374/JU1088	10.45	170
CB3474/KR314	17.4	313
CB3474/MY2	12.1	189

Figure 5.11 Speed distributions of parental and F1 cross progeny populations not exposed to drug. Box and whisker plots represent complete range of speed values recorded by the NemaRx system from minimum to maximum. Horizontal lines within boxes represent median speed and the corresponding values for each population are given in the table to the right of the plots. a.u= arbitrary units; **** indicates p <0.0001. All populations were compared to the N2 strain and except in the case of CB347/KR314 were not significantly different to N2 (p>0.05).

Chapter 6: General Discussion

6.1 Significance of findings

The objective of this thesis was to identify novel mutations or mechanisms that can confer benzimidazole resistance in nematodes. Due to the practical experimental limitations of parasitic nematodes, the model organism *C. elegans* was used. Previous studies on drug resistance and mode of action have used chemically mutagenised strains of *C. elegans* to identify mutations in the nematode genome that can give rise to anthelmintic resistance. In this thesis I have explored a different approach: to use wild strains that are naturally resistant to an anthelmintic (in this case benzimidazoles) to investigate the polymorphisms responsible for this resistance. I hypothesised that this approach may potentially identify a different set of resistance mutations to those identified by previous mutagenesis studies and may identify polymorphisms that are less likely to confer a fitness cost in natural field situations.

In this study, I screened a number of wild *C. elegans* strains with a high degree of genetic diversity (Figure 1.7) for resistance to the anthelmintic albendazole and identified seven strains (KR314, MY2, JU1171, MY14, MY16, JU1088 and GXW1) that showed varying levels of benzimidazole resistance relative to the susceptible phenotype of the N2 reference strain. Since amino acid substitutions in the β-tubulin drug target are the currently known mechanism of benzimidazole resistance in both parasites and chemically mutagenised *C. elegans* strains, I sequenced the *ben-1* gene which encodes this protein in *C. elegans*. This was done in order to determine if there were polymorphisms in this gene in the wild strains relative to the reference N2 sequence which could potentially explain the resistant phenotype of these strains. Indeed this was the case and therefore I proceeded to perform genetic complementation experiments to confirm the functional significance of these polymorphisms.

In this investigation I discovered three naturally occurring non-synonymous amino acid substitutions and a deletion in the *C. elegans* β-tubulin (BEN-1) that can confer benzimidazole resistance. The amino acid substitutions produced by the non-synonymous polymorphisms are: (1) an alanine to proline change at residue 185 (A185P) in strains MY2, MY14, MY16 and JU1171; (2) a methionine to isoleucine change at residue 257 (M257I) in strain JU1088 and (3) an aspartic acid to asparigine change at residue 404 (D404N). In addition a single base pair deletion in codon 71 which leads to a frame shift resulting in a premature stop codon at amino acid 119 in the KR314 strain was also identified (Figure 4.8). A number of main conclusions and implications can be driven from these results:

1. The *ben-1* mutations giving rise to benzimidazole resistance in *C. elegans* in this study have not been reported in parasitic nematodes.
2. The polymorphisms commonly giving rise to resistance in parasitic nematodes (at positions 167, 198 and 200 in the trichostrongylids) have not been found in the wild populations of *C. elegans* examined. This suggests that the β-tubulin substitutions giving rise to benzimidazole resistance may be different in different nematode species.
3. Benzimidazole resistance occurs in natural *C. elegans* populations suggesting selection occurs in the environment. This could be the result of either anthropomorphic environmental contamination with pharmaceuticals or by naturally occurring compounds in the environment.
4. The main causal polymorphisms of benzimidazole resistance in natural *C. elegans* populations are mutations in the β-tubulin gene *ben-1*. These include a frame shift deletion which suggests that worms can tolerate a loss of the *ben-1* β-tubulin gene in natural populations demonstrating its functional redundancy even in the natural environment and,

5. The β-tubulin substitutions identified in *C. elegans* likely have secondary structural consequences in the protein and therefore likely have effects on the three dimensional structure and drug binding

I will discuss each of these conclusions in turn in the sections (6.2 to 6.5) that follow.

6.2 The benzimidazole resistance polymorphisms identified in *C. elegans* in this study are unusual relative to those previously reported in other organisms

The striking feature of the findings from my research is that all of the causal polymorphisms identified in this study have not been previously reported in field resistance of benzimidazoles in any other type of organisms including nematodes or fungi. The only instance where a mutation at the same residue identified in this study (Ala to Pro at 185) is even remotely associated with benzimidazole resistance is in a laboratory mutant strain of fungi. Even here, it is an alanine to serine change (A185S) and it is only found in combination with three other codon changes at residues 179 (Val179Gly), 190 (His190Asn) and 198 (Glu198Ala) (Albertini, Gredt et al. 1999). Even the opposite scenario is true in this case, in that neither do any of the mutations identified so far in parasitic species exist in the wild *C. elegans* strains resistant to benzimidazoles. In fact, neither do these mutations appear in any of the remaining strains not included in this study but have had their whole genomes sequenced through the million mutations project (Table 6.2A) (Thompson, Edgley et al. 2013).

In parasitic nematodes, the mutations associated with benzimidazole resistance are F200Y, E198A and F167Y (Table 6.1) (Kwa, Veenstra et al. 1994; Ghisi, Kaminsky et al. 2007; Rufener, Kaminsky et al. 2009), while in fungi in addition to the F200Y, E198A (also E198Q, E198G, E198K, E198V) and F167Y changes, substitutions at residues 6, 50 and 240 have also been implicated in field resistance (Fujimura, Kamakura et al. 1992; Jung, Wilder et al. 1992; Koenraadt, Somerville et al. 1992; Albertini, Gredt et al. 1999; Ma, Yoshimura et al. 2003). Furthermore, mutations at residues 198 and 200 have also been correlated with benzimidazole resistance in parasitic protozoa such as *T.vaginalis* and *G.intestinalis* (Katiyar, Gordon et al. 1994).

It is quite intriguing that parasitic nematodes would share causal mutations of benzimidazole resistance with distant species such as fungi and protozoa, but not with another nematode such as *C. elegans*. This may simply be a coincidence given that only a small subset of strains was included in my study (16 of the 200 or so isolated to date). Furthermore only 40 of the 200 strains isolated so far have had their whole genomes sequenced and one cannot entirely rule out the possibility that the causal polymorphisms found in parasites may occur in at least some of the 160 remaining strains.

On the contrary, this could mean that there may be differences in the actual dynamics of interaction of benzimidazoles with the β-tubulin in *C. elegans* to that of its parasitic counterparts. This is hinted at in the study by Kwa et al. (1995) where the β-tubulin isotype-1 gene of *H. contortus* when heterologously expressed in the *C. elegans* mutant strain resistant to benzimidazole, failed to restore susceptibility in the presence of benomyl (but did so in the presence of thiabendazole). Hence, the researchers suggested that the β-tubulins from these two species have varying affinity towards benomyl (Kwa, Veenstra et al. 1995).

Another possible explanation is that the mutations in *C. elegans* are present by chance (discussed below) whereas those in the two parasitic species (both helminths and fungi) occur due to selection pressure and are thus more localised. Since *C. elegans* is a non-parasitic species it may be exposed much less selection pressure from drug exposure compared to the parasitic nematode species.

One of the important implications of my results is that they provide functional evidence that the substitutions A185P and M257I found in the wild *C. elegans* strains can confer benzimidazole resistance under field conditions and point to additional mutations that might be found in parasitic species. Although with regard to benzimidazole resistance in some veterinary parasites many consider it a situation where it is like ‘closing the barn door after the horse has already bolted’, there are still many parasite species, including those of humans, in which the situation with benzimidazole resistance is less clear. Researchers developing molecular tools for detection of resistance in human hookworms tend to focus on the regions around the P200, P198 and P167 residues that have been associated with benzimidazole resistance in veterinary parasites (Prichard 2007; Diawara, Schwenkenbecher et al. 2013). However, in *N.americanus*, *A.duodenale* and *A.lumbricoides* the F200Y substitution was not detected by pyrosequencing of this region (Albonico, Wright et al. 2004; Diawara, Drake et al. 2009). My results suggest that it is important to examine other regions of β-tubulin drug target and provide a number of candidate mutations that could be screened for. Therefore, it is recommended that the potential importance of point mutations other than that at residue 200 be explored for human parasites (Albonico, Engels et al. 2004).

6.3 Speculations on the origin of benzimidazole resistance polymorphisms in wild *C. elegans* populations

6.3.1 Insights from parasite population genetics

In parasitic helminth populations, resistance mutations are believed to arise by way of four distinct processes: (1) a pre-existing (ancient) mutation may give rise to multiple divergent alleles through mutation and selection. Upon drug exposure, the increase in frequency of the individuals carrying these alleles will depend on how long the allele had pre-existed before drug exposure and the fitness cost associated with it upon drug exposure; (2) a novel mutation may arise just prior to or following drug exposure. This mutation will then give rise to alleles with less sequence divergence compared to (1); (3) multiple novel resistance mutations may arise on different genetic backgrounds or (4) mutations may be acquired from a different population following migration (Gilleard and Beech 2007).

Support for the notion of pre-existing resistance alleles comes from several early studies. In an *in vitro* selection study it was shown that ivermectin resistance appears in as few as three generations in *H. contortus* (Coles, Rhodes et al. 2005) , while RFLP hybridization studies for the β-tubulin isotype-1 locus with susceptible and resistant field isolates of *H. contortus* and *T. columbriformis* showed reduction in hybridizing fragments in the resistant population compared to the susceptible suggesting selection (Roos, Boersema et al. 1990; Grant and Mascord 1996). However, the assumption of pre-existence of resistance alleles is only valid if it is backed up by the knowledge that the susceptible population had never previously been exposed to the drug (Gilleard and Beech 2007).

Generally in parasitic populations, the presence of the same resistant allele in independent (or geographically separated) populations indicate that it is possibly ancient while presence of unique alleles in a population suggests spontaneous or independent origins for the mutations (Silvestre and Humbert 2002). However, there is a possibility that the presence of an allele in multiple populations could also be due to the lack of genetic differentiation between the populations (Gilleard and Beech 2007).

Speculations regarding the origin of mutations can also be drawn from genome-wide studies since the selection pressure acting on a genetic locus can have an effect on the genetic variation patterns in the area flanking it. This is called a ‘genetic foot print of selection’ and is influenced by genetic hitchhiking and meiotic recombination. In the absence of recombination, there will be a decrease in variation around a particular locus under selection leading to linkage disequilibrium. However, in the event that meiotic recombination takes place, markers associated with the locus under selection may be exchanged with a different haplotype based on the physical distance from this locus (Hartl and Clark 1997). In addition, with large effective population sizes (as seen with parasitic nematodes), recombination tends to break linkage between markers and resistant alleles within a very short period of time. In this case, the same resistance allele (if pre-existing) can occur in different genetic backgrounds. However, with spontaneous mutations (such as those arising due to an external selective force), there would not be much opportunity for recombination to break the linkage between the resistance allele and markers associated with it. This leads to reduction in variability in the flanking region (Hermisson and Pennings 2005). This has been observed with premethamine treated *Plasmodium falciparum* (Nash, Nair et al. 2005). In parasitic nematodes, benzimidazole resistance mutations F200Y, E198A and F167Y are quite ubiquitous. Thus in these populations the presence of the same resistance mutation on different haplotypes is probably due to a combination of factors such as large effective population sizes and the mutations themselves having the possibility of being pre-existing, spontaneous or acquired through migration (Gilleard and Beech 2007; Messer and Petrov 2013).

6.3.2 Possible scenarios for *C. elegans*

Following from the discussion of population genetics of resistance mutations in parasitic nematodes above, speculations can be made for the origin of benzimidazole resistance mutations in *C. elegans* strains identified in this thesis. *C. elegans* has a recombination rate of 3 cM/Mb (higher than sexually dimorphic organisms) possibly due to its hermaphroditic life style (Barnes, Kohara et al. 1995) and is estimated that the nuclear DNA mutation rate in *C. elegans* is around 9×10^{-9} which is roughly two mutations per genome per generation given the size of the genome (Denver, Morris et al. 2004). These values indicate that novel polymorphisms would be arising in the *C. elegans* genome at a considerably high rate. Thus, benzimidazole resistance polymorphisms identified in this project may be simply present as part of the standing genetic variation. Alternatively, they may be present due to selection pressure resulting from exposure to benzimidazoles in the environment. This notion is supported by studies that address the routes by which anthelmintics enter and persist in the environment. In the subsections that follow I address each of these possibilities (ancient/spontaneous) for the origin of resistance polymorphisms in *C. elegans* based on existing evidence for each of the scenarios.

6.3.2.1 Are the benzimidazole resistance polymorphisms related phylogenetically or geographically?

In section 6.3.1 it was noted that generally the presence of the same resistance allele in geographically separated locations implies that it is ancient (or that there is lack of genetic differentiation between populations) whereas the presence of unique alleles indicates that they may be more recent and more likely to have arisen due to selective pressures. The substitutions identified in *C. elegans* tend to fall into both these categories, i.e. there are shared as well as unique polymorphisms.

Three strains from Germany (MY2, MY14 and MY16) carry the same A185P change in β-tubulin as does one strain from Chile (JU1171) (Table 1.2 and Figure 6.1). The other two mutations (M257I) and (G404N) appear to be unique to the strains in which they were identified (JU1088 from Japan and GXW1 from China respectively), and so is the deletion in the strain from Vancouver (KR314). This uniqueness is apparent when looking at whole genome sequencing data for the 40 wild isolates included in the million mutations project (Thompson, Edgley et al. 2013) available at http://genome.sfu.ca/mmp/search.html. Thus, inferences regarding the existence of these mutations may be drawn with regard to phylogeny or location of isolation.

Studies carried out to infer the phlyogenetic relationships among wild *C. elegans* strains include genome-wide microsatellite (Haber, Schüngel et al. 2005) and RAD (Andersen, Gerke et al. 2012) marker based analyses. The former included a total of 58 wild strains while the latter included all 200 strains isolated to date. The strains used in my project were part of one or both of these studies (Table 1.2) and have thus been assigned microsatellite genotypes or isotype groups (the term " isotype" was used in the RAD marker based study to refer to a unique genome-wide haplotype). Both studies have revealed interesting patterns of genomic diversity among the strains, which in the context of this thesis has been useful to speculate on the origin of the resistance conferring polymorphisms.

Sharing of the A185P substitution among *C. elegans* strains

Of the strains that carry the A185P substitution, strains MY2 and MY14 (from Roxel and Mecklenbeck Germany respectively) have the same microsatellite genotype (EU2) while also falling into the same isotype set (MY23) along with the JU1171 strain from Chile (Table 1.2 and Figure 6.1). However, the MY16 strain has a unique microsatellite genotype (EU6) and also falls into a unique isotype set (MY16), although both isotypes MY16 and MY23 are within the same clade (Figure 1.7). According to these results it may be inferred that these strains may share the A185P substitution due to close phylogeny (Figures 1.7 and 6.1). Strain JU1171 may be a result of long range dispersal and colonisation of either the MY2 or MY14 strains. This is possible due to the fact that *C. elegans* is mostly found in human-associated habitats and also in association with invertebrate species (snails, slugs, myriapods, isopods, insects and mites) primarily in the form of dauers. This enables long range dispersal (which in addition is also be facilitated by birds or other mammals) followed by their classic 'boom and bust' style colonisation of the new habitats (Félix and Braendle 2010).

Of the 97 isotype groups identified in the RAD study, two groups included strains from distant geographic locations, one of them being the MY23 haplotype which includes the MY2 and MY14 strains from Germany as well as the JU1171 strain from Chile (Figure 6.1) (Andersen, Gerke et al. 2012). While the researchers also suggest that this observation may be due to long range dispersal events, they also allude to the possibility of a simple case of strain confusion. However, this scenario may be unlikely as previously explained (Section 4.5.2) and the long range disposal hypothesis is still a possibility. Although exact out crossing rates for *C. elegans* in natural habitats are not known it is possible that migration events supplemented by rare out crossing may lead to identical genotypes over geographically separated locations which may explain this sharing of mutations across distant geographic locations (Sivasundar and Hey 2003). A more comprehensive analysis of sharing of variations among strains in genes on different chromosomes will be able to provide more definitive conclusions.

Unique substitutions and deletions among *C. elegans* strains

The rest of the benzimidazole resistance conferring mutations (M257I, D404N and 1bp deletion at codon 71) identified in my project appear to be unique to the strains (JU1088, GXW1, KR314 respectively) that carry them (Table 6.2A). However, they may well be present in strains for which sequence data is not available, since only 40 of the 200 or so isolated to date have had their genomes sequenced (Andersen, Gerke et al. 2012; Thompson, Edgley et al. 2013).

The JU1088 strain (M257I) falls into a unique isotype set and does not share haplotypes with any of the 200 other strains (Andersen, Gerke et al. 2012). Since this mutation does not occur in any other strains either, it may be a recent event and possibly occurred due to spontaneous mutation. The GXW1 strain (D404N) was not included in any of the genotyping studies and neither does it share this mutation with any of rest of the 39 isolates whose genomes were sequenced (Thompson, Edgley et al. 2013). Also these are the only strains isolated from Japan and China respectively. Isolation of additional strains from these locations and comparison of the variations found in these strains and also looking at variations shared between these and others strains from other geographic locations would be informative.

Strain KR314 which has a frame-shift mutation in the β-tubulin locus, has a unique microsatellite genotype (NA8) (Haber, Schüngel et al. 2005) and falls into a unique isotype set (Andersen, Gerke et al. 2012). Like in the case of the previous strains, this is the only one isolated from the whole of Canada.

Of the wild strains studied in the million mutations project four other strains (CB4856, ED3021, MY6 and JU360) also have deletions in the β-tubulin gene (Table 4.4). The CB4856 strain has a ten base pair deletion leading to an in frame deletion of amino acids 440-442. This strain was included in my project and was characterised as having a susceptible phenotype. The ED3021 (isotype ED3005) and MY6 (isotype MY18) strains have the same 2bp deletion at codon 58 (premature stop at 68) while the JU360 strain has a 9bp deletion at codon 322. These three strains were not examined within my project and thus comments on their susceptibility to benzimidazoles cannot be made. ED3021 and MY6 are from Scotland and Germany respectively. Unlike the previous set (MY2, MY14, and

JU1171) where all variations were shared (Table 4.3), analysis of non-synonymous variations in genes close to *ben-1* for these two strains shows that MY6 contains only a subset of the mutations in the ED3021 strain (data not shown). However, the two strains contain very closely related haplotypes reflective of their common ancestry (Figure 1.7) (Andersen, Gerke et al. 2012).

Inferences from the distribution of polymorphisms among *C. elegans* strains

Considering the facts discussed in the above sections, it appears that the sharing of mutations in *C. elegans* is more dependent on phylogeny rather than location of isolation (Figures 1.7 and 6.1). The inferences are also in support of the life style of this species which involves long range dispersal followed by rapid colonisation (Félix and Braendle 2010), resulting in the presence of the same resistance allele in different geographic locations. Thus it appears as though the resistance polymorphisms are part of the neutral genetic variation (alleles are pre-existing). This being said, the presence of unique resistance polymorphisms and deletions do not support this notion. Furthermore, as noted for parasitic nematodes, the pre-existent allele hypothesis is only valid if one is absolutely sure that the populations in which they are found have never been exposed to anthelmintics. However, given the ubiquitous nature of *C. elegans* and the presence of benzimidazole residues in the environment (as discussed below) one cannot be absolutely sure that these strains come from completely naive populations. Therefore, based on the information discussed in this section it is difficult to make a conclusion regarding the origin of benzimidazole resistance polymorphisms in these *C. elegans* strains since both scenarios seem equally likely.

6.3.2.2 Are the resistance polymorphisms the result of selection by benzimidazoles or related compounds or simply present as part of the standing genetic variation?

As noted earlier, there are two possible explanations for the presence of benzimidazole resistance polymorphisms in the *ben-1* gene

(i) They are present as part of the neutral standing genetic variation

(ii) They are present as the result of selection by benzimidazole drugs or related compounds

Inferences based on the geographic distribution of benzimidazole resistance conferring polymorphisms

In parasitic species the same amino acid substitutions in the β-tubulin drug target (F200Y, E198A and F167Y) that give rise to benzimidazole resistance can be found in many different geographic locations. However, based on the sequence information available for 40 wild isolates, resistance polymorphisms do not appear as ubiquitously in *C. elegans.* The A185P substitution is only found in some of the strains from Germany and one from Chile while the other two substitutions M257I (in JU1088 from Japan) and D404N (in GXW1 from China) appear to be unique to those strains. Even the deletions in the *ben-1* gene are mostly unique to the strains they are found in. This may be an indication that the occurrences of these mutations are probably by chance and were rapidly fixed in the genome due to the hermaphroditic life style of *C. elegans.* However, as indicated earlier, sequence information is only available for 40 of the 200 wild strains isolated thus far and one cannot predict that these polymorphisms will not be present in the 160 isolates whose genomes remain to be sequenced. Once this information is available more solid inferences can be made regarding this hypothesis of the presence of substitutions as part of the standing genetic variation.

The possibility of selection pressure cannot be ruled out as I was unable to find any solid evidence for chances of these strains being exposed to benzimidazoles given the lack of knowledge regarding the history of these strains (Dr. Schulenburg pers. comm). Hence, given the widespread use of benzimidazoles (discussed below) there is indeed a possibility that these strains may have encountered benzimidazoles at some point in their life history which led to the appearance and fixation of these polymorphisms. However, since *C. elegans* is a non-parasitic species, there is also the question of whether these strains even though they encounter benzimidazoles, it would be on a scale similar to those encountered by their parasitic counterparts. Investigating the levels of benzimidazole residues in *C. elegans* isolation locations may provide further insights in this regard. This will in turn help to make more informed conclusions about whether these polymorphisms were indeed selected for.

Inferences based on the ubiquitous nature of *C. elegans*

Since *C. elegans* is such a global species, benzimidazole resistance associated mutations should be a more common occurrence had there been selection pressure imposed on its genome as benzimidazoles are widely used in many parts of the world. The fact that only very few strains out of those isolated to date exhibit resistance conferring mutations points to the fact that these occurrences are more than likely coincidental. However, this depends on the amount of benzimidazoles encountered by the strains and whether they are high enough to exert selection pressure.

Mutations conferring resistance to avermectin and the bacterium that produces it, *Streptomyces avermetilis*, are thought to be maintained by long term balancing selection (advantage in the presence of bacterium but fitness cost in terms of brood size in its absence) since the bacterium and *C. elegans* both share a common habitat in soil (Ghosh, Andersen et al. 2012). Whether such a scenario is applicable to benzimidazoles is questionable since benzimidazoles are chemically synthesised and thus may not be as commonly encountered by *C. elegans* as a habitat sharer such as *S. avermetilis*.

Inferences based on the location of the *ben-1* gene on chromosome III

On the other hand, adaptive evolution or positive selection is not uncommon in *C. elegans* and is seen for example with chemoreceptor (*srz*) gene families. The observed non-synonymous to synonymous mutation ratios >1 for this family led to the deduction of positive selection driven by ligand binding because most changes were found in the extracellular ligand binding domain (Thomas, Kelley et al. 2005). Similar observations regarding chemoreceptor genes (MATH-BTB, F-box lectin and serpine chemoreceptor genes involved in environmental response and innate immunity) were obtained also from a study that looked at copy number variations in the genomes of wild *C. elegans* strains (Maydan, Lorch et al. 2010). The researchers observed that deletions in these genes accounted for 83% of the total indels identified in this study (Maydan, Lorch et al. 2010). In fact, these genes are located in the arms of chromosomes where increased recombination is afforded. Clustered genes (mostly have no detectable RNAi phenotype and coding for nematode specific secreted and transmembrane proteins, detox systems, innate immunity components) in fact are said to occur more frequently in chromosome arms with higher recombination rate and therefore undergo rapid evolution upon changing environmental circumstances (Thomas 2006). This has also been the case with traits such as abamectin resistance, pathogen avoidance and hybrid incompatibility. The genes that control these traits are thought to lie in the arms of chromosomes because it affords more variation. Therefore in is suggested that such traits are deliberately placed in those regions for adaptive flexibility (Rockman and Kruglyak 2009; Andersen, Gerke et al. 2012; Ghosh, Andersen et al. 2012).

A similar observation was also noted from the million mutations project data where whole genome sequencing of wild isolates showed that indels are frequent in the chromosome arms where rapidly evolving genes are located (Thompson, Edgley et al. 2013) possibly allowing for the quick adaptability required with the *C. elegans* life style. Since *ben-1* is located in the left arm of chromosome III it may be undergoing considerable evolution. To make more informed conclusions, non-synonymous to synonymous mutation ratios in this region need to be investigated in order to find evidence for selection. For instance genome-wide approaches such as RAD tagging may be undertaken on *C. elegans* populations that show phenotypic resistance and those that do not. Evidence for selection can then be identified in the form of valleys of reduced polymorphism centred across the *ben-1* locus or regions of linkage disequilibrium centered on the *ben-1* locus relative to other areas of the genome in resistant strains as opposed to susceptible ones.

Inferences based on the presence of anthelmintics in the environment and the possibility of them exerting selection pressure on wild *C. elegans* populations

Several studies have addressed the possibility of the presence of anthelmintics in the environment by means such as emissions during spraying of crops, contaminations associated with industrial production, presence in animal feed, excretions by treated animals etc. Benzimidazoles are used as antifungal agents as well as anthelmintics and therefore they can reach the environment by all the pathways mentioned above. Specifically, thiabendazole and benzimidazole carbamates such as carbendazim are used pre and post harvest and are either applied directly to the soil or sprayed over crop fields (Wu, Li et al. 2009) and it is suggested that the extensive use of these compounds may lead to their accumulation in the environment (Torres-Padron, Aufartova et al. 2010). Given the natural habitat of *C. elegans* (rotting fruit) and the isolation locations of many of these strains such as soil and compost heaps (Table 1.2) it seems likely that these strains may be exposed to the drug. This in turn may have imposed selective pressures and caused these resistance polymorphisms to occur.

Furthermore, based on heavy usage and sales of anthelmintics it is suggested that residues can easily enter the environment and indeed have been detected globally in soil and natural waters (Wall and Strong 1987; Boxall and Long 2005). Most notably, anthelmintics that have high potential of entering the environment include, fenbendazole, ivermectin, levamisole, morantel and triclabendazole (Boxall, Kolpin et al. 2003). These residues enter the environment predominantly through the excretion in urine and faeces of livestock or washing off of topical treatments (Boxall, Kolpin et al. 2003).

Once they enter the environment, depending on the pharmacokinetics of the drug (i.e. how and to what extent the drug is metabolised), a mixture of both parent compound and metabolite will enter and persist in the environment (Boxall, Fogg et al. 2004). Although considerable number of studies have addressed the fate and environmental consequences of ivermectin which show that it is quite persistent in the environment (Lumaret, Galante et al. 1993; Halling-Sørensen, Nors Nielsen et al. 1998), there is a paucity of such data for benzimidazoles. However, it is known that benzimidazoles are photo-sensitive and easily oxidizable (Horvat, Babić et al. 2012). Thus it may be unlikely that these compounds would persist in the environment for long periods of time so as to exert selection pressures in which case selection would not be strong for resistance polymorphisms in *C. elegans* strains. Therefore, more elaborate environmental studies regarding the presence and fate of benzimidazoles are required to make more informed speculations.

Another important area of research is to look for 'footprints of selection' in the *C. elegans* genome (specifically in this case at the *ben-1* locus and flanking regions) as alluded to at the end of the previous section. A recent study suggests that ivermectin resistance may be a trait maintained by balancing selection in wild *C. elegans* isolates (Ghosh, Andersen et al. 2012). A naturally occurring 4 amino acid deletion in the *glc-1* gene of many wild *C. elegans* strains was associated with ivermectin resistance in this study. Analysis of polymorphisms at this locus between resistant and susceptible strains (53 in total) revealed that,

(1) CB4856 sequence was highly divergent (30 times greater than the expected 1/840bp compared to N2

(2) non-synonymous to synonymous polymorphism ratio was <1 and only 6 distinct haplotypes existed

(3) SNPs flanking the *glc-1* locus were in complete linkage disequilibrium

(4) N2 and CB4856 like alleles were found worldwide with no geographic structuring

(5) strains resistant to avermectin were also resistant to the bacterium that produces it (*S. avermetilis*), but tend to have a smaller brood size in its absence.

Therefore, it was concluded that since the bacterium is cosmopolitan and soil-dwelling and so is *C. elegans*, the bacterium and the secondary metabolite it secretes (avermectin) may be exerting a selection pressure on this nematode which causes the resistance conferring mutation to be maintained by balancing selection.

Based on this study on ivermectin resistance, if *C. elegans* populations were under constant selection pressure from benzimidazole residues in the environment, one would expect to see similar scenarios with respect to the *ben-1* locus. In a preliminary attempt to address one of the aspects mentioned in the avermectin study, deriving from million mutations project data I found that of the total of 9 polymorphisms identified at the *ben-1* locus among 40 wild *C. elegans* strains only two are synonymous while the rest are non-synonymous (Table 6.2A). Of the 7 non-synonymous polymorphisms, according to data from my project at least 4 are capable of conferring varying levels of benzimidazole resistance (Table 6.2B and C). Drawing from this, given that most non-synonymous polymorphisms impart resistance it would be a remarkable coincidence if they are occurring by chance and not due to selection by drug pressure playing a role.

A recent study based on genome-wide RAD markers suggests that the patterns of genetic variation seen in *C. elegans* chromosome II and III (where the *ben-1* gene is located) do not show footprints of positive selection, whereas those on chromosomes I, IV, V and X (based on the existence of rare variants and shared haplotypes across genomes of many strains) have undergone strong selective sweeps due to positive selection (Figure 6.2). Thus while strong selective forces due to the dramatic changes associated with human populations may explain variation in chromosome I, IV, V and X, it is suggested that this is possibly not the case for chromosome II and III (Andersen, Gerke et al. 2012). Since the *ben-1* gene is located on chromosome III this alludes to the possibility that the benzimidazole resistance polymorphisms identified in this project may be part of the standing genetic variation that have been fixed due to recurrent population bottlenecks. However, the possibility of selection pressure exerted by the presence of benzimidazoles in the environment on a subset of strains (as identified in this project) cannot be completely ruled out. Therefore, further studies focusing on the *ben-1* and flanking loci investigating evidence of selection need to be undertaken to make conclusive statements.

Overall deductions for the presence of benzimidazole resistance polymorphisms in wild *C. elegans* strains

Considering the information presented in sections 6.3.2.2.1 through 6.3.2.2.4 it is difficult to make any definitive conclusions about the origins of the benzimidazole resistance polymorphisms in *C. elegans* populations without further studies as repeatedly emphasized throughout the discussion. Genome-wide studies to look at variations in and around the *ben-1* locus of more strains (both susceptible and resistant to benzimidazole) that indicate signatures of selection in this region (as done for the *glc-1* region implicated in avermectin resistance by Ghosh et al 2012), screening of more wild strains to characterise their benzimidazole phenotype and assessment of the presence of benzimidazole residues in *C. elegans* sampling locations are some areas of research that will prove informative in terms of the selection pressure hypothesis.

6.4 Evidence for functional redundancy of *ben-1* β-tubulin in *C. elegans*

Deletions in genes are fixed into the genomes of wild *C. elegans* strains due to the consistent bottlenecks experienced by this species as they depend on ephemeral resources for survival (Félix and Braendle 2010). In the laboratory, *C. elegans* strains that have deletions in the *ben-1* gene are perfectly viable. This was demonstrated by Driscoll et al. (1989) using chemically mutagenised strains. Strains with 28 different mutations in the *ben-1* gene (five of which were partial or complete deletions of the gene) were healthy when grown on agar plates under standard laboratory conditions. The researchers thus suggested that the *ben-1* gene is redundant and completely dispensable under laboratory conditions.

As addressed in the introduction of this thesis, mutations that allow survival under laboratory conditions may not necessarily occur in the wild due to fitness costs potentially associated with the loss of function of these genes. However, I have identified a *ben-1* deletion predicted to be a null mutation in a wild natural population; the KR314 strain in which a 1bp deletion in the *ben-1* gene leading to a premature stop codon is present. As discussed in Chapter 4, mRNAs that have a stop signal >50bp upstream of the last splice junction are degraded by the nonsense-mediated RNA decay machinery and therefore the KR314 strain possibly lacks the BEN-1 β-tubulin. Data from the million mutations project reveal that strains CB4856, ED3021, MY6 and JU360 also carry deletions in the *ben-1* gene (Thompson, Edgley et al. 2013). The presence of multiple strains with null mutations of the *ben-1* gene in the wild suggests it does not confer a major fitness cost (assuming that benzimidazoles are not common in the environment) and suggests its function may well be redundant under natural conditions.

Studies that have looked at pseudogene evolution patterns suggest that the *C. elegans* genome may have a high rate of spontaneous deletions and may be under selection for smaller genome size (Robertson 2000; Witherspoon and Robertson 2003; Denver, Morris et al. 2004). Although it is expected that when it comes to coding sequences, amplifications should be favoured over deletions, the primarily selfing reproductory mode of *C. elegans* coupled with the ‘boom-and-bust’ lifestyle that leads to constant population bottlenecks may be leading to smaller effective population sizes where selection against deletions cannot take place leading to their fixation (Félix and Braendle 2010; Maydan, Lorch et al. 2010). In addition, gene loss is also considered the most common form of adaptation (over SNPs or changes in regulatory mechanisms) when a novel selection pressure is encountered. The gene harbouring deletions then supposedly remains in the genome (unless it is a complete deletion) available for reversion (Olson 1999). This could be the case with these strains in that they may have encountered benzimidazole or some other type of selective force at some point which drove the fixation of these deletions in the *ben-1* gene. It may be that *ben-1* serves a function and it is advantageous to the organism but is not absolutely essential. However, in the face of benzimidazole selection it may have been more advantageous to lose this gene. In other words, the *ben-1* gene may confer a fitness advantage in the absence of drug exposure but a fitness cost in the presence of drug. Redundancy of this gene may be a further contributing factor in that it may be relatively easily dispensed with.

6.5 Structural changes in the BEN-1 β-tubulin due to changes in the amino acid sequence

Identification of substitutions within the drug binding site facilitates the further understanding of the drug mode of action and mechanism of resistance which in turn affords the possibility of designing new and more effective drugs. In this section I will firstly provide some background information on the structure of β-tubulin. Secondly, I discuss some currently available simulation models which provide insights into the structural consequences of amino acid substitutions present in parasitic species that are resistant to benzimidazoles. Finally, drawing insight from these models and other resources, I include speculations on how the polymorphisms identified in this thesis may affect the structure of β-tubulin and thus affect drug binding and lead to a resistant phenotype.

6.5.1 The β-tubulins: structural subunits of microtubules

Microtubules are polymers of heterodimers consisting of α and β tubulin monomers (Figure 1.2). These monomers share nearly 40% sequence identity and are about 450 amino acids in length while also possessing identical secondary structures consisting of a core of two β-sheets (made of 4 and 6 strands) surrounded by 12 α-helices (Nogales, Wolf et al. 1998). Each monomer has three domains: The amino (N) terminal (residues 1-205), intermediate (residues 206-381) and the highly acidic carboxy (C) terminal (residue 381 to stop) (Figure 6.3). The C terminal domain has a hyper-variable region towards the end which varies among the different β-tubulin monomer isotypes and across species (Ludueña 1997). The N, intermediate and C domains of the β-tubulin monomer can also be characterised as nucleotide (GTP) binding, drug binding and motor (kinesins) or microtubule associated protein (MAP) binding domains respectively (Nogales, Wolf et al. 1998).

6.5.2 The benzimidazole binding domain of β-tubulin and possible effects of resistance mutations on the structure of the binding pocket

Within the β-tubulin drug binding domain, three different regions are identified: the colchicine site, taxol site and vinblastine site (Downing 2000). Early competitive binding studies suggested that benzimidazoles bind to the colchicine site of β-tubulin (Lacey 1988). Similar observations were made from other studies that showed competitive inhibition of colchicine binding to β-tubulin in the presence of benzimidazoles (Friedman and Platzer 1978; Friedman and Platzer 1980; Jimenez-Gonzalez, De Armas-Serra et al. 1991). Additional evidence for this phenomenon comes from more recent homology modelling studies that show that the colchicine binding site partly overlaps with that of the benzimidazole (Aguayo-Ortiz, Méndez-Lucio et al. 2013).

6.5.2.1 The colchicine binding site and its relevance to benzimidazole binding

The colchicine binding site involves the regions of amino acids 1-46 and 214-241 in rat brain tubulin and although these regions are far apart in the primary sequence, drug-induced conformational changes are hypothesised to occur reducing the distance between these regions (Figure 6.4) (Uppuluri, Knipling et al. 1993). The phenomenon of drug-induced conformational changes is further supported by the fact that a plethora of anti-microtubule drugs with variable structure are able bind to the same site on the β-tubulin monomer (Nogales, Wolf et al. 1998). In fact, reports suggest that colchicine binds at the interface of α-β tubulins and has a stabilising effect on the heterodimer and that the induced conformational change interferes with the addition of these heterodimers to the microtubule lattice (Downing 2000). Such information regarding the colchicine binding site and knowledge of β-tubulin residues that are altered in resistant organisms led to hypotheses regarding the benzimidazole binding site.

6.5.2.2 The benzimidazole binding site inferred from current X-ray crystallographic models

The notion that benzimidazoles bind to monomers pre-polymerisation is supported by binding studies that used recombinant tubulin from parasitic protozoa and demonstrated that the incorporation of the drug-tubulin complex onto the growing end of the microtubule prevents further elongation and results in its depolymerisation. However, the drugs did not affect the integrity of the already assembled microtubules (MacDonald, Armson et al. 2004). This observation clarifies that resistance is due to alterations in drug binding to β-tubulin monomers and not due to altered microtubule stability as previously presumed.

The benzimidazole binding site is thought to be located at the N terminal and intermediate domain interface. The drug is specifically thought to bind within a pocket formed by two β-strands-S5 and S6 and an α-helix-H7 (Figure 6.3) and the binding of the drug is believed to invoke an inter-domain movement (N-terminal relative to the intermediate domain) i.e. a drug induced conformational change within the β-tubulin occurs (Nogales, Wolf et al. 1998; Downing 2000; Robinson, McFerran et al. 2004). The C terminal domain which interacts with kinesins also needs to rotate in order to prevent intra molecular steric hindrance due to rotation of the N terminal domain relative to the intermediate domain (Robinson, McFerran et al. 2004). However, the C-terminal domain seems to have no direct role in drug binding. This is evident from results from my project as well as early studies in yeast. The CB4856 strain was included in my project however it does not show phenotypic resistance. Data from the million mutations project indicate that this strain has a 3 amino acid deletion (440-442) in the C-terminal region (Thompson, Edgley et al. 2013). Mutagenesis studies in yeast has shown that truncation of part of the C-terminal (amino acid 431 onwards) leads to super-sensitivity to benzimidazole drugs such as thiabendazole and carbendazim (Matsuzaki, Matsumoto et al. 1988). Since this region is hyper-variable across isotypes and species, polymorphisms in this region also may be well tolerated functionally. This may explain why these organisms are susceptible to benzimidazoles regardless of having a deletion in the coding region.

6.5.2.3 Effect of benzimidazole resistance mutations resulting in amino acid substitutions on drug binding

Although mutations that confer benzimidazole resistance are distributed along the length of the β-tubulin molecule suggesting that resistance is due to altered microtubule stability rather than drug binding, a certain residue cluster (165-200) confers markedly higher levels of resistance implicating their involvement in drug binding (Downing 2000). Four regions of β-tubulin-residues-6, 165-167, 198-200, and 241- have been implicated in benzimidazole activity through studies with laboratory mutant strains of fungi such as *Aspergillus nidulans* and *Sacchromyces cerevisiae* (Thomas, Neff et al. 1985; Jung, Wilder et al. 1992). Mutations in the β-tubulin implicated in benzimidazole resistance in parasitic nematodes and fungi (at residues 6, 50, 134, 165, 167, 198, 200 and 257) apparently form a cluster buried within the protein has raised some controversy about their direct involvement in drug binding (Figure 6.5) (Robinson, McFerran et al. 2004). However, benzimidazoles are thought to bind to tubulin in a biphasic manner, which involves a first step that is slow with low affinity binding followed by a second higher affinity binding step apparently brought about by the conformational change induced in the target molecule in the first binding step (Downing 2000). It is believed that this conformational change makes these residues accessible to the drug and thus may explain their involvement in resistance when mutated (Robinson, McFerran et al. 2004).

X-ray crystallographic structures of parasite β-tubulins

Until recently, the lack of availability of crystallographic structures of parasite β-tubulin has hampered the elucidation of benzimidazole resistance with regard to the structural changes incurred by the causal substitutions at the benzimidazole binding site. Many studies have recently been published that have presented the research community with homology models for β-tubulin of *H. contortus* and *Fasciola hepatica* (Robinson, McFerran et al. 2004; Aguayo-Ortiz, Méndez-Lucio et al. 2013), *Trichinella spiralis* (Aguayo-Ortiz, Méndez-Lucio et al. 2013) and *B.malayi* (Sharma, Pan et al. 2012) to name a few. The *Trichinella spiralis, H. contortus* and *Fasciola hepatica* β-tubulin models were built using the *Ovis aries* and bovine models through homology modelling and residue replacement. The benzimidazole binding site proposed in these studies include residues implicated in benzimidazole resistance in helminths (167, 198 and 200) and fungi (50, 134, 165, and 257) along with hydrophobic residues Leu240, Leu250, Leu253 and Phe266 and hydrophilic residues Thr237 and Asn256.

Effects of benzimidazole resistance mutations inferred from X-ray crystallographic structures

The major interacting forces in benzimidazole binding to β-tubulin are hydrophobic interactions and hydrogen (H) bonds. Leu250, Leu253 and Met257 form a hydrophobic pocket that is capable of interacting with the R_2 carbamate group of benzimidazoles (Figure 1.1). Hydrophobic interactions with the benzimidazole ring itself are speculated to occur with Tyr50, Phe167 and Phe200 (Robinson, McFerran et al. 2004). These interactions facilitate the internalisation and stabilisation of the benzimidazole molecule within the β-tubulin. Therefore, resistance due to mutations in these residues is suggested to be due to closure of this binding pocket or modification of interactions between drug and protein (Aguayo-Ortiz, Méndez-Lucio et al. 2013).

Upon comparison of β-tubulin sequences across species susceptible and resistant to benzimidazoles, variations mainly occurred at amino acids residues 165, 167, 198 and 200 and mutations in these residues are speculated to cause changes in the spatial arrangement of the drug within the β-tubulin binding pocket (Aguayo-Ortiz, Méndez-Lucio et al. 2013). According to the *H. contortus* model, residues 165 and 200 face each other and depending on the side chains, are capable of forming a H-bond across the base of the binding pocket (Robinson, McFerran et al. 2004). In a susceptible helminth, residue 200 is a phenylalanine which is incapable of hydrogen bonding with the serine at P165 and thus grants access to the drug into the binding pocket (Figure 6.6). However, in resistant organisms (and also in mammalian β-tubulin), residue 200 is a tyrosine where the oxygen atom (a donor), allows the formation of this hydrogen bond, blocking access of the drug into the pocket.

The hydroxyl groups in Tyr167 or Tyr200 in resistant organisms increases the hydrophilicity of the binding pocket and also interfere with the internalisation of the benzimidazole molecule by forming hydrogen bonds with the adjacent residues. Specifically, Tyr167 is proposed to interact with Gln134 and Tyr200 with Gln198 (Aguayo-Ortiz, Méndez-Lucio et al. 2013). Gln198 is considered an anchor point for benzimidazole in the first step of binding. It makes two H-bonds with the drug-one with the H in the R_2 carbamate group and the other with the H in the benzimidazole ring itself (Figure 6.7A) (Qiu, Xu et al. 2011). The E198A mutation results in loss of this inter-molecular hydrogen bonding (Figure 6.7B) as well as an increase in the size of the binding pocket allowing the drug to slip out (Aguayo-Ortiz, Méndez-Lucio et al. 2013). Furthermore, in resistant organisms interaction between Tyr200 and Gln198 changes the orientation of the Gln side chain preventing the interaction with the benzimidazole molecule (Figure 6.7C) which in turn prevents the internalisation of the benzimidazole molecule into the binding pocket.

H-bonding between the R_2 methyl carbamate group in the benzimidazole molecule and residue Gln134 helps in the internalisation of the drug within the binding pocket facilitating contact with Gln198 (Figure 6.8A). In β-tubulins with an altered 134th residue this cannot occur which likely reduces the internalisation of the drug leading to resistance.

Benzimidazoles with a H-bond acceptor group (carbonyl or sulphoxide) at C_5 can bond with Cys239 (Figure 6.8B) which is next to residues 240 and 241 also implicated in drug resistance in fungi (Thomas, Neff et al. 1985; Albertini, Gredt et al. 1999) in that mutations at P240 or 241 may get in the way of H bond formation between Cys239 and benzimidazole molecule. The Cys239-C_5 interaction stabilises the drug within the binding pocket while also reducing the distance between the Thr165 and the carbamate group oxygen facilitating the H-bond formation between these two groups (Figure 6.8B). Benzimidazole drugs that consist of ether or thioether groups at C_5 (e.g. albendazole) form weaker hydrogen bonds possibly explaining the higher efficacy of their metabolites (e.g. albendazole sulphoxide).

Additionally, residue 165 also directly interacts with the R_2 side chain of the benzimidazole molecule (Jung and Oakley 1990) while the aromatic side chain in residue 167 phenylalanine is supposedly involved in hydrophobic bonding with the C_1 substituent of benzimidazole (Li, Katiyar et al. 1996). The presence of cysteine, serine or threonine at residue 165 allows hydrogen bond formation which may play a role in further stabilising the benzimidazole molecule within the binding pocket (Aguayo-Ortiz, Méndez-Lucio et al. 2013).

6.5.3 Possible impact of substitutions identified in this project on benzimidazole binding

From the information presented above, it is evident how benzimidazole resistance mutations identified in parasites may affect interactions of the drug with β-tubulin. In Chapter 5, I have discussed the possible secondary structural changes that may occur due to the A185P, M257I and D404N mutations in the β-tubulin. However, further modelling studies are required to draw more definitive conclusions regarding the impacts of these substitutions on its three dimensional structure. This being said some inferences can be made based on the information discussed in the previous section and other studies.

The β-tubulin secondary structure consists of 2 β-sheets surrounded by 12 α-helices and residue 185 falls within helix 5 (H5) (Nogales, Wolf et al. 1998). A proline is unable to hydrogen bond with adjacent residues (due to the lack of –NH group) which is a requirement for the formation of α-helices and incorporation of a proline residue into a helix is rather cumbersome (Strehlow, Robertson et al. 1991). A change from alanine to proline therefore can create a major 'kink' in the three dimensional structure of β-tubulin. In fact it is noted that in Ribonuclease A, when the proline residue falls towards the inner region (which is the case here as residue 185 is right in the middle of H5 of the β-tubulin), the helix is effectively truncated (Strehlow, Robertson et al. 1991). This in turn will have an effect on the dynamics of drug binding since the drug binding domain is thought to be formed by β-strands S5 and S6 and helix H7 (Robinson, McFerran et al. 2004). Residue 185 is within H5 which is between β-strands S5 and S6 (Figure 6.3) (Nogales, Wolf et al. 1998) and it would be expected that formation of the drug binding pocket will be dramatically affected by the A185P mutation.

According the model built by Robinson et al (2004), residue 257 is involved in the formation of the hydrophobic pocket into which the carbamate moiety of the benzimidazole drug fits. Methionine consists of a $–SCH_3$ side chain which affords the possibility of forming a hydrogen bond with the carbamate group which may aid in securing the drug within the binding pocket. With the change from methionine to isoleucine (-CH_3) this possibility is lost due to the absence of the S atom (Appendix A). Thus, the drug may ‘slip out of’ the binding pocket easily. However this H bond may not be essential for the binding which would be consistent with the lower level of resistance afforded to the JU1088 strain carrying the M257 mutation compared to the strains carrying the A185P substitution. In addition, a methionine to isoleucine change is shown to alter the catalytic property of certain proteases where an increase in the Michaelis constant (which is inversely proportional to the substrate affinity) is seen for mutants with a methionine to isoleucine change close to the binding site (Hege and Baumann 2001; Pérez, Kerrigan et al. 2007). Since the methionine residue at position 257 of β-tubulin is conserved across species (Aguayo-Ortiz, Méndez-Lucio et al. 2013), it is possible that this residue is important for specific three dimensional structural features and therefore binding kinetics.

The D404N substitution is in the C terminal region which seems to have no role in the binding of benzimidazole drugs as discussed earlier. Furthermore, no dramatic changes in the secondary structural level are predicted (Figure 4.17C). Thus, it is not surprising that the strain carrying this mutation displays even lower levels of resistance compared to the JU1088 strain (Figure 4.16) in that the mutation may not be having any effect on drug binding.

6.6 Concluding remarks and future directions

The work of this thesis has identified three naturally occurring amino acid substitutions in the *ben-1* β-tubulin (A185P, M257 and D404N) which confer varying levels of benzimidazole resistance in wild *C. elegans* populations. These polymorphisms are yet to be identified in parasitic nematode species and highlight the importance of investigating the role of polymorphisms other than F200Y, E198A and F167 in benzimidazole resistance particularly in human parasites. Although this work does not definitively identify the underlying reasons for the origin of these resistance polymorphisms in a non-parasitic, cosmopolitan species such as *C. elegans*, the findings are highly suggestive of selection pressure due to the presence of benzimidazole residues in the environment. Further extension of this study by looking for signatures of selection across the genomes of *C. elegans* populations both susceptible and resistant to benzimidazoles and estimation of D_N/D_S ratios will be fruitful in further confirming this suggestion. In addition to the three amino acid substitutions, naturally occurring deletions in the *ben-1* gene were also identified in wild *C. elegans* populations which demonstrate the functional redundancy of this protein even in the wild. This observation also leads to an interesting implication: while the gene may confer fitness advantages in populations not exposed to the drug, under conditions of selection pressure exerted by benzimidazoles the loss of this gene may offer a fitness advantage. Finally, the amino acid substitutions identified in benzimidazole resistant wild *C. elegans* strains are predicted to result in structural changes at the secondary level. However, these are very preliminary observations and therefore further modelling studies are required to predict the possible effects of these polymorphisms on benzimidazole binding to β-tubulin.

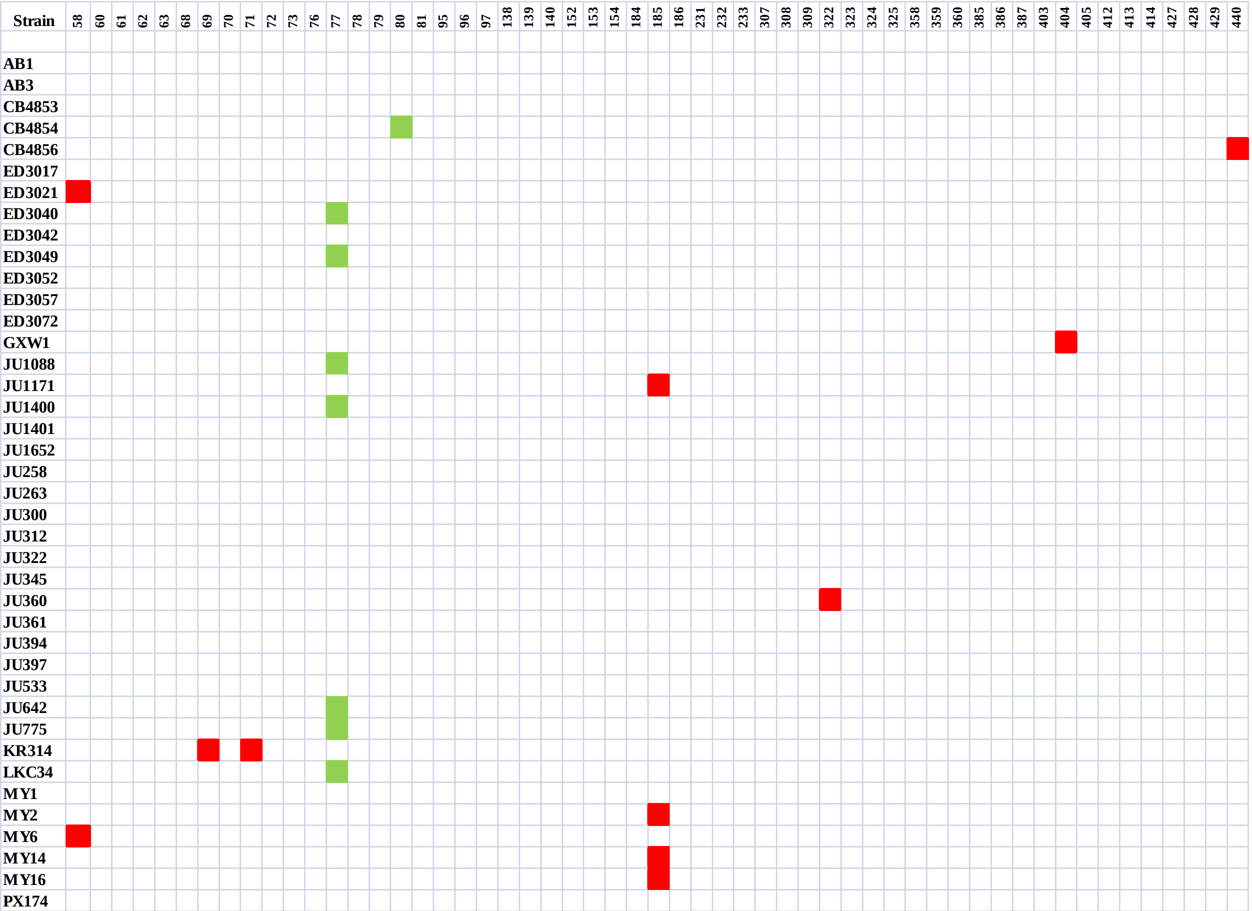
6.7 Chapter 6: Figures and Tables

Table 6.1 Occurrence of benzimidazole resistance conferring polymorphisms in parasitic nematodes

Amino acid substitution	Parasitic species	Clades
F200Y	*H.contortus, T.colubriformis, T.circumcinta, C.onchophora, O.ostertagi, C.nassatus N.americanus, A.duodenale*, W.bancrofti*	V, III
E198A	*H.contortus, N.americanus**	V
F167Y	*H.contortus, T.circumcincta, C.catinatum, N.americanus**	V

*No clear evidence of association with field resistance; Table adapted from James et al (2009).

Table 6.2A Polymorphisms in the BEN-1 β-tubulin of 40 wild *C. elegans* strains



Numbers on the top row indicate amino acid positions. The numbers are not continuous and only relevant residues with synonymous (green) and non-synonymous (red) polymorphisms and a few adjacent ones are shown. For comparison, a similar figure showing distribution of polymorphisms in artificially mutagenised strains is included in Appendix F. The figure is based on data downloaded from the million mutations project website at http://genome.sfu.ca/mmp/.

Table 6.2B Non-synonymous polymorphisms in the *ben-1* locus of wild *C. elegans* strains

Strain	Polymorphism	Effect	Type	Benzimidazole phenotype
GXW1	C->T	D404N	missense	Resistant (low)
JU1088	C->T	M257I	missense	Resistant (moderate)
JU1171, MY14, MY16, MY2	C->G	A185P	missense	Resistant
KR314	T->C	E69G	missense	Resistant
	CT->C	119 stop	deletion, frame shift	Resistant
CB4856	GCATCTCCAT->G	DGDA443A	coding exon, deletion	Susceptible
ED3021, MY6	TTG->T	68 stop	deletion, frame shift	Not tested
JU360	ACATACGTC->A	441 stop	deletion, frame shift	Not tested

Table 6.2C Synonymous polymorphisms in the *ben-1* locus of wild *C. elegans* strains

Strain	Polymorphism	Effect	Benzimidazole phenotype
CB4854	T->C	P80P	Susceptible
ED3040, ED3049, JU1088, JU1400, JU642, JU775,LKC34	G->T	R77R	Susceptible (except JU1088)

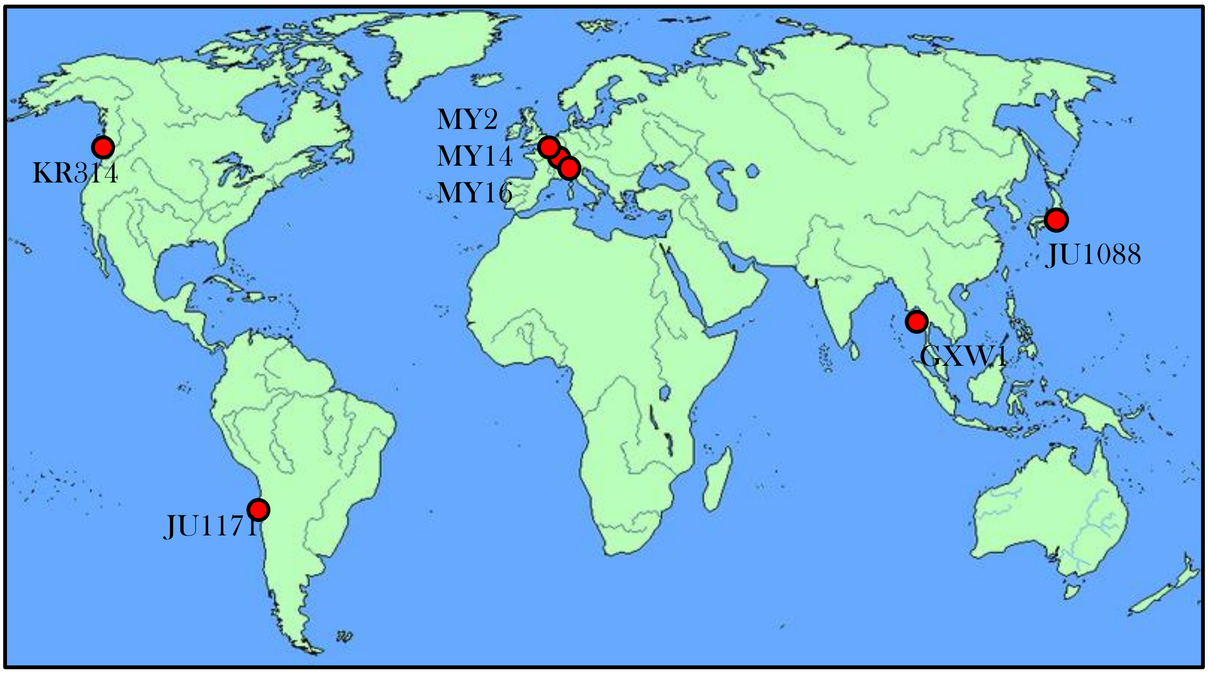


Figure 6.1 Geographic distribution of wild *C. elegans* strains resistant to benzimidazoles. Strains MY2 (from Roxel, Germany) , MY14 and MY16 (from Mecklenbeck, Germany) and JU1171 (from Concepcion, Chile) all carry the A185P substitution ; strain JU1088 (from Kakegawa, Japan) carries the M257 substitution and strain GXW1 (from Wuhan city, China) carries the D404N substitution in the β-tubulin drug target. The KR314 strain (from Vancouver, Canada) possibly lacks the β-tubulin drug target altogether.

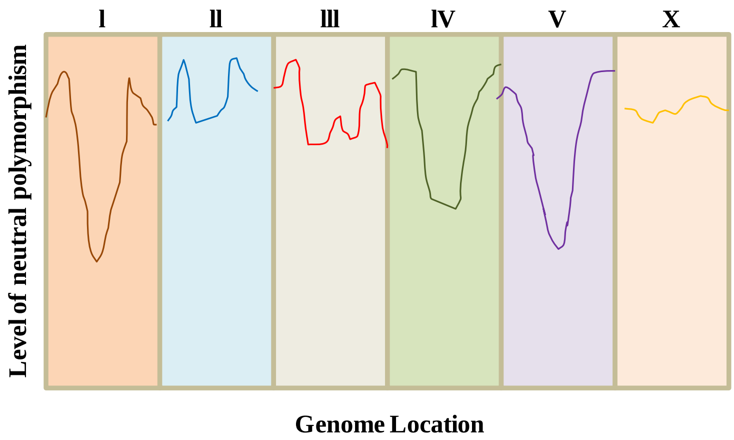


Figure 6.2 Schematic representation of the neutral polymorphisms along the *C. elegans* genome. Patterns of genetic variation along the length of chromosomes I, IV and V show signatures of positive selection in the form of valleys of reduced polymorphisms whereas the variation along the length of chromosomes II, III and X represents a more uniform distribution of polymorphisms. Figure adapted from Andersen et al (2012).

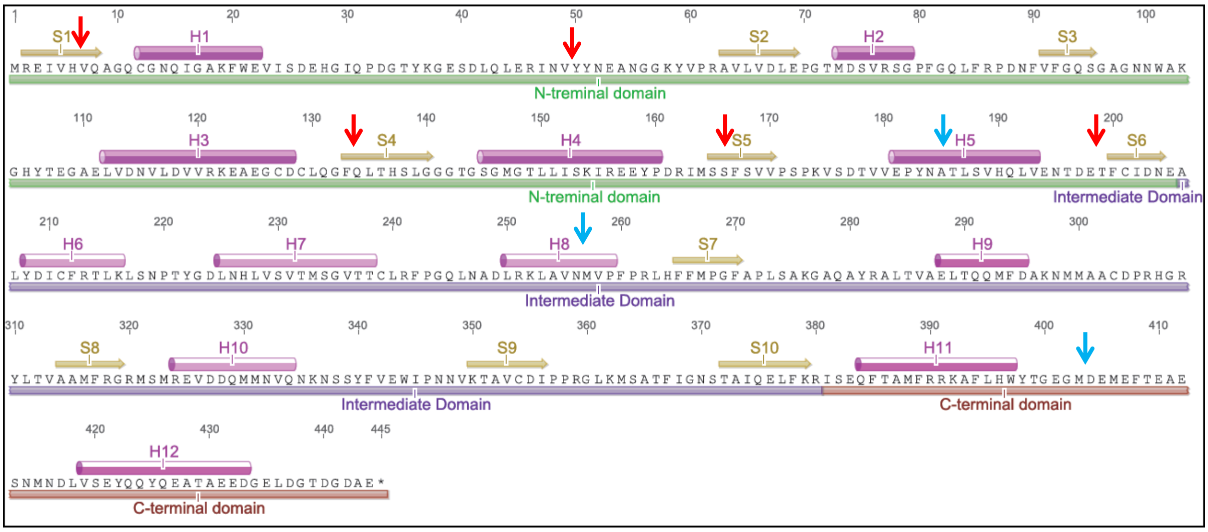


Figure 6.3 Predicted secondary structure of nematode β-tubulin. Helix regions (H) are annotated by purple cylinders and β-strands (S) by yellow arrows. The benzimidazole binding pocket is believed to be formed by β-strands S5, S6 and α-helix H7. The positions of secondary structural elements are approximate. The N-terminal (residues 1-205), Intermediate (residues 206-380) and C terminal (residues 381 to stop) domains are annotated as green, blue and brown regions respectively. The residues where substitutions are encountered in parasitic nematode and fungi (6, 50, 134, 165&167, 198&200) are denoted by red arrows. The residues that are replaced in benzimidazole resistant *C. elegans* strains identified in this thesis (185, 257, and 404) are denoted by blue arrows. Figure adapted from Nogales et al. (1998).

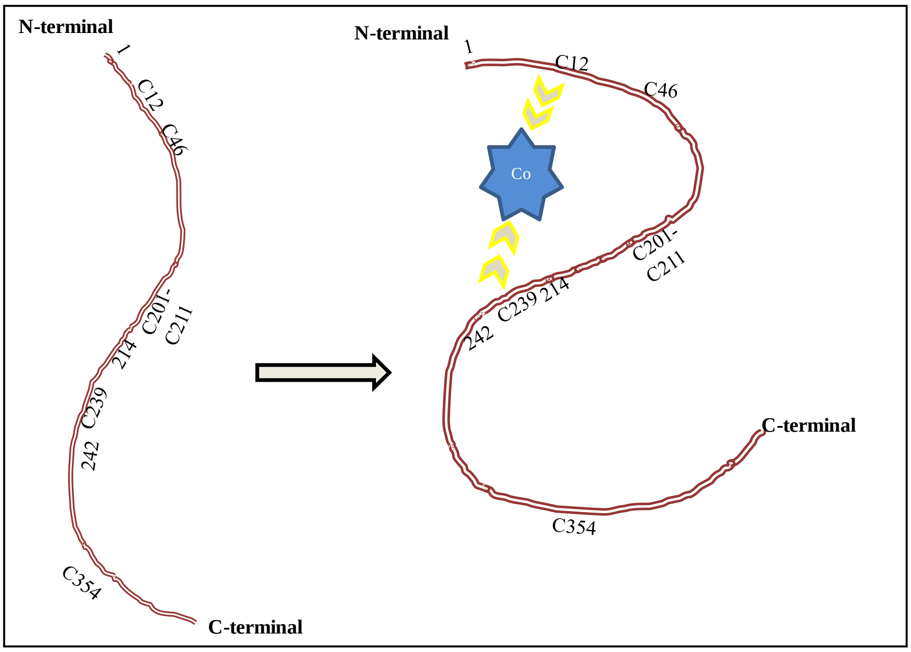


Figure 6.4 Colchicine binding to rat-brain β-tubulin. The colchicine (Co-blue star) induces conformational changes in the spacial structure of the β-tubulin molecule which brings regions 1-46 and 214-241 closer enabling interaction and binding. Cysteine residues are denoted by a C preceding residue number. Figure adapted from Uppuluri et al (1993).

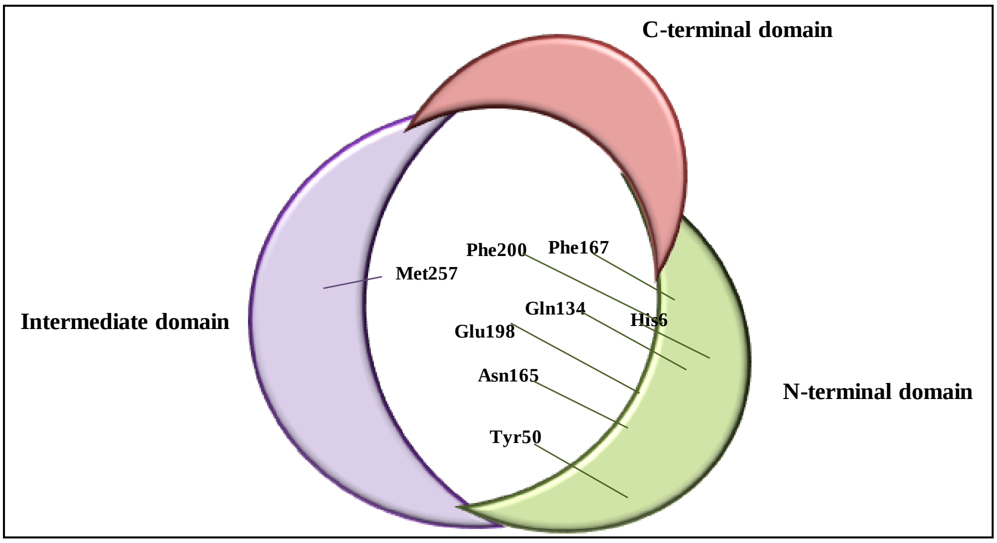


Figure 6.5 Positions of residues implicated in benzimidazole resistance in parasitic nematodes and fungi within the three dimensional structure of β-tubulin. Relative positions of residues are shown in the N-terminal (green), intermediate (purple) and C terminal (magenta) domains (the crescent shapes represent the spatial area occupied by the three domains). Figure adapted from ribbon diagram of porcine β-tubulin in Robinson et al (2004).

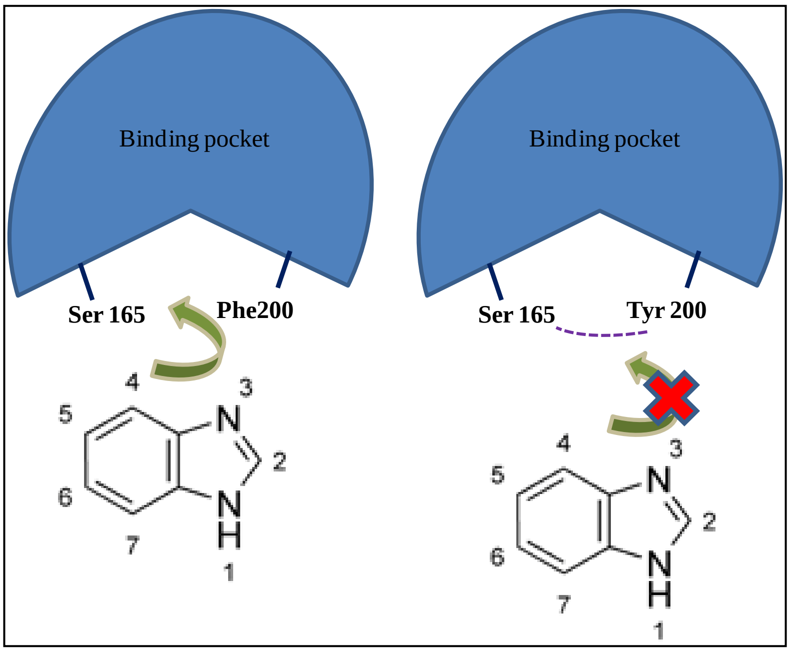


Figure 6.6 Effect of F200Y mutation on drug binding. The Ser165 and Tyr200 residues in resistant organisms can interact with each other preventing the internalisation of the benzimidazole molecule into the binding pocket.

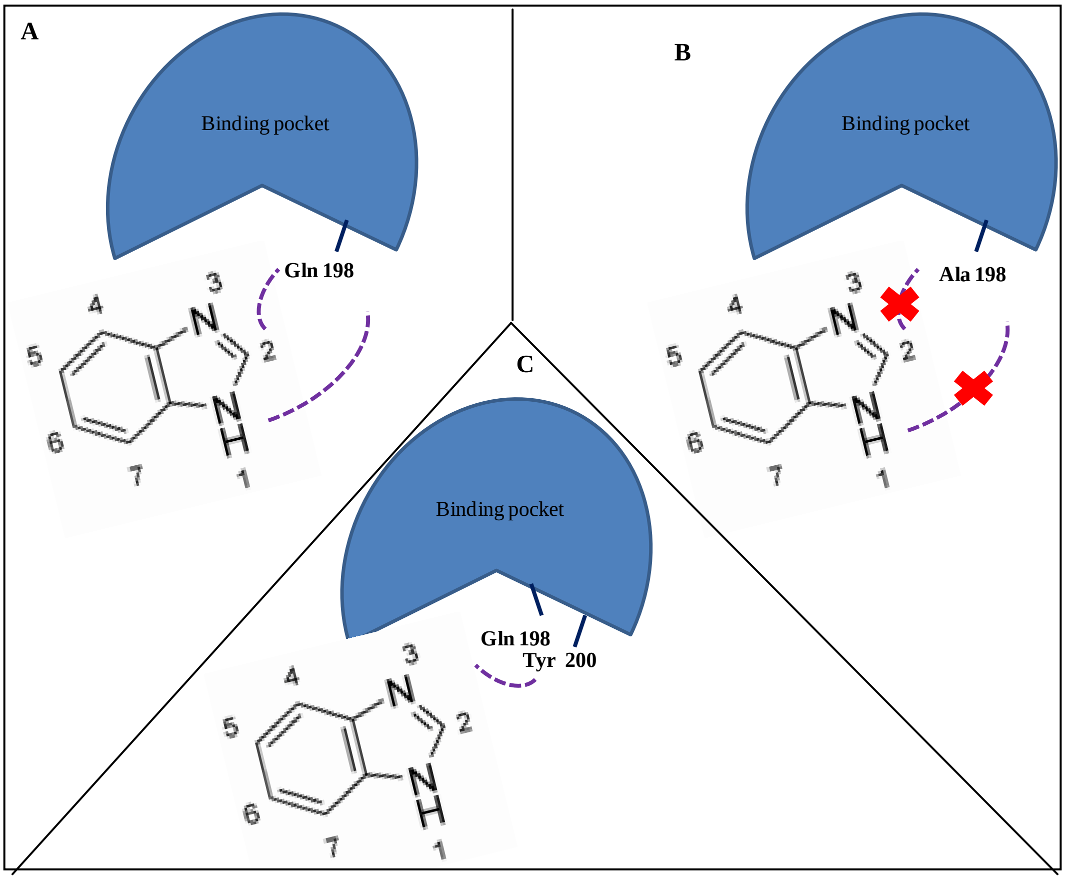


Figure 6.7 Effect of E198A and F200Y mutations on drug binding. The Gln198 residue in wild type protein is able to form hydrogen bonds with the R2 carbamate group and benzimidazole ring hydrogen (A), whereas the Ala198 is not able to do so affecting the internalisation of the benzimidazole molecule (B). In addition Tyr200 can form a hydrogen

bond with the adjacent Gln198 changing its orientation and preventing this double hydrogen bonding (C).

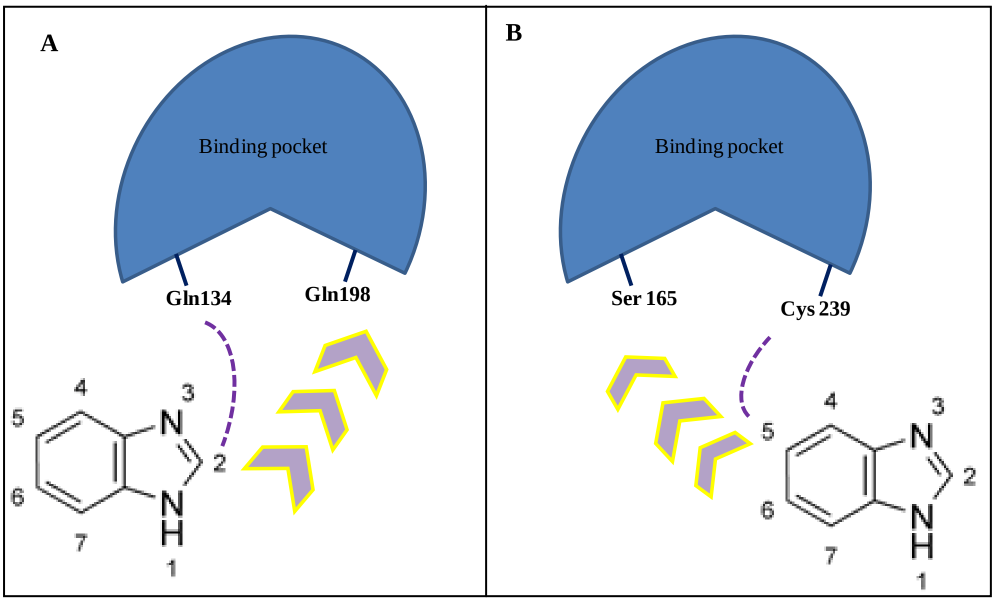
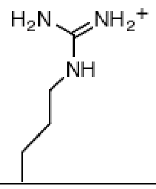
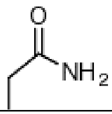
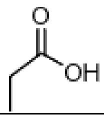
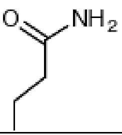
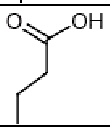
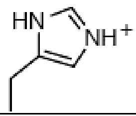
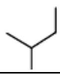
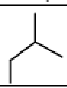
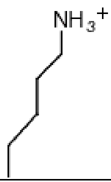
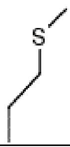
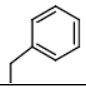
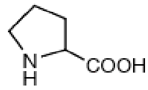
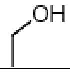
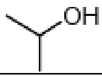
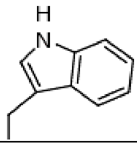
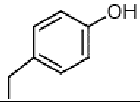
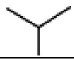


Figure 6.8 Additional interactions aiding in the internalisation of the benzimidazole molecule. Gln134 interacts with the R_2 carbamate group facilitating contact with Gln198 (A). Cys239 interacts with the R_5 carbonyl or suphoxide group and helps internalise the ligand and it turn the interaction between Ser165 and the R_2 carbamate group by reducing the distance between them (B).

Appendix A: Amino acid codes and side chains

Full name	Three-letter code	One letter code	Side chain
Alanine	**Ala**	**A**	**-CH_3**
Arginine	**Arg**	**R**	
Asparigine	**Asn**	**N**	
Aspartic acid	**Asp**	**D**	
Cysteine	**Cys**	**C**	
Glutamine	**Gln**	**Q**	
Glutamic acid	**Glu**	**E**	
Glycine	**Gly**	**G**	**-H**
Histidine	**His**	**H**	
Isoleucine	**Ile**	**I**	
Leucine	**Leu**	**L**	
Lysine	**Lys**	**K**	
Methionine	**Met**	**M**	

Phenylalanine	**Phe**	**F**	
Proline	**Pro**	**P**	 *****
Serine	**Ser**	**S**	
Threonine	**Thr**	**T**	
Tryptophan	**Trp**	**W**	
Tyrosine	**Tyr**	**Y**	
Valine	**Val**	**V**	

*****complete amino acid structure

Appendix B: Set up of benzimidazole exposure assays for wild *C. elegans* strain

B.1 Albendazole sensitivity of strains AB1, CB3191 and JU322 (Section 3.2.1)

L1 counts:

Strain	Count 1	Count 2	Count 3	Average (L1/µL)
N2	24	32	23	26.3
CB3474	52	68	63	61
AB1	13	18	8	13
CB3191	11	14	13	12.7
JU322	24	43	25	30.7

Master mix components:

Master mix	Average Worm Count/uL	Worm Culture Volume (mL)	NGM (mL)	10%OP50 (uL)	Streptomycin (uL)
N2	26.3	0.6084	14.42	960	16
CB3474	61	0.2623	14.76	960	16
AB1	13	1.231	13.79	960	16
CB3191	12.7	1.260	13.76	960	16
JU322	30.7	0.5212	14.5	960	16

B.2 Albendazole sensitivity of strains CB4853, JU258 and JU263 (Section 3.2.2)

L1 counts:

Strain	Count 1	Count 2	Count 3	Average (L1/µL)
N2	49	60	70	59.67
CB3474	28	27	22	25.67
CB4853	45	55	72	57.33
JU258	79	66	62	69
JU263	27	47	30	34.67

Master mix components:

Master mix	Average Worm Count/uL	Worm Culture Volume (uL)	NGM (mL)	10%OP50 (uL)	Streptomycin (uL)
N2	59.7	268	14.8	960	16
CB3474	25.7	622.6	14.4	960	16
CB4853	57.3	279.2	14.7	960	16
JU258	69	231.9	14.8	960	16
JU263	34.7	461.1	14.6	960	16

B.3 Albendazole sensitivity of strains CB4854, CB4856 and CB4858 (Section 3.2.3)

L1 counts:

Strain	Count 1	Count 2	Count 3	Average (L1/µL)
N2	13	15	20	16
CB3474	10	8	13	10.33333
CB4854	8	8	10	8.666667
CB4856	6	9	7	7.333333
CB4858	15	11	10	12

Master mix components:

Master mix	Average Worm Count/uL	Worm Culture Volume (uL)	NGM (mL)	10%OP50 (uL)	Streptomycin (uL)
N2	16	718.75	10.1	690	11.5
CB3474	10.3	1116.5	9.7	690	11.5
CB4854	8.7	1321.8	9.5	690	11.5
CB4856	7.3	1575.3	9.2	690	11.5
CB4858	12	958.3	9.8	690	11.5

For this particular assay, since the L1 counts were very low the total mastermix volume was kept at 11.5mL (instead of 16mL) and each well of the 6 well plate received a 1.75mL aliquot of this mastermix (instead of

B.4 Albendazole sensitivity of strains KR314 and MY2 (Section 3.2.4)

L1 counts:

Strain	Count 1	Count 2	Count 3	Average (L1/µL)
N2	160	240	160	186.67
CB3474	99	105	96	100
KR314	70	66	60	65.33
MY2	75	68	70	71

Master mix components:

Master mix	Average Worm Count/uL	Worm Culture Volume (uL)	NGM (mL)	10%OP50 (uL)	Streptomycin(uL)
N2	186.7	85.7	14.9	960	16
CB3474	100	160	14.9	960	16
KR314	65.3	245	14.8	960	16
MY2	71	225.4	14.8	960	16

Appendix C: Visual observations of benzimidazole exposure assay plates

C.1 Albendazole sensitivity of strains AB1, CB3191 and JU322 (Section 3.2.1)

Strain	[ABZ]	Observations	Worms
N2	DMSO	Long thick ; moving fast	63
	0.00075	long thick; ends moving	122
	0.0015	long thick; ends moving	138
	0.003	long thick curled; ends moving	91
	0.006	shorter thicker; ends moving slower	53
	1.13	short thin mostly completely paralysed	14 (18)
CB3474	DMSO	longer thinner moving fast some twisted on self	107
	0.00075	longer thinner moving fast some twisted on self	83
	0.0015	thinner still unaffected	49
	0.003	thinner tend to have a curling movement	63
	0.006	still unaffected	66
	1.13	very thin long	19(112)
AB1	DMSO	lesser number long thick unaffected	25(77)
	0.00075	thinner; ends moving	53
	0.0015	ends moving; more affected	54
	0.003	mostly curled up; very slow movement	81
	0.006	shorter thinner; completely paralysed	96
	1.13	very small thin; completely paralysed	30(22)
CB3191	DMSO	long thin; fast swimming; some twisted	97
	0.00075	long thin; slow movement	134
	0.0015	some completely paralysed; mostly ends moving	180
	0.003	curled up shorter thicker; ends moving	147
	0.006	shorter thicker curled; ends moving very slowly	96
	1.13	very tiny (like AB1) thin curled; completely paralysed	8(8)
JU322	DMSO	very thin long; swimming fast	110
	0.00075	ends moving fast vibration some swimming; lots of dauers	150
	0.0015	appear thicker; ends moving slow; no swimmers	217
	0.003	curled; ends moving very slow	202
	0.006	some paralysed ;mostly static; some ends moving	223
	1.13	curled up; ends moving slowly	29(23)

C.2 Albendazole sensitivity of strains CB4853, JU258 and JU263 (Section 3.2.2)

Strain	[ABZ]	Observations
N2	DMSO	long thick; unaffected; not swimming; lack of food?
	0.00075	slightly moving
	0.0015	ends moving
	0.003	curled up mostly; ends moving
	0.006	shorter thicker; ends slightly moving; mostly paralysed
	1.13	all paralysed some ends moving
CB3474	DMSO	long thick; swimming
	0.00075	unaffected
	0.0015	unaffected
	0.003	unaffected
	0.006	unaffected
	1.13	longer thinner; unaffected
CB4853	DMSO	unaffected; longer
	0.00075	ends moving; some curled
	0.0015	ends moving
	0.003	mostly paralysed; one/two swimmers
	0.006	shorter thicker
	1.13	mostly paralysed; curled; ends moving in some
JU258	DMSO	long thin; unaffected; fast swimmers
	0.00075	ends moving
	0.0015	ends moving; curled
	0.003	curled; ends moving
	0.006	ends moving
	1.13	mostly completely paralysed; ends moving
JU263	DMSO	unaffected; longer thinner
	0.00075	slightly affected
	0.0015	mostly static; ends moving
	0.003	ends moving
	0.006	ends moving very slowly
	1.13	mostly static; some ends moving

C.3 Albendazole sensitivity of strains CB4854, CB4856 and CB4858 (Section 3.2.3)

Strain	[ABZ]	TAV	Observations
N2	DMSO	000	Long thick mostly swimming some stiff movement
	0.00075	001	slow swimmers/ends moving mostly
	0.0015	002	mostly static some ends moving
	0.003	003	mostly paralysed very few ends moving
	0.006	004	all paralysed
	1.13	005	all paralysed
CB3474	DMSO	006	Unaffected; some stiff?
	0.00075	007	unaffected
	0.0015	008	unaffected
	0.003	009	unaffected; some stiff
	0.006	010	mostly unaffected; some stiff
	1.13	011	unaffected
CB4854	DMSO	012	unaffected some stiff
	0.00075	013	mostly static; some ends moving
	0.0015	014	mostly stiff; some L1s swimming
	0.003	015	all paralysed
	0.006	016	all paralysed
	1.13	017	all paralysed
CB4856	DMSO	018	Unaffected; some stiff (lack of liquid?)
	0.00075	019	mostly ends moving
	0.0015	020	mostly stiff; some C like movement
	0.003	021	some ends moving
	0.006	022	some ends moving
	1.13	023	static
CB4858	DMSO	024	unaffected
	0.00075	025	slow S like movement
	0.0015	026	ends moving
	0.003	027	ends moving
	0.006	028	mostly stiff some slow movement
	1.13	029	mostly stiff look like sticks (dauers?) less of curled worms

C.4 Albendazole sensitivity of strains KR314 and MY2 (Section 3.2.4)

Strain	[ABZ]	Observations
N2	DMSO	Unaffected
	0.00075	ends moving fast; mostly curled
	0.0015	ends moving
	0.003	ends moving; some like circles
	0.006	ends moving very slowly
	1.13	completely paralysed
CB3474	DMSO	long thinner compared to N2; unaffected
	0.00075	unaffected; some twisted on self
	0.0015	unaffected; some twisted on self
	0.003	unaffected; some twisted on self
	0.006	unaffected
	1.13	unaffected
KR314	DMSO	unaffected; very fast swimmers
	0.00075	unaffected; very fast swimmers
	0.0015	unaffected; very fast swimmers
	0.003	unaffected; very fast swimmers
	0.006	unaffected; very fast swimmers
	1.13	unaffected; slower swimmers
MY2	DMSO	unaffected; slower swimmers compared to KR314
	0.00075	mostly unaffected; slow swimmers; some ends moving
	0.0015	still swimming quite fast
	0.003	still swimming quite fast
	0.006	still swimming quite fast
	1.13	still swimming quite fast

Appendix D: Complete coding sequences of the *ben-1* locus of wild *C. elegans* strains resistant to albendazole and mutant strain CB3474: *ben-1 (e1880) III**

D.1 KR314: *mab-23 (e2518) V ben-1* genomic sequence (complete coding sequence)

ATGAGAGAAATTGTTCACGTTCAAGCCGGACAATGTGGTAATCAAATCGGAGCC
AAGTTCTGGGAAGTGATATCCGATGAGCATGGGATCCAGCCTGATGGAACTTAT
AAGGGAGAAAGTGATTTGCAGTTGGAAAGAATCAATGTCTACTATAATGAGGCT
AATGGTGAGAAATTTAGCTTTTTATTCGATTTTCAGATTCTGTTTGAATATAATA
AAGCAAAATGTTCCGAAATTTTCTTTAACTTCCAATTTTCAATATTTATTGCTCAA
TCGCAAAATTTGTTCGAGTGTTGCGCAATAAATACGGTGCCCGGTCTCGACACGA
CTTTTGTTGACAACGAAAGGTCGTGCGCCTTTAAAGGATACTGTAGCTTCAAACT
TTTGTGGCAGCGGGCTTTGCATTAGTTTTCATAGTTTTTTGATTGATAAATGTGTA
CTTATGTATTTTTTTATTAAAAACTCAAATATTTATTACACATTTTAACAAATTAA
TTCTGCAAATTATGAGAATGAACGGAAGATATATTGCCAGAGACACTATTACCG
GTACAGAGAGTGTAGATAGTTAGAGAGTGACAGACATACGGGAACCTATGGGGC
GGGGCGCGCGGAAGAGAAGATTTGTGTCGTAAATCGACACAAATCGTCTCTTCC
GCGCGCCCCGCCCCATTGGGTCCCGGATGTCTGTCACTCTCTAACTATATACACT
CTCTGTACCGGTAATACGAACGTAGGAATCGTGGTATTTTGAGAGACAAAACCA
CCGGCGTATACTAGTTTCTTGCACACTTTTTCGGTTGAATTAGGATTTTAGTTAGT
CAAACAAAAGCTCAAAGACAGCTTTCTGAAATTCACGTTTACAAATTCACGAAT
AGTTATTTTTATAGACCCATTCTGATGAAATTTCAGTATATCTGTTTCTCCCATTTT
TCGATTCTATAAACGTGTGGTGTCTCTTGCGCATTCGCGCTCTATAGCAACCAATT
TTTTTCAATTTTTATTTTTTCTTTCCAAAGATAAATCTTCGATTAAGACCTCATTTT
TGTTGGTTTTAAAAAAAAAAATTTTTAAATTAATTTAAAATTTAAAATTTAATTCT
CTCAATGTGAATTACCAAAATTCACTAATTGTTACCATTTTCAGGCGGCAAATAT
GTCCCACGCGCTGTTCTCGTCGATCTTGGGCC-GGAACCATGGATTCTGTCCGCT
CTGGACCATTCGGCCAGCTGTTCCGTCCAGATAACTTTGTGTTCGGACAATCCGG
AGCCGGAAACAACTGGGCCAAGGGTCACTACACCGAAGGAGCCGAACTTGTCGA
TAATGTGCTCGACGTAGTTCGAAAAGAGGCTGAAGGATGTGATTGTCTTCAAGG
ATTCCAGTTGACTCATTCACTTGGAGGAGGAACTGGATCTGGAATGGGAACTCTT
CTCATTTCGAAAATCCGTGAAGAGTATCCAGATAGAATTATGAGTTCTTTCTCGG
TTGTTCCGTCGCCAAAGGTTGGATTAATTGAATTTAATGAATATTTTAAAACTAA
TAATTAAAATTCAGGTCTCGGACACAGTCGTCGAGCCATACAACGCTACTCTTTC
TGTCCACCAGCTCGTTGAAAATACCGATGAGACTTTCTGCATTGACAACGAGGCT
CTTTATGATATCTGCTTCAGAACCCTCAAGCTTTCAAATCCAACTTATGGAGATCT
CAATCATCTTGGTAAGATTTTCTTTTATTTTTTATTATTTTTTCTATTTTAACTTTCA
ATTATTTCAGTTTCCGTTACAATGTCCGGAGTCACCACGTGCCTCCGCTTCCCAGG
ACAACTCAATGCTGATCTCCGCAAACTTGCAGTCAACATGGTTCCATTCCCACGT
CTTCACTTCTTTATGCCAGGATTTGCTCCATTGTCAGCTAAAGGAGCACAAGCGT
ACCGTGCACTTACGGTCGCCGAGCTTACCCAGCAGATGTTTGACGCCAAGAATAT
GATGGCTGCCTGTGATCCGAGACACGGACGTTACCTGACTGTAGCTGCAATGTTC

CGTGGACGTATGTCAATGAGAGAAGTCGATGATCAAATGATGAATGTGCAGAAT
AAGAACTCTTCGTATTTTGTTGAATGGATTCCGAATAATGTGAAGACTGCGGTCT
GTGATATTCCACCACGTGGGCTCAAGATGTCGGCCACTTTTATTGGAAACTCGAC
AGCTATTCAGGAGTTGTTCAAGAGAATCTCTGAACAATTTACAGGTACTATTATG
GAGACGCGGAAAAGTTCATTTTATTGCGGATTCGAAAGCGAAAACGCGAAAAAA
TTATCGACTTTTCACCACATGTCGGATTTTATAGCGAAAATTATCGATTTTTCAGA
ACATAAAAAAAAACGAGAAAAATTTCAATATTTTATCGAATTTGGTATATTTTAA
ATTTAAAAAAATCATAATTTTTCTCAATTTTATGAAATTTTCAGCAATTTTTTGGT
TAAAGTTTGGTAAATTTGGGTTAAAAACATGAATTATAGGCGAAATTCGAAAAA
GAATTTCCAATTATCGAAAAATCTAAAAAAAAAAAAATTTTNCTATATTTTTTTTT
CATAATTTTCCCCTAACTCTGTCCAAATTTCAGCAATGTTCCGCCGCAAGGCTTTC
CTCCATTGGTATACTGGCGAAGGAATGGACGAGATGGAATTCACTGAAGCTGAG
AGTAATATGAACGATCTTGTCTCAGAATACCAGCAATACCAGGAAGCAACTGCA
GAGGAAGATGGAGAACTTGATGGAACTGATGGAGATGCTGAATAG

D.2 MY2 *ben-1* genomic sequence (complete coding sequence)

ATGAGAGAAATTGTTCACGTTCAAGCCGGACAATGTGGTAATCAAATCGGAGCC
AAGTTCTGGGAAGTGATATCCGATGAGCATGGGATCCAGCCTGATGGAACTTAT
AAGGGAGAAAGTGATTTGCAGTTGGAAAGAATCAATGTCTACTATAATGAGGCT
AATGGTGAGAAATTTAGCTTTTTTATTCGATTTTCAGATTCTGTTTGAATATAATA
AAGCAAAATGTTCCGAAATTTTCTTTAACTTCCAATTTTCAATATTTATTGCTCAA
TCGCAAAATTTGTTCGAGTGTTGCGCAATAAATACGGTGCCCGGTCTCGACACGA
CTTTTGTTGACAACGAAAGGTCGTGCGCCTTTAAAGGATACTGTAGCTTCAAACT
TTTGTGGCAGCGGGCTTTGCATTAGTTTTCATAGTTTTTTGATTGATAAATGTGTA
CTTATGTATTTTTTTATTAAAAACTCAAATATTTATTACACATTTTAACAAATTAA
TTCTGCAAATTATGAGAATGAACGGAAGATATATTGCCAGAGACACTATTATCTC
TGTACCGGTAATACGAACGTAGGAATCGTGGTATTTTGAGAGACAAAACCACCG
GCGTATACTAGTTTCTTGCACACTTTTTCGGTTGAATTAGGATTTTAGTTAGTCAA
ACAAAAGCTCAAAGACAGCTTTCTGAAATTCACGTTTACAAATTCACGAATAGTT
ATTTTTATAGACCCATTCTGATGAAATTTCAGTATATATGTTTCTCCCATTTTTCG
ATTCTATAAACGTGTGGTGTCTCTTGCGCATTCGCGCTCTATAGCAACCAATTTTT
TTCAATTTTTATTTTTTCTTTCCAAAGATAAATCTTCGATTAAGACCTCATTTTTGT
TGGTTTTAAAAAAAAAATTTTTTAAATTAATTTAAAATTTAAAATTTAATTCTCTC
AATGTGAATTACCAAAATTCACTAATTGTTACCATTTTCAGGCGGCAAATATGTC
CCACGCGCTGTTCTCGTCGATCTTGAGCCAGGAACCATGGATTCTGTCCGCTCTG
GACCATTCGGCCAGCTGTTCCGTCCAGATAACTTTGTGTTCGGACAATCCGGAGC
CGGAAACAACTGGGCCAAGGGTCACTACACCGAAGGAGCCGAACTTGTCGATAA
TGTGCTCGACGTAGTTCGAAAAGAGGCTGAAGGATGTGATTGTCTTCAAGGATTC
CAGTTGACTCATTCACTTGGAGGAGGAACTGGATCTGGAATGGGAACTCTTCTCA
TTTCGAAAATCCGTGAAGAGTATCCAGATAGAATTATGAGTTCTTTCTCGGTTGT
TCCGTCGCCAAAGGTTGGATTAATTGAATTTAATGAATATTTTAAAACTAATAAT

TAAAATTCAGGTCTCGGACACAGTCGTCGAGCCATACAACCCTACTCTTTCTGTC
CACCAGCTCGTTGAAAATACCGATGAGACTGCATTGACAACGAGGCTCTTTATGA
TATCTGCTTCAGAACCCTCAAGCTTTCAAATCCAACTTATGGAGATCTCAATCAT
CTTGGTAAGATTTTCTTTTATTTTTTATTATTTTTTCTATTTTAACTTTCAATTATTT
CAGTTTCCGTTACAATGTCCGGAGTCACCACGTGCCTCCGCTTCCCAGGACAACT
CAATGCTGATCTCCGCAAACTTGCAGTCAACATGGTTCCATTCCCACGTCTTCAC
TTCTTTATGCCAGGATTTGCTCCATTGTCAGCTAAAGGAGCACAAGCGTACCGTG
CACTTACGGTCGCCGAGCTTACCCAGCAGATGTTTGACGCCAAGAATATGATGGC
TGCCTGTGATCCGAGACACGGACGTTACCTGACTGTAGCTGCAATGTTCCGTGGA
CGTATGTCAATGAGAGAAGTCGATGATCAAATGATGAATGTGCAGAATAAGAAC
TCTTCGTATTTTGTTGAATGGATTCCGAATAATGTGAAGACTGCGGTCTGTGATAT
TCCACCACGTGGGCTCAAGATGTCGGCCACTTTTATTGGAAACTCGACAGCTATT
CAGGAGTTGTTCAAGAGAATCTCTGAACAATTTACAGGTACTATTATGGAGACGC
GGAAAAGTTCATTTTATTGCGGATTCGAAAGCGAAAACGCGAAAAAATTATCGA
CTTTTCAGAAAATCAAAAAATTTAAAAAAATTAAAATGTTTATTATATTTTGTAT
CCAAAAATTACAAAAATTCTCATTAAAGGCTTTTTGAAAATTAAATATTGAAAAA
AGTTGCTGGAAACGTGCTCCACCGGGAGGCGAAATTGTGAATCCAAATGTTTTAC
CAAATTMTCAGCCATTMTTTCGTCTTTTTTGTGATTTTTTTCCGTTTAAAAAAAAA
TTTCGTGTCAAAACGATTTTTTCACCAATTTCGACATAATTTTTTCAACGAACAGC
ATCACAAAAATGAATAAAATCGGCAAAAAAATTCAAAAAACCTTTTAATTTTCA
GTTGTTTTTTTTATTGAAAATTGGCGAAACTAATTGAAACATTCTAAATTTACGGT
ATTAAATATCCAACATGTCGGATTTTATAGCGAAAATTATCGATTTTTCAGAACA
TAAAAAAAAACGAGAAAAATTTCAATATTTTATCGAATTTGGTATATTTTAAATT
TAAAAAAATCATAATTTTTCTCAATTTTATGAAATTTTCAGCAATTTTTTGGTTAA
AGTTTGGTAAATTTGGGTTAAAAACATGAATTATAGGCGAAATTCGAAAAAGAA
TTTCCAATTATCGAAAAATCTAAAAAAAAAAAAAAATTTTCTATATTTTTTTTTCAC
AATTTTCCCCTAACTCTGTCCAAATTTCAGCAATGTTCCGCCGCAAGGCTTTCCTC
CATTGGTATACTGGCGAAGGAATGGACGAGATGGAATTCACTGAAGCTGAGAGT
AATATGAACGATCTTGTCTCAGAATACCAGCAATACCAGGAAGCAACTGCAGAG
GAAGATGGAGAACTTGATGGAACTGATGGAGATGCTGAATAG

D.3 CB3474 [*ben-1(e1880)*] *ben-1* genomic sequence (complete coding sequence)

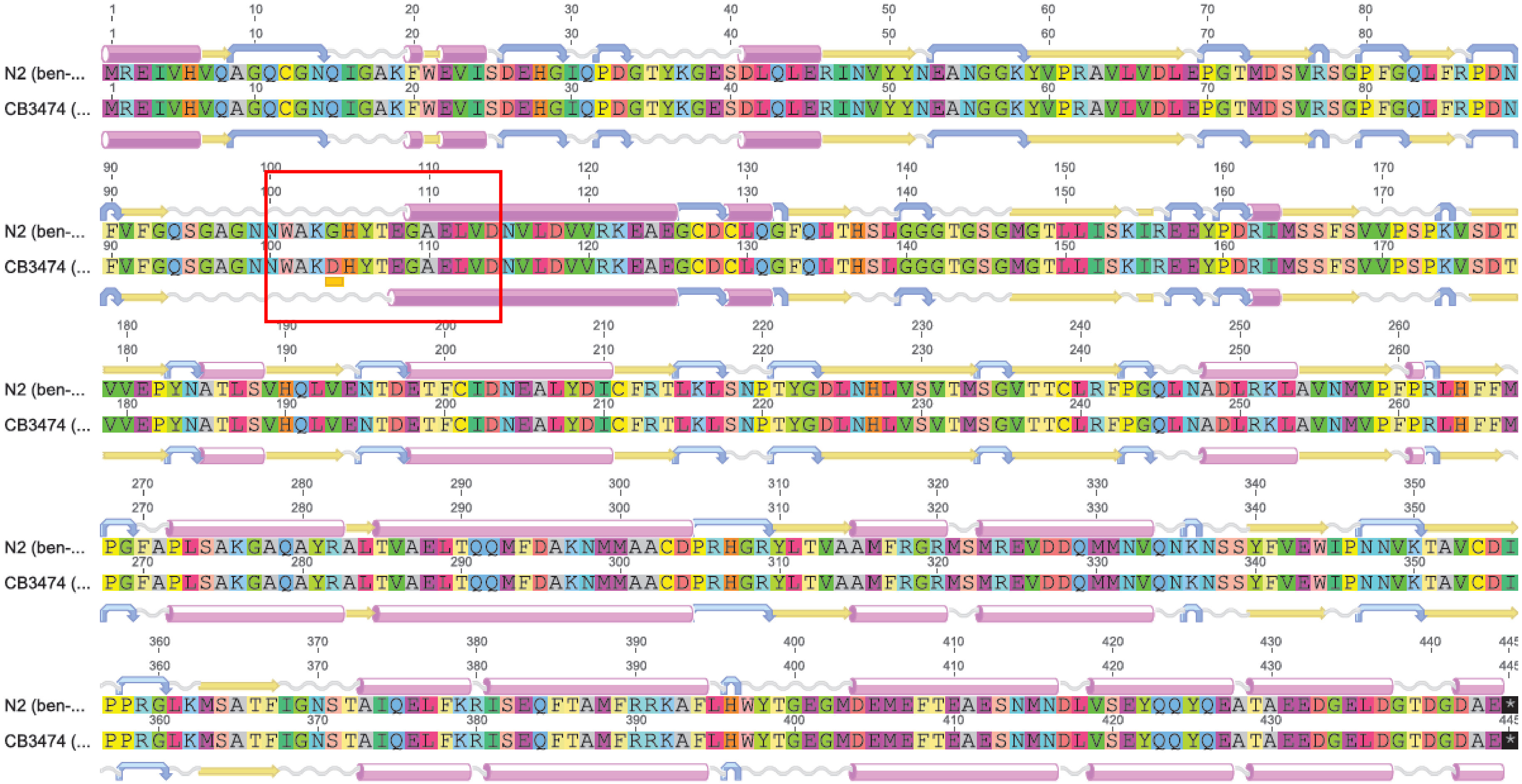
ATGAGAGAAATTGTTCACGTTCAAGCCGGACAATGTGGTAATCAAATCGGAGCC
AAGTTCTGGGAAGTGATATCCGATGAGCATGGGATCCAGCCTGATGGAACTTAT
AAGGGAGAAAGTGATTTGCAGTTGGAAAGAATCAATGTCTACTATAATGAGGCT
AATGGTGAGAAATTTAGCTTTTTTATTCGATTTTCAGATTCTGTTTGAATATAATA
AAGCAAAATGTTCCGAAATTTTCTTTAACTTCCAATTTTCAATATTTATTGCTCAA
TCGCAAAATTTGTTCGAGTGTTGCGCAATAAATACGGTGCCCGGTCTCGACACGA
CTTTTGTTGACAACGAAAGGTCGTGCGCCTTTAAAGGATACTGTAGCTTCAAACT
TTTGTGGCAGCGGGCTTTGCATTAGTTTTCATAGTTTTTTGATTGATAAATGTGTA
CTTATGTATTTTTTTATTAAAAACTCAAATATTTATTACACATTTTAACAAATTAA
TTCTGCAAATTATGAGAATGAACGGAAGATATATTGCCAGAGACACTATTAMCC
GGTACAGAGAGTGTAGATAGTTAGAGAGTGACAGACATACGGGAACCTATGGGG
CGGGGCGCGCGGAAGAGAAGAAACAAAAGCTCAAAGACAGCTTTCTGAAATTC
ACGTTTACAAATTCACGAATAGTTATTTTTATAGACCCATTCTGATGAAATTTCAG
TATATCTGTTTCTCCCATTTTTCGATTCTATAAACGTGTGGTGTCTCTTGCGCATTC
GCGCTCTATAGCAACCAATTTTTTTCAATTTTTATTTTTTCTTTCCAAAGATAAAT
CTTCGATTAAGACCTCATTTTTGTTGG-TTTTAAAAAAAAAATTTTTTAAATTAATT
TAAAATTTAAAATTTAATTCTCTCAATGTGAATTACCAAATTCACTAATTGTTACC
ATTTTCAGGCGGCAAATATGTCCCACGCGCTGTTCTCGTCGATCTTGAGCCAGGA
ACCATGGATTCTGTCCGCTCTGGACCATTCGGCCAGCTGTTCCGTCCAGATAACT
TTGTGTTCGGACAATCCGGAGCCGGAAACAACTGGGCCAAGGATCACTACACCG
AAGGAGCCGAACTTGTCGATAATGTGCTCGACGTAGTTCGAAAAGAGGCTGAAG
GATGTGATTGTCTTCAAGGATTCCAGTTGACTCATTCACTTGGAGGAGGAACTGG
ATCTGGAATGGGAACTCTTCTCATTTCGAAAATCCGTGAAGAGTATCCAGATAGA
ATTATGAGTTCTTTCTCGGTTGTTCCGTCGCCAAAGGTTGGATTAATTGAATTTAA
TGAATATTTTAAAACTAATAATTAAAATTCAGGTCTCGGACACAGTCGTCGAGCC
ATACAACGCTACTCTTTCTGTCCACCAGCTCGTTGAAAATACCGATGAGACTTTC
TGCATTGACAACGAGGCTCTTTATGATATCTGCTTCAGAACCCTCAAGCTTTCAA
ATCCAACTTATGGAGATCTCAATCATCTTGGTAAGATTTTCTTTTATTTTTTATTAT
TTTTTCTATTTTAACTTTCAATTATTTCAGTTTCCGTTACAATGTCCGGAGTCACCA
CGTGCCTCCGCTTCCCAGGACAACTCAATGCTGATCTCCGCAAACTTGCAGTCAA
CATGGTTCCATTCCCACGTCTTCACTTCTTTATGCCAGGATTTGCTCCATTGTCAG
CTAAAGGAGCACAAGCGTACCGTGCACTTACGGTCGCCGAGCTTACCCAGCAGA
TGTTTGACGCCAAGAATATGATGGCTGCCTGTGATCCGAGACACGGACGTTACCT
GACTGTAGCTGCAATGTTCCGTGGACGTATGTCAATGAGAGAAGTCGATGATCA
AATGATGAATGTGCAGAATAAGAACTCTTCGTATTTTGTTGAATGGATTCCGAAT
AATGTGAAGACTGCGGTCTGTGATATTCCACCACGTGGGCTCAAGATGTCGGCCA
CTTTTATTGGAAACTCGACAGCTATTCAGGAGTTGTTCAAGAGAATCTCTGAACA
ATTTACAGGTACTATTATGGAGACGCGGAAAAGTTCATTTTATTGCGGATTCGAA
AGCGAAAACGCGAAAAAATTATCGACTTTTCAGAAAATCAAAAAATTTAAAAAA
ATTAAAATGTTTATTATATTTTGTATCCAAAAATTACAAAAATTCTCATTAAAGG
CTTTTTGAAAATTAAATATTGAAAAAAGTTGCTGGAAACGTGCTCCACCGCCGTT

AGGTATTTGGAGGCGAAATTGTGAATCCAAATGTTTTACCAAATTTCAGCCATTT
TTCGTCTTTTTTGTGATTTTTTTCCGTTTAAAAAAAAATTTCGTGTCAAAACGATT
TTTTCACCAATTTCGACATAATTTTTTCAACGAACAGCATCACAAAAATGAATAA
AATCGGCAAAAAAATTCAAAAAACCTTTTAATTTTCAGTTGTTTTTTTATTGAAA
ATTGGCGAAACTAATTGAAACATTCTAAATTTACGGTATTAAATATCCAACATGT
CGGATTTTATAGCGAAAATTATCGATTTTTCAGAACATAAAAAAAAACGAGAAA
AATTTCAATATTTTATCGAATTTGGTATATTTTAAATTTAAAAAAATCATAATTTT
TCTCAATTTTATGAAATTTTCAGCAATTTTTTGGTTAAAGTTTGGTAAATTTGGGT
TAAAAACATGAATTTAGGCGAAATTCGAAAAAGAATTTCCAATTATCGAAAAAT
CTAAAAAAAAAAAAAATTTTCTATATTTTTTTTTCATAATTTTCCCCTAACTCTGT
CCAAATTTCAGCAATGTTCCGCCGCAAGGCTTTCCTCCATTGGTATACTGGCGAA
GGAATGGACGAGATGGAATTCACTGAAGCTGAGAGTAATATGAACGATCTTGTC
TCAGAATACCAGCAATACCAGGAAGCAACTGCAGAGGAAGATGGAGAACTTGAT
GGAACTGATGGAGATGCTGAATAG

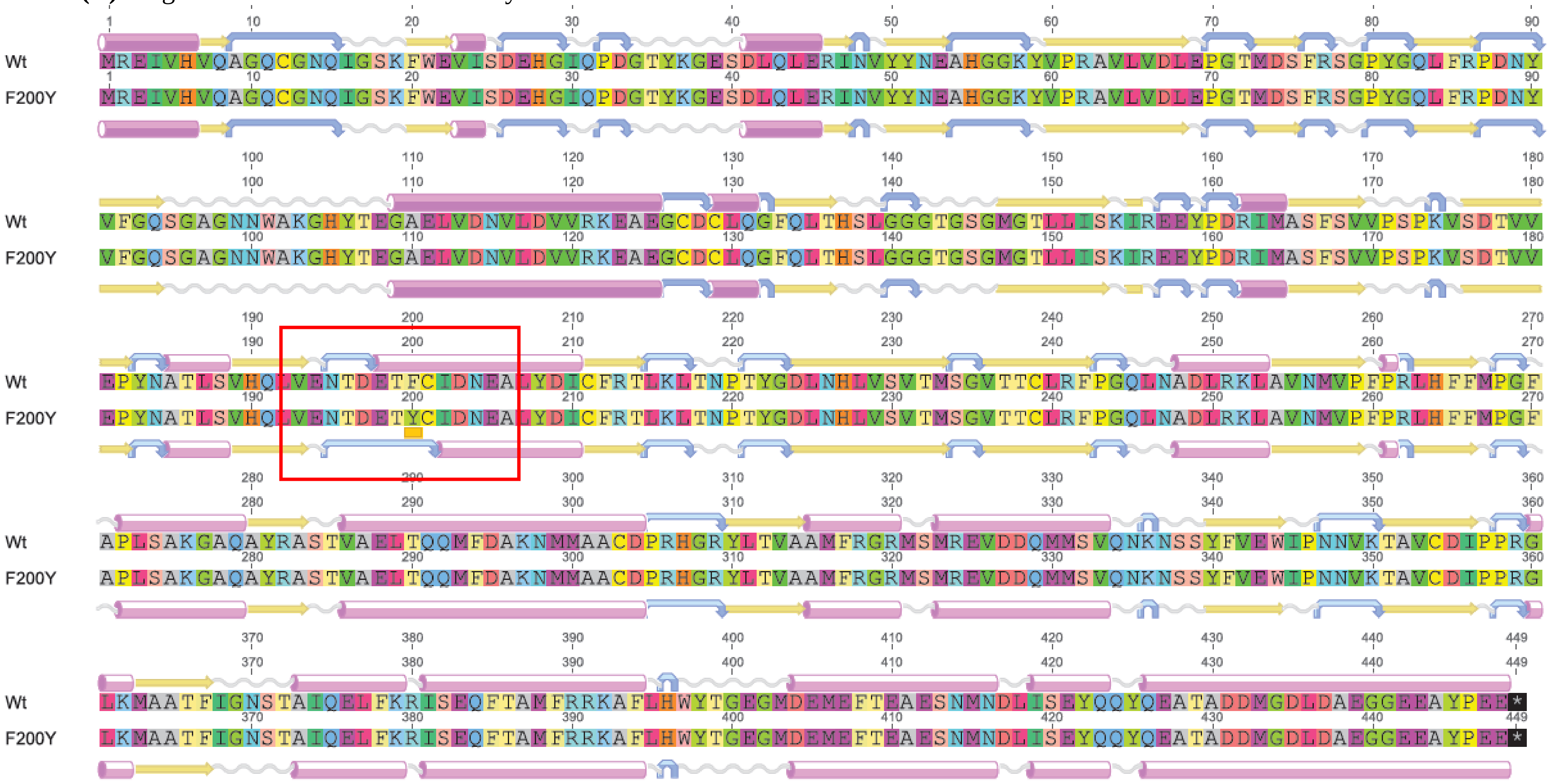
*The exonic regions are highlighted in green and in all cases the complete coding sequence of the *ben-1* locus was obtained. Nucleotide substitutions with respect to the N2 sequence are highlighted in yellow and deletions are highlighted in red (indicated by a hyphen). The intronic sequences are not complete. Only those available after sequencing are shown.

Appendix E: Secondary structure predictions and protein alignments

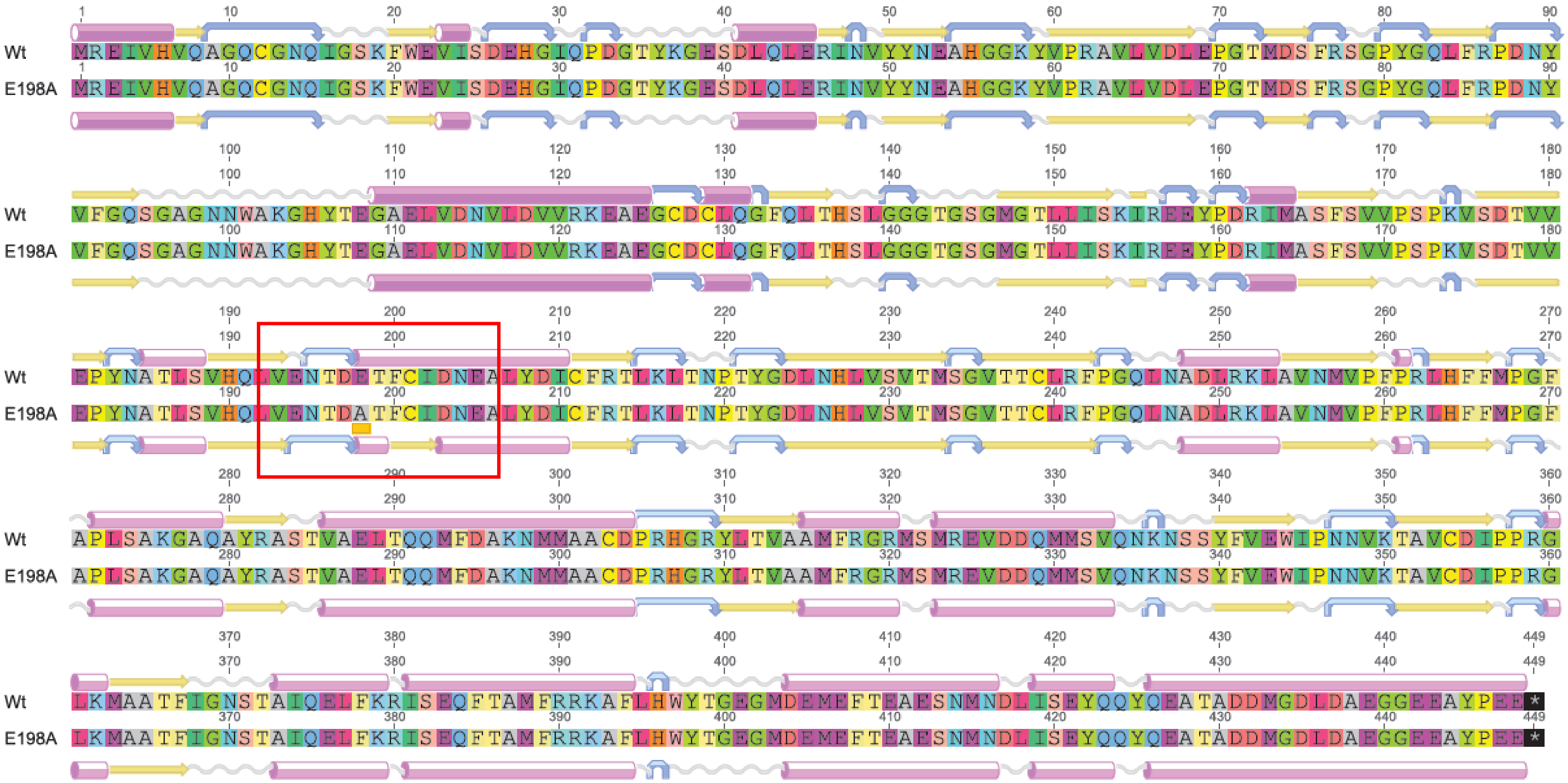
E.1 Secondary structure prediction for *ben-1* β-tubulin of CB3474: *ben-1 (e1880)* III. Region of conflict is indicated by a red box.



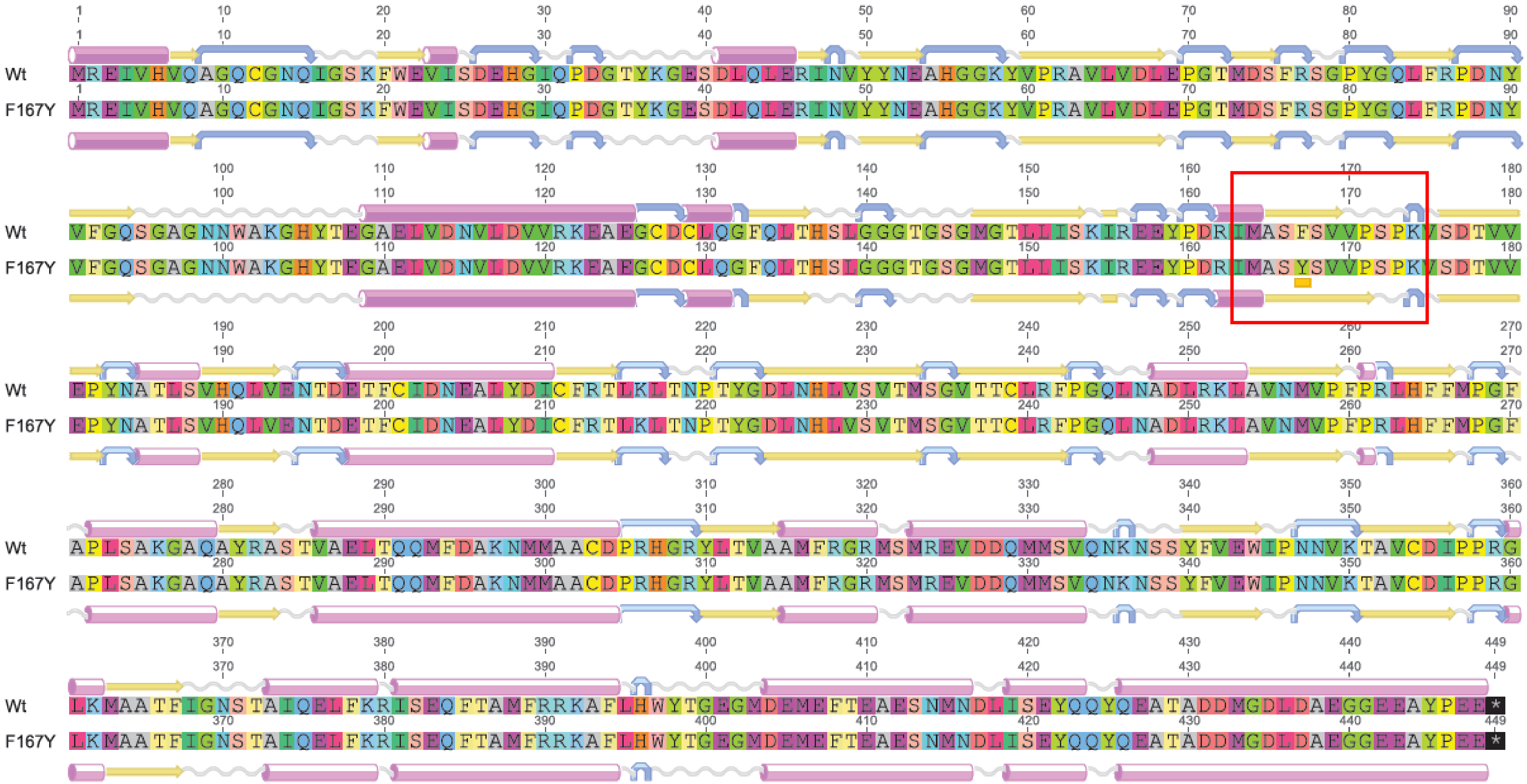
E.2 Secondary structure predictions for *H. contortus* isotype-1 β-tubulin with resistance polymorphisms F200Y (A), E198A (B) and F167Y (C). Regions of conflict are indicated by red boxes.



A



B



C

E.3 Alignment of *C. elegans ben-1* and *H. contortus* isotype-1 β-tubulins

*Areas that differ are indicated by red boxes

Appendix F: Polymorphisms in the BEN-1 β-tubulin of artificially mutagenised *C. elegans* strains created for the million mutations project

Strain	61	62	63	68	69	70	71	72	73	76	77	78	79	80	81	95	96	97	138	139	140	152	153	154	184	185	186	231	232	233	307	308	309	322	323	324	325	358	359	360	385	386	387	403	404	405	412	413	414	427	428	429	440	441	442
VC20230																																																						■	
VC20260																							■																																
VC20512							■																																																
VC20620																																				■																			
VC20791																	■																																						
VC30043																																																■							
VC30200																																							■																
VC40295																																										■													
VC40387																																																			■				
VC40476								■																																															
VC40560																				■																																			
VC40563																																■																							
VC40563																														■																										
VC40655		■																																																					

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