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Characterization and comparative analysis of the complete *Haemonchus contortus* β-tubulin gene family and implications for benzimidazole resistance in strongylid nematodes

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ABSTRACT

Parasitic nematode β-tubulin genes are of particular interest because they are the targets of benzimidazole drugs. However, in spite of this, the full β-tubulin gene family has not been characterized for any parasitic nematode to date. *Haemonchus contortus* is the parasite species for which we understand benzimidazole resistance the best and its close phylogenetic relationship with *Caenorhabditis elegans* potentially allows inferences of gene function by comparative analysis. Consequently, we have characterized the full β-tubulin gene family in *H. contortus*. Further to the previously identified *Hco-tbb-iso-1* and *Hco-tbb-iso-2* genes, we have characterized two additional family members designated *Hco-tbb-iso-3* and *Hco-tbb-iso-4*. We show that *Hco-tbb-iso-1* is not a one-to-one orthologue with *Cel-ben-1*, the only β-tubulin gene in *C. elegans* that is a benzimidazole drug target. Instead, both *Hco-tbb-iso-1* and *Hco-tbb-iso-2* have a complex evolutionary relationship with three *C. elegans* β-tubulin genes: *Cel-ben-1*, *Cel-tbb-1* and *Cel-tbb-2*. Furthermore, we show that both *Hco-tbb-iso-1* and *Hco-tbb-iso-2* are highly expressed in adult worms; in contrast, *Hco-tbb-iso-3* and *Hco-tbb-iso-4* are expressed only at very low levels and are orthologous to the *Cel-mec-7* and *Cel-tbb-4* genes, respectively, suggesting that they have specialized functional roles. Indeed, we have found that the expression pattern of *Hco-tbb-iso-3* in *H. contortus* is identical to that of *Cel-mec-7* in *C. elegans*, being expressed in just six "touch receptor" mechano-sensory neurons. These results suggest that further investigation is warranted into the potential involvement of strongylid isotype-2 β-tubulin genes in mechanisms of benzimidazole resistance.

1. Introduction

Infection with parasitic nematodes causes serious disease in a wide variety of organisms (Jackson, 1993). Control of these infectious diseases is primarily through the use of chemotherapeutic anthelmintic drugs (Jackson, 1993). However resistance to anthelmintics is widespread for a variety of nematodes of veterinary importance and is an emerging concern for several parasites of human importance (Jackson, 1993; Sargison et al., 2001; Bartley et al., 2003; Gilleard and Beech, 2007). Of the anthelmintic drug classes, resistance to the benzimidazoles is the most widespread and has been the most extensively investigated at the molecular level.

The major mode of action of benzimidazole drugs is hypothesized to involve the disruption of the polymerization of tubulin dimers that comprise microtubules (Lacey, 1988; Prichard, 2001; Robinson et al., 2004). Microtubules provide intracellular support for the cytoskeleton and their disruption has been shown to be the causative effect of many drug classes (Lacey, 1988; Wilson et al., 1999). Benzimidazole drugs have been shown to bind with high affinity to tubulin in vitro (Kohler and Bachmann, 1981), however the molecular detail of the benzimidazole–tubulin interaction within nematodes has not been fully resolved, with different

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potential models being proposed (Oxberry et al., 2001; Prichard, 2001; Robinson et al., 2004). Although the precise benzimidazole binding site is a contentious issue, conclusive in vivo evidence has shown that β-tubulin is the major benzimidazole target in nematodes; from genetic studies in *Caenorhabditis elegans*, all 28 different ethyl methanesulfonate (EMS)-induced mutations that conferred benzimidazole resistance to the organism mapped to a single locus; β-tubulin *Cel-ben-1* (Driscoll et al., 1989).

There is compelling evidence that mutations in β-tubulin genes are associated with benzimidazole resistance for a number of parasitic nematode species (Kwa et al., 1993a; Prichard, 2001). The most detailed work to date has been conducted on the parasitic nematode of sheep, *Haemonchus contortus*, in which two β-tubulins genes, *isotype-1* and *isotype-2*, have been identified (Geary et al., 1992; Kwa et al., 1993b). Subsequent work has also identified closely related genes in a number of other strongylid nematode parasites (Njue and Prichard, 2003). The major research focus in *H. contortus* and other related nematodes has been to investigate the relationship between the *isotype-1* β-tubulin gene and benzimidazole resistance. Roos et al. (1990) demonstrated a reduced number of restriction fragment length polymorphism (RFLP) fragments for the β-tubulin *isotype-1* locus in benzimidazole-resistant *H. contortus* populations relative to susceptible populations and this was later shown to be due to changes in allelic polymorphism, rather than loss of a benzimidazole-susceptible gene (Beech et al., 1994). Further work showed that the only sequence polymorphism that consistently differed between the drug-susceptible and -resistant populations of *H. contortus* was a phenylalanine to a tyrosine substitution at amino acid position 200 of the polypeptide encoded by the β-tubulin *isotype-1* gene (F200Y) (Kwa et al., 1993b). Subsequent work has consistently shown an association of this polymorphism with the benzimidazole-resistant phenotype in *H. contortus* and several other trichostrongylid nematode species (Clark et al., 2005; Von Samson-Himmelstjerna et al., 2007; Rufener et al., 2009). The functional ability of the F200Y substitution in *H. contortus* β-tubulin ISOTYPE-1 to confer benzimidazole resistance was shown using *C. elegans* as a heterologous expression system (Kwa et al., 1995). Transgenic expression of *H. contortus isotype-1* alleles encoding a phenylalanine residue at position 200 of the polypeptide increased the susceptibility of the benzimidazole-resistant *Cel-ben-1* mutant *C. elegans* to the drug, whereas alleles encoding a tyrosine at this position did not. More recently, benzimidazole resistance in *H. contortus* has also been associated with mutations resulting in single substitutions of other amino acids of the β-tubulin ISOTYPE-1 protein (F167Y and E198A), although functional studies on these mutations have not yet been performed (Von Samson-Himmelstjerna et al., 2007). There has been less research on the potential role of the *isotype-2* β-tubulin gene in benzimidazole resistance. However, deletion of the *isotype-2* gene has been correlated with benzimidazole resistance in field strains (Kwa et al., 1993a; Beech et al., 1994) and in vitro selection for resistance has also been shown to select for an E198A substitution in β-tubulin ISOTYPE-2 (Rufener et al., 2009). This provides an interesting contrast to benzimidazole resistance in fungi and *C. elegans*: parasitic trichostrongylid nematodes are currently the only group of organisms for which mutations in more than one β-tubulin locus have been implicated in this resistance mechanism (Von Samson-Himmelstjerna et al., 2007). Moreover, selection of the F200Y-encoding single nucleotide polymorphism (SNP) of *H. contortus* has also been suggested to correlate with resistance to the macrocylcic lactone, ivermectin (Eng et al., 2006).

Despite the importance of β-tubulin genes as drug targets and their involvement in anthelmintic resistance, the full gene family has not been characterized in any parasitic nematode species to date. For *H. contortus*, *isotype-1* and *isotype-2* are the only identified β-tubulin genes and the *isotype-1* gene has generally been assumed to be the *Cel-ben-1* orthologue (Geldhof et al., 2006). However there has been no systematic attempt to identify additional gene family members from *H. contortus*, or to determine relationships with members of the β-tubulin gene family in *C. elegans* and other Clade V nematodes.

Consequently, we have undertaken a detailed characterization of the *H. contortus* β-tubulin gene family using a combination of molecular cloning and bioinformatic analysis of the available genome sequence data (http://www.sanger.ac.uk/resources/downloads/helminths/haemonchus-contortus.html). This has revealed two additional β-tubulin genes that we have designated *Hco-tbb-isotype-3* and *Hco-tbb-isotype-4*. Here we present a comparative analysis of the full *H. contortus* gene family with that of other Clade V nematodes for which good quality finished or draft genomes are available, and discuss relationships with the functionally characterized genes in the model organism, *C. elegans*.

2. Materials and methods

2.1. Haemonchus contortus β-tubulin gene nomenclature

Prior to this project, full-length complementary DNA (cDNA) sequence data was available for two previously characterized *H. contortus* β-tubulin genes, *isotype-1* (M76493) and *isotype-2* (M76491 and M76492) (Geary et al., 1992). In addition, the full genomic locus for the *H. contortus isotype-1* gene was available (X67489.1). A variety of different nomenclatures have been used for these genes (Geary et al., 1992; Kwa et al., 1993b; Beech et al., 1994; Geldhof et al., 2006). In order to avoid confusion with previous literature, we have used the same system of nomenclature as employed by Kwa et al. (1993b) for the current and newly identified *H. contortus* β-tubulin genes (i.e. *Hco-tbb-iso-1*; Table 1).

2.2. Cloning and sequencing of the Hco-tbb-iso-2 locus

cDNA sequence from the *Hco-tbb-iso-2* locus is available, but the genomic sequence had not been previously reported. Primer sequences specific to the *Hco-tbb-iso-2* locus were designed from the available cDNA sequence to amplify overlapping products from genomic DNA template of *H. contortus* strain MHco3 (ISE) (primer sequences in Supplementary data S1). Each product was cloned into the pCR-4 TOPO vector (Invitrogen, UK) and sequenced in triplicate for three individual plasmid clones (Eurofins MWG Operon, Germany). A consensus sequence was assembled using the ContigExpress application of VectorNTI (Invitrogen), onto which the gene structure was manually annotated using the available *Hco-tbb-iso-2* cDNA sequences and canonical eukaryotic splice sites (Geary et al., 1992).

2.3. Sequence of the Hco-tbb-iso-3 locus

A Basic Local Alignment Search Tool (BLAST) searchable *H. contortus* bacterial artificial chromosome (BAC) end genomic sequence database was generated by the Wellcome Trust Sanger Institute, UK (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus). TBLASTN searching of this database with each of the six *C. elegans* β-tubulin polypeptide sequences identified a BAC end (haem_ends15g16.q1kT7) containing β-tubulin sequence. This BAC was selected for complete sequencing and finishing (Pathogen Sequencing Unit, Wellcome Trust Sanger Institute). Sequence from this BAC forms part of the largest contiguous sequence (contig) fragment generated to date from the *H. contortus* genome sequencing project (408,911 bp (Redman et al., 2008; Laing et al., 2011)). The β-tubulin locus, which is near the middle of this sequence, was manually annotated and designated *Hco-tbb-iso-3*.

Table 1
Haemonchus contortus β-tubulin nomenclature.

GenBank accession number		Previous nomenclature used	Nomenclature to be used in this analysis
gDNA	cDNA		
X67489	M76493	*Hc-isotype-1*[a,b] 1.1.1 *Hc-tub-8-9*[c] 1.1.2 *Hc-gru-1*[a] *Hc-ben-1*[d]	*Hco-tbb-iso-1*
HE604100	M76491 M76492	*Hc-isotype-2*[a,b] *Hc-tub-12-16*[c] *Hc-tub-12-164*[b]	*Hco-tbb-iso-2*
HE604101	HE604103	N/A	*Hco-tbb-iso-3*
HE604102	HE604104	N/A	*Hco-tbb-iso-4*

gDNA, genomic DNA.
[a] Kwa et al. (1993b).
[b] Beech et al. (1994).
[c] Geary et al. (1992).
[d] Geldhof et al. (2006).

2.4. Sequence of the Hco-tbb-iso-4 locus

Each of the six *C. elegans* β-tubulin polypeptide sequences were used as query in TBLASTN searching >800 Mb of *H. contortus* genomic sequence within a database that contains all Sanger sequenced shotgun reads generated for this project thus far (http://130.209.234.35/blast/blast.html). Only reads that gave a *C. elegans* β-tubulin polypeptide as a top hit in reciprocal BLASTX searching of *C. elegans* Wormpep (release WS150, http://130.209.234.35/blast/blast.html) were considered as real hits. A total of 88 reads passed these criteria.

Each of these reads were aligned to the current genome assembly of *H. contortus* (ftp://ftp.sanger.ac.uk/pub/pathogens/Haemonchus/contortus/genome/) that represents 76.6% (82.7%) full (partial) CEGMA sequences (Parra et al., 2007). Of the 88 β-tubulin shotgun sequence reads, 79 mapped to the *Hco-tbb-iso-1*, *Hco-tbb-iso-2* or *Hco-tbb-iso-3* loci of this assembly. Each of the remaining nine reads aligned to a single locus on contig06084. The β-tubulin locus near the middle of this sequence was manually annotated and designated *Hco-tbb-iso-4*.

2.5. cDNA sequencing for the Hco-tbb-iso-3 and Hco-tbb-iso-4 genes

PCR primers were designed to regions flanking the predicted initiation and termination codons of the *Hco-tbb-iso-3* locus, and the predicted 3' transcribed region of the *Hco-tbb-iso-4* locus (primer sequences given in Supplementary data S1). PCR products were obtained using *H. contortus* strain MHco3 (ISE) L3 and adult stage cDNA as template (Redman et al., 2008). Products were cloned into vector pCR-4 (Invitrogen) and sequenced in triplicate for three plasmid clones (Eurofins MWG Operon). The sequences for each gene were independently assembled (ContigExpress, Invitrogen). The *Hco-tbb-iso-3* cDNA sequence flanked both the initiation and termination codons, indicating that the complete protein-coding region for this locus had been obtained. A total of 792 bp cDNA sequence flanking the *Hco-tbb-iso-4* termination codon was obtained by 3′ rapid amplification of cDNA ends (RACE), allowing determination of the C-terminal region of this putatively translated β-tubulin polypeptide.

2.6. Mapping Illumina transcriptome reads to the H. contortus β-tubulin cDNA sequences

We have previously sequenced 38 million 76 bp Illumina reads from adult *H. contortus* MHco3 (ISE) cDNA to aid annotation of the *H. contortus* genome (Laing et al., 2011). These reads were mapped against the four *H. contortus β-tubulin* cDNA sequences with the Burrows-Wheeler Aligner program (BWA) and the number of reads aligned to each were counted and normalized to the length of the cDNA (Li and Durbin, 2009). This permitted a comparison of constitutive expression of the four genes in the adult worm.

2.7. The H. contortus genome contains four β-tubulin loci

All β-tubulin sequences described for eukaryotic species share 80–100% amino acid sequence identity (Njue and Prichard, 2003). Greater than 30 β-tubulin polypeptide sequences from a range of eukaryotic species were used as query in TBLASTN searching of the *H. contortus* genome assembly (ftp://ftp.sanger.ac.uk/pub/pathogens/Haemonchus/contortus/genome/) and each polypeptide aligned only to the four *H. contortus* β-tubulin loci.

As the assembly is incomplete, we also investigated whether any high-throughput sequencing reads support the existence of any additional β-tubulin loci within the *H. contortus* genome. Thirty-eight million 76 bp paired-end Illumina reads generated from MHco3 (ISE) cDNA were mapped to the assembly (ftp://ftp.sanger.ac.uk/pub/pathogens/Haemonchus/contortus/genome/) using BWA (Li and Durbin, 2009). These reads represent approximately 54-fold coverage of the *H. contortus* genome and mapped with even coverage to the four β-tubulin loci at >90% sequence identity.

Additionally, cDNA sequence for each of the four *H. contortus* β-tubulin isotypes were mapped to 346 Mb of 454-sequence reads using Nucmer (ftp://ftp.sanger.ac.uk/pub/pathogens/Haemonchus/contortus/genome/). Thirty-one reads were identified in total, showing >80% identity to at least one of the four cDNA sequences. Twenty-five of these reads mapped to scaffolds in the *H. contortus* genome assembly (ftp://ftp.sanger.ac.uk/pub/pathogens/Haemonchus/contortus/genome/) representing the four β-tubulin loci. The remaining six 454-sequence reads that were not found in the assembly matched exonic sequence of only one of the β-tubulin isotypes at 100% identity. Each of these reads showed much lower identity (70–80%) to flanking intronic sequence of the respective β-tubulin isotype, presumably due to non-coding polymorphism, explaining their exclusion from the assembly.

Thus, we have concluded, from a variety of different analyses, that the *H. contortus* genome contains a total of four β-tubulin loci.

2.8. Identification of β-tubulin loci from other species of Rhabditida, Trichinella spiralis, Saccharomyces cerevisiae and Aspergillus clavatus

The β-tubulin gene models for *Caenorhabditis briggsae*, *Caenorhabditis brenneri*, *C. elegans*, *Caenorhabditis japonica*, *Caenorhabditis remanei* and *Pristionchus pacificus* were obtained from Wormbase (http://www.wormbase.org/, release WS225, date June 2011). Their gene structures were manually inspected, leading us to edit two *C. japonica* sequences (CJA07130 and CJA06141), one *C. briggsae* sequence (CBG16137) and one *C. remanei* sequence (CRE25447). Coding sequences (CDS) annotated as β-tubulin from species of the suborder Strongylida were downloaded from EMBLCDS (Leinonen et al., 2010). To confirm the annotation, we aligned these sequences with confirmed α- and β-tubulins using the Needleman–Wunsch algorithm (Needleman and Wunsch, 1970) and accepted the highest pairwise score as the correct annotation. For subsequent analyses, only species for which both ISOTYPE-1-like and ISOTYPE-2-like sequences were available were used: *Ancylostoma caninum*, *Cooperia oncophora*, and *Cylicocyclus nassatus*. For an outgroup to the evolutionary analysis, the genomes of the Clade I nematode *T. spiralis* and the fungi *S. cerevisiae* and *A. clavatus* were searched (Supplementary Table S1). Proteins were named based on *C. elegans* nomenclature and top reciprocal similarity scores (Supplementary Table S2).

2.9. Phylogenetic analysis

The β-tubulin CDS were aligned with the translation alignment algorithm implementation of Geneious (version 5.4 created by Biomatters. Available from http://www.geneious.com), using MAFFT (G-INS-i, Blosum-62, Gap-open:1.53, Offset value:0.123) (Katoh et al., 2005). The C-terminus of the β-tubulin protein contains predominantly acidic amino acids and is highly variable in terms of composition and length. This was excised for the subsequent tree building. Modelling rate heterogeneity with TREE-PUZZLE revealed that the third codon position was often strongly heterogeneous, making homoplasy likely (Schmidt and von Haeseler, 2007). Therefore, we excluded the third codon-position from subsequent analysis. Phylogenetic model assignment and maximum likelihood trees were performed with 1,000 bootstrap replicates, using MEGA (version 5.10) (Tamura et al., 2011).

2.10. Hco-tbb-iso-3 expression pattern analysis

Polyclonal antibodies were generated against the peptide DEEPAETFEAE corresponding to the variable C-terminal region of the Hco-TBB-ISO-3 polypeptide. Two rabbits were immunized and boosted with the peptide at days 0, 21, 42, 74, 91 and 105 and IgG antibody was immunoaffinity purified from terminal bleed serum (130 days) and pooled from both rabbits for use in immunolocalisation studies (animal care, immunisations and sampling undertaken by CovalAb, Cambridge, UK). For immunofluorescent antibody staining, exsheathed *H. contortus* L3s were fixed and stained using the Finney-Ruvkun method (Finney and Ruvkun, 1990). Purified primary antibody was used at a 1:20 dilution and the secondary antibody (Alexa Flour 448 goat anti-rabbit IgG, Invitrogen) at 1:200. Secondary antibody only controls were negative for the described expression pattern.

An *Hco-tbb-iso-3* promoter GFP fusion construct was made using the PCR fusion protocol described by Hobert (2002). Two thousand and fifty-six bp of sequence immediately upstream from the *Hco-tbb-iso-3* ATG start site was PCR amplified from *H. contortus* genomic DNA (primer sequences in Supplementary data S1), and fused to the *gfp* gene, including synthetic introns, and the unc-54 3′ untranslated region (UTR) from Fire vector pPD95.67 (Fire et al., 1990). Transgenic lines were created by microinjection into the *C. elegans* N2 germline, using the method of Mello et al. (1991) with the plasmid pRF-4 as a co-transformation marker to identify transgenic worms. Expression patterns were visualized using a Zeiss, Axioscop 2 plus microscope. Images were collected and processed using ImprovisionOpenlab software (http://www.improvision.com).

3. Results

3.1. The H. contortus β-tubulin gene family consists of four members

Three full-length *H. contortus* cDNA sequences putatively encoding β-tubulin polypeptides, one corresponding to *Hco-tbb-iso-1* and two to *Hco-tbb-iso-2*, have been previously published (Table 1) (Geary et al., 1992). The full-length genomic sequence corresponding to one of these, *Hco-tbb-iso-1*, has also been previously documented (Table 1) (Kwa et al., 1993b). We set out to characterize the full *H. contortus* β-tubulin gene family. Firstly, we cloned and sequenced the complete *Hco-tbb-iso-2* genomic locus. This was achieved by PCR amplification from genomic DNA of the *H. contortus* genome strain MHco3 (ISE) (see Section 2.2). We also identified two new β-tubulin genes from bioinformatic analysis of the available *H. contortus* genome sequence databases (http://www.sanger.ac.uk/resources/downloads/helminths/haemonchus-contortus.html). The first of these was identified by searching the BAC end sequence database (*H. contortus* BAC end sequence reads) with *C. elegans* β-tubulin polypeptide sequences. The identified BAC clone was sequenced in its entirety together with several overlapping BACs, and the new β-tubulin gene was designated *Hco-tbb-iso-3*. The fourth *H. contortus* β-tubulin gene was identified within the latest available multi-platform genome assembly; this gene was manually annotated and designated *Hco-tbb-iso-4*.

Transcripts specific to each of the four *H. contortus* β-tubulin genes were amplified from L3 and adult cDNA confirming expression of all four genes in both of these developmental stages (data not shown). The cDNA sequences of *Hco-tbb-iso-3* and *Hco-tbb-iso-4* were cloned and sequenced, allowing polypeptide sequences to be deduced. Although the cDNA from *Hco-tbb-iso-4* was incomplete, next-generation sequence data allowed us to deduce the full-length polypeptide sequence translated from this gene. At the amino acid level, the putative translated polypeptides of the four *H. contortus* β-tubulin genes are 89–94% identical to one another and the cDNA sequences are 74–77% identical (Fig. 1 and Supplementary Tables S3 and S4). All four *H. contortus* putatively translated β-tubulin polypeptides contain the conserved sequence motif GGGTGS that distinguishes α, β and γ tubulin family members (amino acid positions 140–146) (Hesse et al., 1987); and the β-tubulin characteristic auto-regulatory motif, MREI (amino acid positions 1–4) (Cleveland, 1988).

The C-terminal regions, encompassing the final 17 amino acids, are highly variable between the four *H. contortus* β-tubulin polypeptides (Fig. 1). This is a common feature of β-tubulins, both within and between many other eukaryotic species (Sullivan and Cleveland, 1986). The C-terminal region of the β-tubulin polypeptide interacts with the microtubule associated proteins (MAPs) and deletion of this region has been shown to interrupt the correct assembly of microtubules (Dominguez et al., 1990).

3.2. Relative expression levels of the four H. contortus β-tubulin genes in adult worms

To investigate the relative expression levels of the *H. contortus β-tubulin* genes in adult worms, we mapped 38 million, 76 bp Illumina cDNA reads generated from adult MHco3 (ISE) to the four cDNA sequences (Table 2). This revealed that *Hco-tbb-iso-1* and *Hco-tbb-iso-2* are much more highly expressed than *Hco-tbb-iso-3* and *Hco-tbb-iso-4*. *Hco-tbb-iso-1* was highest, followed by *Hco-tbb-iso-2*, *Hco-tbb-iso-4* then *Hco-tbb-iso-3*.

3.3. Genomic structure of the H. contortus β-tubulin genes

We manually annotated the structures of each of the *H. contortus* β-tubulin genes based on the alignment of genomic and cDNA sequences (Fig. 2). The structures of *Hco-tbb-iso-1* and *Hco-tbb-iso-2* are very similar to each other, with all intron positions being identical except for the last intron of *Hco-tbb-iso-1* that is not found in *Hco-tbb-iso-2*. The gene structures of *Hco-tbb-iso-3* and *Hco-tbb-iso-4* share 10 of 12 intron positions and are therefore more similar to each other than either is to *Hco-tbb-iso-1* or *Hco-tbb-iso-2*. However, both of these models are distinct from each other, demonstrated by intron position variation across the entire lengths of the genes (Fig. 2).

The gene models generated for *Hco-tbb-iso-1*, *Hco-tbb-iso-2*, *Hco-tbb-iso-3* and *Hco-tbb-iso-4* were compared with those of the six β-tubulin genes in *C. elegans* (www.wormbase.org; Fig. 2). In all cases, the models of the *H. contortus* β-tubulin genes cover a larger genomic region than those of the *C. elegans* family. All of the exon/intron boundary positions of the *Cel-ben-1*, *Cel-tbb-1*, *Cel-tbb-2* and *Cel-tbb-6* genes are perfectly conserved in *Hco-tbb-iso-1* and *Hco-tbb-iso-2*, although both of the parasite genes have

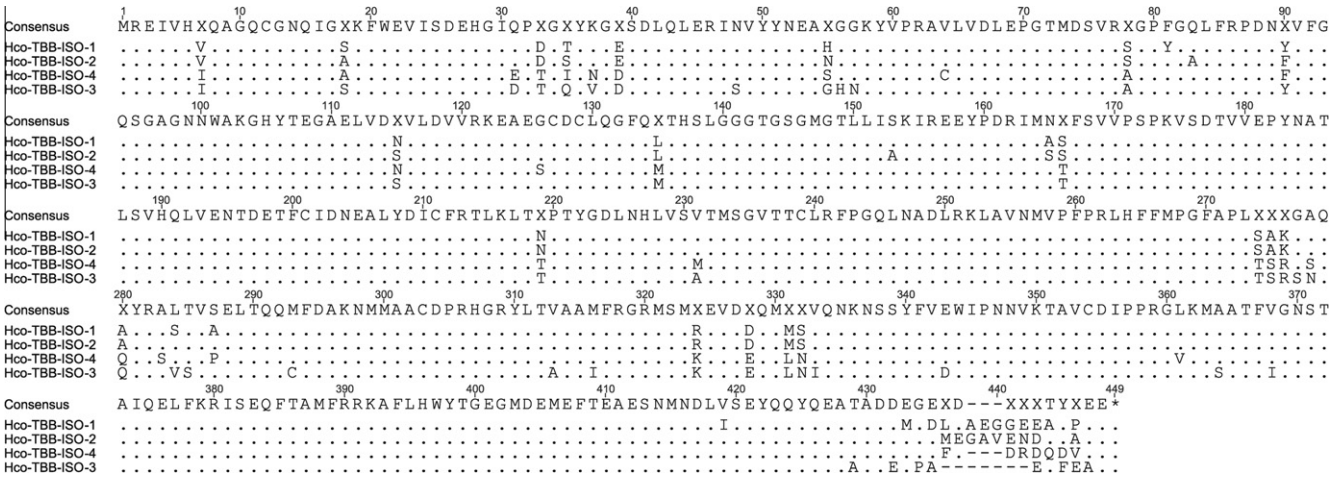

Fig. 1. Alignment of the putative translations of the *Haemonchus contortus* β-tubulin genes for *Hco-tbb-iso-1*, *Hco-tbb-iso-2*, *Hco-tbb-iso-3* and *Hco-tbb-iso-4*.

Table 2
Expression levels of the four *Haemonchus contortus* β-tubulin genes from Illumina sequence read data.

β-tubulin cDNA	cDNA length (kb)	Number of reads mapped	Number of reads mapped per kb
Hco-ttb-iso-1	1.155	10,290	8909.1
Hco-ttb-iso-2	1.323	6,234	4258.2
Hco-ttb-iso-3	1.132	6	5.3
Hco-ttb-iso-4	0.792	6	7.6

additional introns. Just one intron, at p.174, is unique to *Cel-ben-1* within the *C. elegans* gene family and this intron position is conserved in *Hco-tbb-iso-1* and *Hco-tbb-iso-2* but not in the other two *H. contortus* β-tubulin genes. *Cel-mec-7* is the only *C. elegans* β-tubulin gene for which all its exon/intron breakpoints are shared with *Hco-tbb-iso-3*. This includes an intron position unique to *Cel-mec-7* within the *C. elegans* gene family, at codon 62. Finally, the *Hco-tbb-iso-4* model shares more exon/intron break points with *Cel-tbb-4* than any other β-tubulin gene from this free-living nematode. This comparative analysis of gene structures is consistent with the orthologous relationships suggested by the phylogenetic comparisons of the *H. contortus* and *C. elegans* β-tubulin polypeptide sequences described in Section 3.2.

3.4. Comparative analysis of the H. contortus β-tubulin polypeptide sequences with those from Caenorhabditis spp. and P. pacificus

In the canonical phylogeny of the nematodes (Blaxter et al., 1998), *H. contortus* is found within Clade V, a classification it shares with five species of *Caenorhabditis* and *P. pacificus*, for which complete or draft genomes are available (*C. elegans* sequencing consortium, 1998; Stein et al., 2003; Dieterich et al., 2008). We were able to annotate the tubulin genes from these genomes. In addition, specific projects have identified β-tubulin genes from other Clade V species: *A. caninum*, *C. oncophora* (Njue and Prichard, 2003) and *C. nassatus* (Pape et al., 1999). Together, these data offered an excellent opportunity to explore the evolutionary relationships between the *H. contortus* β-tubulin gene family and that of other Clade V nematodes. It was necessary to remove three caenorhabditid genes from the analysis. There were problems with the gene models for *Cre-ben-1* and *Cbr-tbb-1*. In addition, *Cel-tbb-6*, which is likely to be the result of a *C. elegans*-specific duplication, had a nucleotide composition that did not conform to other β-tubulin genes. However, *Cel-tbb-6* contains the M[RK]EI motif, which is both necessary and sufficient to drive β-tubulin auto-regulation (Hesse et al., 1987; Cleveland, 1988; Bachurski et al., 1994; Saussede-Aim et al., 2009), and intron position conservation supports its homology with other nematode β-tubulin genes (Fig. 2). The gene was excluded to remove the potential influence of long-branch attraction. For all genes, the rate of heterogeneity for each nucleotide position was modelled; the third codon position typically showed a high rate, thus the subsequent phylogenetic analysis ignored these and used the first and second codon positions only.

The maximum likelihood phylogenetic reconstruction (model: TN93+G) implies gene duplications at multiple points in the evolution of the Clade V nematodes (Fig. 3). The caenorhabditid genes, *tbb-1*, *tbb-2*, and *ben-1*, formed a monophyletic clade with the trichostrongylid genes, *tbb-iso-1* and *tbb-iso-2*, hereafter termed the *tbb-1*-like clade (Fig. 3 – node A). A second clade joined caenorhabditid genes, *tbb-4* and *mec-7*, with *tbb-iso-3* and *tbb-iso-4* from *H. contortus*, hereafter termed the *tbb-4*-like clade (node B). The precise relationship of these two clades with the single β-tubulin gene from *T. spiralis* could not be resolved. Within the *tbb-1*-like clade, *tbb-1*, *tbb-2* and *ben-1* genes from *Caenorhabditis* spp. form a monophyletic clade (node C), the two *P. pacificus* genes are sister taxa (node D), and the *tbb-iso-1* and *tbb-iso-2* genes from the Trichostrongyloidae are also monophyletic (node E). The *tbb-4*-like clade can be split further into two clades: *tbb-4* (node F) and *mec-7* (node G). Each of these clades followed the accepted nematode species phylogeny.

3.5. Expression pattern of Hco-tbb-iso-3 is consistent with an orthologous relationship with Cel-mec-7 and suggests functional conservation

Immunoaffinity purified IgG directed against the carboxy-terminal 11 amino acids (DEEPAETFEAE) of the *H. contortus* β-tubulin ISOTYPE-3 polypeptide primarily localized to six neuronal cell bodies and associated axonal processes in MHco3 (ISE) exsheathed L3s (Fig. 4B). The positions of these six cell bodies, as well as the length and direction of the axonal processes, closely corresponded to the six touch cell receptors described in *C. elegans* (ALML/R, PLML/R, AVM and PVM) (Fig. 4A and http://www.wormatlas.org/cellid/cellID.htm). Faint expression was also seen in cell bodies consistent with the position of the *C. elegans* FLP, PVD and BDU neurons.

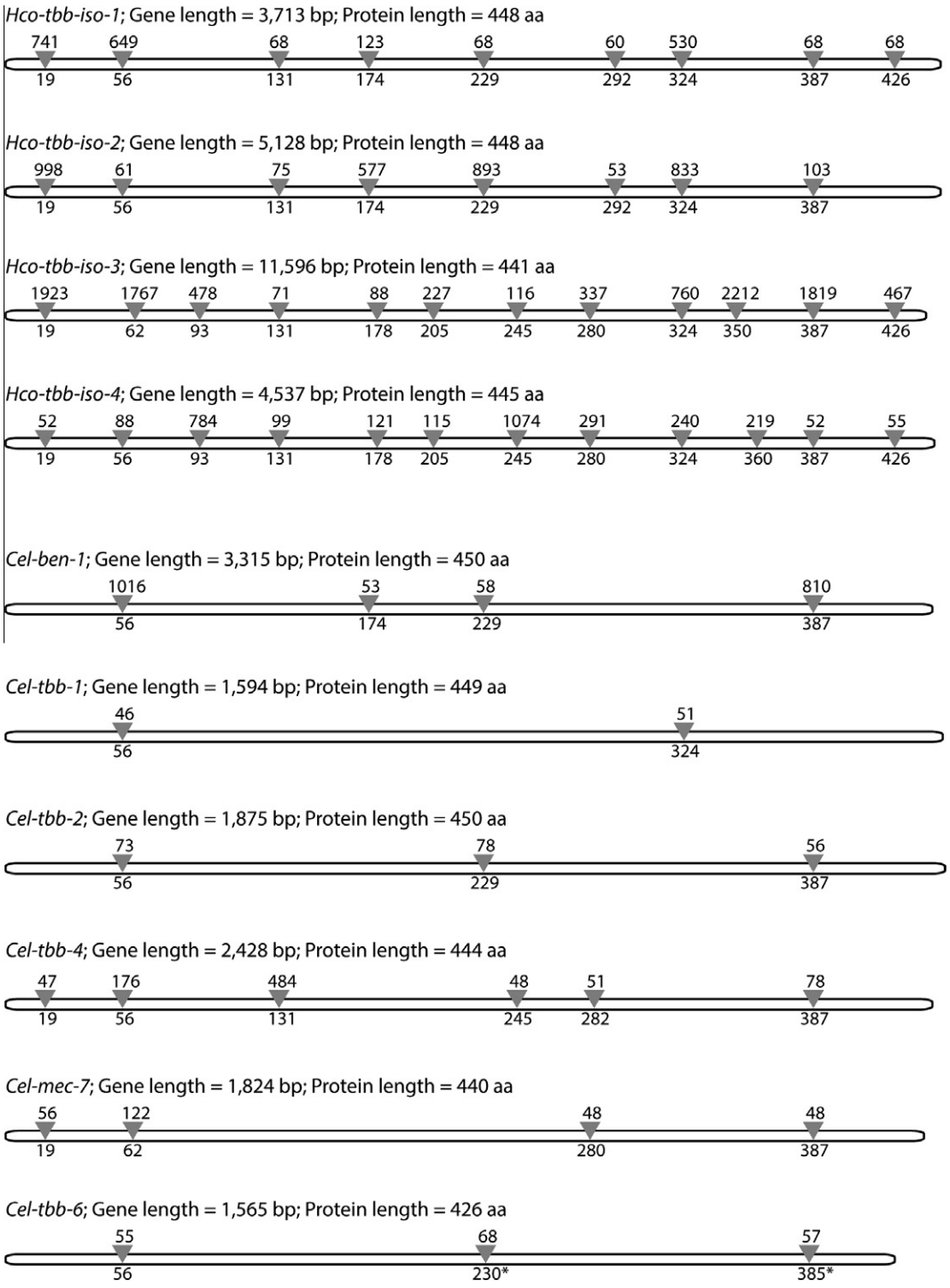

Fig. 2. Comparative analysis of *Haemonchus contortus* (*Hco*) and *Caenorhabditis elegans* (*Cel*) β-tubulin gene models. Manually annotated gene models for the four *H. contortus* and six *C. elegans* β-tubulin genes are shown with protein length in amino acids (aa). The introns are indicated by the grey triangles. The number above each intron is its size (bp) and the number below is the codon position. In *Cel-tbb-6*, the asterisks (∗) signify that an insertion and deletion are responsible for shifting the introns at codons 230 and 385, respectively. In the multiple sequence alignments, these introns align with codons 229 and 387 in other nematode β-tubulins.

Three independent transgenic *C. elegans* lines carrying extrachromosomal arrays containing constructs in which GFP expression was under the control of the 2,056 bp sequence immediately upstream of the *Hco-tbb-iso-3* ATG were examined. GFP expression was confined to these same six cells (Fig. 4C).

4. Discussion

The nematode β-tubulin genes are of particular interest because they are targets of one of the most important classes of anthelmintic drugs, the benzimidazoles (Driscoll et al., 1989; Oxberry et al.,

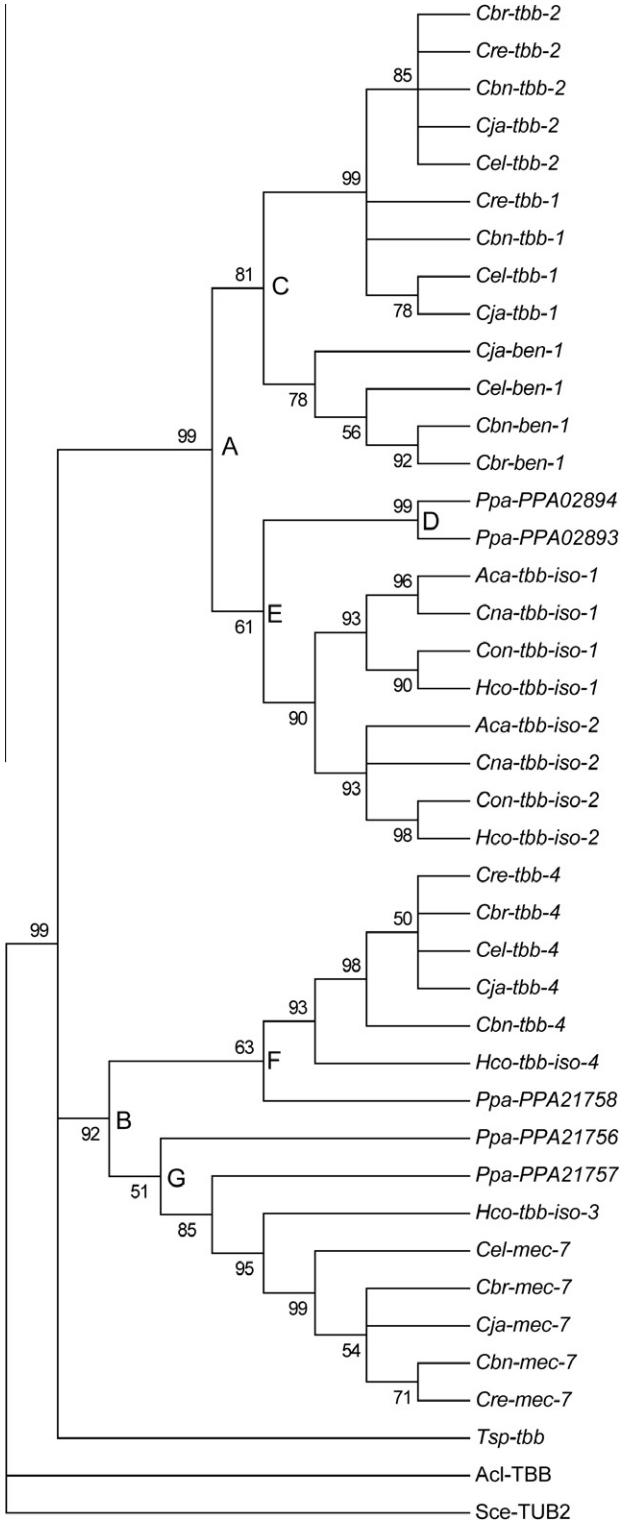

Fig. 3. A maximum likelihood based phylogenetic reconstruction of β-tubulin genes from Clade V nematodes. A single β-tubulin gene could be identified from the Clade I nematode, *Trichinella spiralis* (Tsp). Tubulin genes from *Aspergillus clavatus* (Acl) and *Saccharomyces cerevisiae* (Sce) were chosen as outgroups. Nodes A-G are indicated. Node support was calculated using 1,000 bootstrap replicates and nodes with less than 50% support have been collapsed. *Aca*, *Ancylostoma caninum*; *Cel*, *Caenorhabditis elegans*; *Cbn*, *Caenorhabditis brenneri*; *Cbr*, *Caenorhabditis briggsae*; *Cja*, *Caenorhabditis japonica*; *Cna*, *Cylicocyclus nassatus*; *Con*, *Cooperia oncophora*; *Cre*, *Caenorhabditis remanei*; *Hco*, *Haemonchus contortus*; *Ppa*, *Pristionchus pacificus*.

2001). Mutations in the *isotype-1* β-tubulin gene of *H. contortus* (*Hco-tbb-iso-1*; Table 1), and a number of other strongylid nematodes, play an important role in the mechanism of benzimidazole resistance (Gilleard, 2006; Gilleard and Beech, 2007; Prichard, 2007). *Hco-tbb-iso-1* has also recently been suggested to play a role in ivermectin resistance (Eng et al., 2006). A second β-tubulin gene, designated *isotype-2*, has previously been identified in a number of strongylid nematodes including *H. contortus* (*Hco-tbb-iso-2*) and has been implicated in benzimidazole resistance (Kwa et al., 1993a). Most parasitic nematode research on β-tubulins to date has focused on the role of *Hco-tbb-iso-1*, and to a lesser extent, *Hco-tbb-iso-2*, in benzimidazole resistance. The full β-tubulin gene family has yet to be described for any parasitic nematode and the discovery of new parasitic nematode β-tubulin genes has been largely confined to PCR approaches using primers designed against known *isotype-1* sequences (Gilleard, 2006; Von Samson-Himmelstjerna et al., 2007). Whilst this is a reasonable approach to identify *Hco-tbb-iso-1* homologs from new species, it is intrinsically biased and this presents a number of problems. Firstly, within parasitic genomes, additional, undiscovered, β-tubulin genes could be present that are benzimidazole drug targets, potentially involved in resistance. Secondly, without knowledge of the full gene family, or a phylogenetic framework for nematode β-tubulin genes, it is difficult to infer orthology of genes between species or extrapolate experimental data with any confidence. *Haemonchus contortus* is the parasitic nematode on which the most β-tubulin and anthelmintic resistance research has been conducted to date. Its position in the strongylid nematode clade, and its close phylogenetic relationship with *C. elegans*, make it a valuable model for a large number of parasitic nematodes of medical and veterinary importance (Gilleard, 2004). Consequently, we have characterized the full β-tubulin gene family for this important parasite.

4.1. The *H. contortus* β-tubulin gene family and phylogenetic relationships with *C. elegans*

Our analysis shows that *H. contortus* has a total of four β-tubulin genes; further to the previously identified *Hco-tbb-iso-1* and *Hco-tbb-iso-2* genes, two additional family members have been identified and designated *Hco-tbb-iso-3* and *Hco-tbb-iso-4*. These loci incorporate all β-tubulin containing *H. contortus* genomic sequence reads that have been generated thus far from the genome sequencing project and we are therefore confident that these genes represent the full family for this organism. Consequently, we have explored the phylogenetic relationship of the *H. contortus* β-tubulin gene family with those of Clade V nematodes for which there is an essentially complete genome sequence available (*C. elegans*, *C. briggsae*, *C. remanei*, *C. japonicum*, *C. brenneri* and *P. pacificus*). The model organism, *C. elegans*, is of particular interest since this is currently the only nematode species for which there is experimental data on β-tubulin function. The phylogenetic relationships are consistent with those from genomic structure and intron position comparisons, and these relationships are schematically summarized in Fig. 5. There is uncertainty as to the number of β-tubulin genes in the ancestral nematode. The single gene in *T. spiralis* may reflect the gene complement of the early nematodes; it could equally be the result of gene loss in a reduced sized genome (Mitreva et al., 2011). For simplicity, we shall assume a single β-tubulin gene in the ancestral nematode. In this ancestral nematode, there were at least two gene duplication events, designated as A and C in Fig. 5, which left the most recent common ancestor of *C. elegans* and *H. contortus* with three β-tubulin genes: B, D and E. These were carried through into each lineage following the speciation event that separated them. No further gene duplications in lineages D or E led to the pair of one-to-one orthologous genes: *Hco-tbb-iso-3* and *Cel-mec-7*, and *Hco-tbb-iso-4* and *Cel-tbb-4*. In contrast, the β-tubulin gene on the B lineage underwent several independent duplications: F, G, H and I. The duplication at F gave rise to the

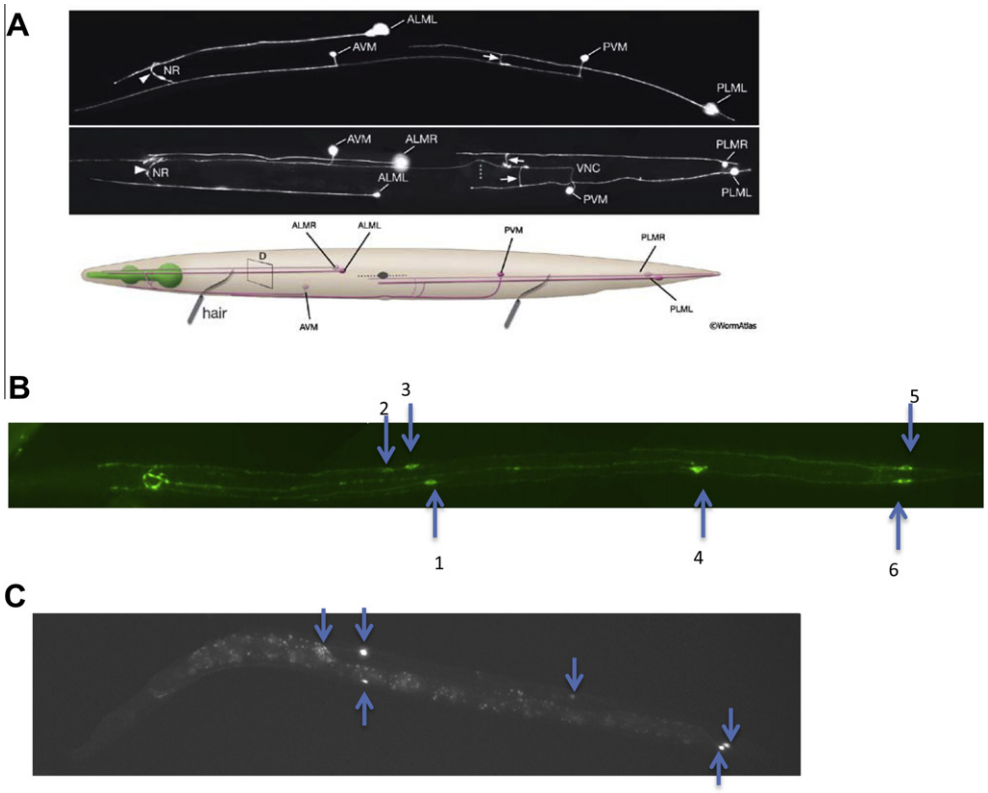

Fig. 4. Expression pattern of *Haemonchus contortus Hco-tbb-iso-3*. (A) Diagrammatic representation of the positions of the touch receptor neurons in which *Caenorhabditis elegans Cel-mec-7* is expressed (adapted from http://www.wormbook.org/). (B) Immunofluorescence staining of an exsheathed *H. contortus* L3 with immunoaffinity purified polyclonal IgG directed against the C-terminal peptide of the *Hco*-TBB-ISO-3 polypeptide. Arrows 1–6 indicate the stained cell bodies that correspond in position to the *C. elegans* touch receptor neuronal cell bodies, ALML/R, PLML/R, AVM and PVM. (C) GFP reporter gene expression in an adult *C. elegans* hermaphrodite. The six cells (arrowed) correspond in position to the *C. elegans* touch receptor neuronal cell bodies, ALML/R, PLML/R, AVM and PVM. Due to the mosaicism inherent in expression patterns generated by extrachromosomal arrays, other individual transgenic worms showed different combinations of GFP expression in these cells. NR, nerve ring; VNC, ventral nerve cord.

paralogues *Hco-tbb-iso-1* and *Hco-tbb-iso-2* in *H. contortus*, while *Cel-ben-1*, *Cel-tbb-1*, *Cel-tbb-2* and *Cel-tbb-6* form a further in-paralogous clade. Our placement of *Cel-tbb-6* as a sister taxon to *Cel-tbb-2* is supported by shared intron positions (Fig. 2).

4.2. Functional inferences for the Hco-tbb-iso-1 and Hco-tbb-iso-2 genes

The genes *Cel-ben-1*, *Cel-tbb-1* and *Cel-tbb-2* are the most highly and widely expressed β-tubulins in *C. elegans* and so are likely to have major roles in microtubule structure and function throughout the organism. However, neither of these genes is essential to *C. elegans* viability in itself. Individually, deletion of *Cel-tbb-1* or *Cel-tbb-2* have little or no deleterious effect on *C. elegans*; however, an embryonic lethal phenotype is observed when both genes are deleted together (Driscoll et al., 1989; Lu et al., 2004). Therefore, *Cel-tbb-1* and *Cel-tbb-2* are broadly functionally redundant with respect to each other but together represent an essential gene pair. These tubulins are key components of meiotic and mitotic spindles in most, if not all, cells in the *C. elegans* embryo. Although they have not been functionally studied post-embryonically, they are widely expressed in larval and adult stage worms, suggesting a similarly important role throughout development (Lu et al., 2004). *Cel-ben-1* is the only β-tubulin targeted by benzimidazole (Driscoll et al., 1989) drugs that cause widespread loss of microtubule integrity and function, producing a lethal effect on the organism. This suggests that *Cel-ben-1* also makes an important and widespread contribution to microtubule structure and function throughout the worm. However, *Cel-ben-1* null mutants are viable and grossly normal, also suggesting functional redundancy, most likely with *Cel-tbb-1* and *Cel-tbb-2* given its phylogenetic relationship with these genes. Hence, *Cel-ben-1*, *Cel-tbb-1* and *Cel-tbb-2* appear to be the β-tubulins that perform the main "housekeeping" role in *C. elegans* microtubule function in most cells of the organism. The paralogous relationship of *Hco-tbb-iso-1* and *Hco-tbb-iso-2* with *Cel-ben-1*, *Cel-tbb-1* and *Cel-tbb-2*, together with them being the only highly expressed β-tubulin genes in *H. contortus* (Table 2) suggests that these two genes are likely to play a similar role in *H. contortus*. Although the phenotype of null mutations of *Hco-tbb-iso-1* is not known, deletions of the *Hco-tbb-iso-2* gene have been described in field populations (Kwa et al., 1993a; Beech et al., 1994), suggesting that this gene is not in itself essential for viability. It therefore seems a reasonable hypothesis that *Hco-tbb-iso-1* and *Hco-tbb-iso-2* perform analogous functions to *Cel-ben-1*, *Cel-tbb-1* and *Cel-tbb-2* in a mutually redundant manner.

4.3. Functional inferences for Hco-tbb-iso-3 and Hco-tbb-iso-4 genes

Cel-mec-7 and *Cel-tbb-4* are not widely expressed in *C. elegans* but have specialized functions in a subset of sensory neurons. *Cel-mec-7* is expressed only in the six touch receptor neurons and null mutations in this gene result in severe touch response abnormalities (Savage et al., 1989). *Cel-tbb-4* has recently been shown to be expressed in a number of ciliated sensory neurons, including the amphids, and null mutants have morphological defects in the sensory cilia resulting in abnormal touch responses (Hurd et al., 2010). Phylogenetic analysis suggests *Hco-tbb-iso-3* and *Hco-tbb-iso-4* are orthologous to *Cel-mec-7* and *Cel-tbb-4*,

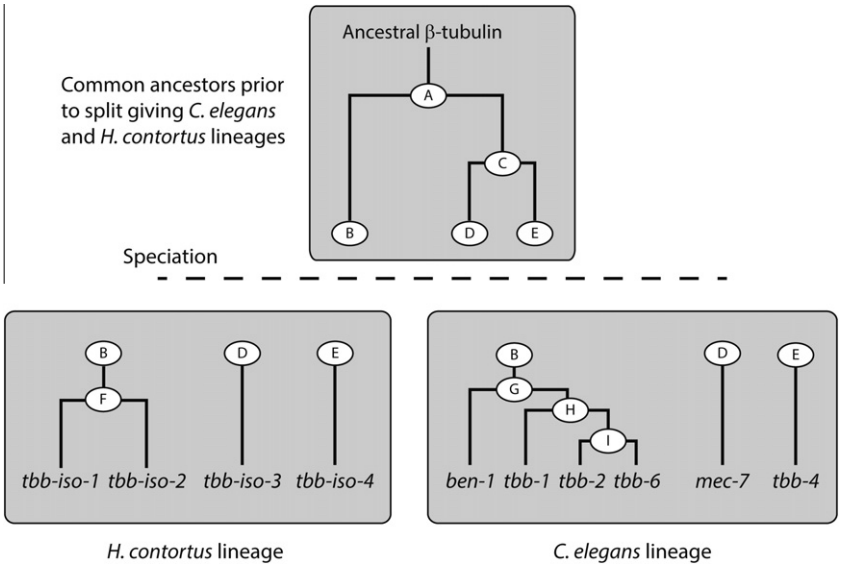

Fig. 5. Proposed model of β-tubulin evolution in nematodes. Gene duplication events are indicated by labeled nodes. The speciation event that separates the *Haemonchus contortus* and *Caenorhabditis elegans* lineages occurs sometime after duplication event 'C'.

respectively, and RNAseq analysis has revealed that both *H. contortus* genes are expressed at a very low level (Table 2), consistent with these genes having similarly specialized roles. Indeed, *Hco-tbb-iso-3* expression is confined to six neurons in *H. contortus* that are anatomically conserved with the six *C. elegans* touch receptor neurons (Fig. 4B). Furthermore, the *Hco-tbb-iso-3* promoter drives expression of a GFP reporter gene in the six touch receptor neurons in transgenic *C. elegans* (Fig. 4C). Hence, the *Hco-tbb-iso-3* and *Cel-mec-7* genes appear to have precisely conserved functions in spite of their highly specialized roles. This is an exquisite example of functional conservation between these two nematode species further supporting the utility of *C. elegans* as a model organism for particular aspects of strongylid nematode biology. Interestingly, we have previously reported a conserved microsyntenic relationship between *Hco-tbb-iso-3* and *Cel-mec-7*, further supporting this orthologous relationship (Laing et al., 2011).

4.4. Implications for benzimidazole mode-of-action and resistance

Saturation mutagenesis and genetic mapping experiments show that *Cel-ben-1* is the only member of the *C. elegans* β-tubulin gene family that is a benzimidazole drug target (Driscoll et al., 1989). Although, as explained above, *Cel-tbb-1* and *Cel-tbb-2* have critical and widespread functions in *C. elegans*, it is not surprising that they are not benzimidazole targets as both contain a tyrosine residue at position 200 in wild type worms (a tyrosine residue at this position is thought to prevent drug binding) (Chambers et al., 2010). Although it is possible that the other *C. elegans* β-tubulin polypeptides (Cel-TBB-4, Cel-MEC-7 and Cel-TBB-6) may bind benzimidazole drugs (since they have phenylalanine at P200), they have more specialized functions and so disruption of microtubules containing these β-tubulins may have much more subtle effects on the worm. Therefore, the model of benzimidazole mode of action and resistance in *C. elegans* is as follows: in wild type worms microtubules in many tissues throughout the worm are comprised of combinations of Cel-BEN-1, Cel-TBB-1 and Cel-TBB-2 monomers (in association with alpha tubulin monomers). Benzimidazole drugs consequently bind to Cel-BEN-1 monomers, that are present in microtubules throughout the worm, "capping" the microtubules at the associating end while the microtubules continue to dissociate at the opposite end leading to a loss of integrity (Lacey, 1990). In *Cel-ben-1* null mutants, the microtubules are comprised of just Cel-TBB-1 and Cel-TBB-2 monomers and so would not bind the drug leading to a resistance phenotype.

Clearly then, it is an important question which of the *H. contortus* β-tubulin genes are orthologs and/or functional homologs of *Cel-ben-1*. It has commonly been assumed that the *isotype-1* gene of strongylid nematodes is the *Cel-ben-1* orthologue, with *Hco-tbb-iso-1* even being referred to as "*Hc-ben-1*" (Geldhof et al., 2006). This is because the *isotype-1* gene has been commonly associated with benzimidazole resistance in strongylid parasites and *Hco-tbb-iso-1* has been shown to complement the *Cel-ben-1* gene in transgenic *C. elegans* (Kwa et al., 1995). This has led to an almost exclusive focus on mutations in this particular gene for potential mechanisms of benzimidazole resistance in parasitic nematode populations. However, *Hco-tbb-iso-1* and *Hco-tbb-iso-2* have a paralogous relationship with *Cel-ben-1* (i.e. neither is more closely related to *Cel-ben-1* than the other). Given that *Hco-tbb-iso-2* has already been implicated in benzimidazole resistance (Kwa et al., 1993a; Rufener et al., 2009), this analysis suggests that a more detailed investigation into the role of the ISOTYPE-2 β-tubulin is warranted in cases of benzimidazole resistance in strongylid nematode parasites. On the other hand, the orthologous relationship of *Hco-tbb-iso-3* and *Hco-tbb-iso-4* with *Cel-mec-7* and *Cel-tbb-4*, respectively, makes these unlikely candidates for involvement in benzimidazole resistance in *H. contortus*, or other strongylid nematodes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2012.12.011.

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