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# The astacin metalloprotease moulting enzyme NAS-36 is required for normal cuticle ecdysis in free-living and parasitic nematodes

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## SUMMARY

Nematodes represent one of the most abundant and species-rich groups of animals on the planet, with parasitic species causing chronic, debilitating infections in both livestock and humans worldwide. The prevalence and success of the nematodes is a direct consequence of the exceptionally protective properties of their cuticle. The synthesis of this cuticle is a complex multi-step process, which is repeated 4 times from hatchling to adult and has been investigated in detail in the free-living nematode, *Caenorhabditis elegans*. This process is known as moulting and involves numerous enzymes in the synthesis and degradation of the collagenous matrix. The *nas-36* and *nas-37* genes in *C. elegans* encode functionally conserved enzymes of the astacin metalloprotease family which, when mutated, result in a phenotype associated with the late-stage moulting defects, namely the inability to remove the preceding cuticle. Extensive genome searches in the gastrointestinal nematode of sheep, *Haemonchus contortus*, and in the filarial nematode of humans, *Brugia malayi*, identified NAS-36 but not NAS-37 homologues†. Significantly, the *nas-36* gene from *B. malayi* could successfully complement the moult defects associated with *C. elegans nas-36*, *nas-37* and *nas-36/nas-37* double mutants, suggesting a conserved function for NAS-36 between these diverse nematode species. This conservation between species was further indicated when the recombinant enzymes demonstrated a similar range of inhibitable metalloprotease activities.

**Key words:** *Haemonchus contortus*, *Brugia malayi*, *Caenorhabditis elegans*, astacin metalloprotease, moulting, cuticle.

## INTRODUCTION

Parasitic nematode infections are amongst the most widespread diseases worldwide, causing significant debilitating infections in both humans and livestock. Nematode species in grazing animals, such as *Haemonchus contortus* and *Teladorsagia circumcincta* in sheep and *Ostertagia ostertagi* in cattle, are major causes of economic loss in the livestock industry worldwide, having a detrimental effect on the farmers' livelihoods. Currently, the only treatment is by anthelmintics belonging to one of 3 classes: the benzimidazoles (e.g., albendazole), the imidazothiazoles (e.g., levamisole) and the macrocyclic lactones (e.g., ivermectin) (McKeller and Jackson, 2004). However, in the nematodes of livestock, triple resistance has now developed against the drugs in

each of these 3 classes. Hence, treatment of these infections has now become problematic in many parts of the world, notably in Australia and Scotland (Bartley *et al.* 2006; Besier, 2007), further emphasizing the need to identify novel drug targets and develop new classes of anthelmintics. Three new anthelmintic drugs have recently become available commercially. Monepantel has demonstrated significant anthelmintic activity, with a different mechanism of action, against the nematodes of sheep (Ducray *et al.* 2008; Kaminsky *et al.* 2008), Profender is a cyclooctadepsipeptide known as emodepside, which is now used for the treatment of nematodes and cestodes of cats (Harder *et al.* 2003; Altreuther *et al.* 2005) and tribendimidine has a broad spectrum of anthelmintic activity against nematodes of humans and animals (Xiao *et al.* 2005). Although these 3 drugs have only become available recently, they have limitations, particularly in the case of tribendimidine, which has the same mechanism of action as levamisole and pyrantel (Hu *et al.* 2009), indicating that this drug would not be of use in areas in which nematode resistance to levamisole or pyrantel exists. However, it could be used as a replacement for the benzimidazoles or macrocyclic lactones. Thus, although some new anthelmintic drugs have now become available

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† Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers FJ812519 (*H. contortus nas-36*) and FJ812520 (*B. malayi nas-36*).

commercially, their use is presently restricted, suggesting the need to identify further novel targets for more drugs.

All nematodes are encased in a collagenous exoskeleton, known as the cuticle, which is responsible for maintaining the body morphology, protecting the nematode from the external environment and allowing mobility (Page and Johnstone, 2007). This resilient cuticle restricts growth and, therefore, must be successively shed and re-synthesized. All nematodes undergo 4 cuticle moults from the L1 hatchling to the mature adult. The moulting process replaces the cuticle from the previous stage with a new cuticle, and occurs in 3 steps: (i) lethargus, in which the general activity of the organism decreases; (ii) apolysis, in which the old cuticle separates from the hypodermis; and (iii) ecdysis, in which the old cuticle is completely shed and the worm emerges as the next stage with a new cuticle (Singh and Sulston, 1978). Moulting is an integral and highly specialized process, both for parasitic and free-living nematodes; thus, disruption of this developmental pathway would prevent completion of the nematode life cycle, and therefore represents an ideal target for infection control.

The process of moulting during larval development has been found to involve various proteases, such as cysteine proteases and metalloproteases, in a wide range of free-living (e.g., *Caenorhabditis elegans*; Hashmi *et al.* 2004) and parasitic nematodes, including *Onchocerca volvulus* (Lustigman *et al.* 1996, 2004), *Brugia malayi* (Guiliano *et al.* 2004), *B. pahangi* (Hong *et al.* 1993; Guiliano *et al.* 2004), *Dirofilaria immitis* (Richer *et al.* 1992), *H. contortus* (Gamble *et al.* 1996) and *Ascaris suum* (Rhoads *et al.* 1997, 1998). Several studies have indicated that these enzymes are required for the complete shedding of the old cuticle during moulting (Lustigman *et al.* 1996, 2004; Guiliano *et al.* 2004). In the presence of specific inhibitors, the L3 stage from diverse species remain viable and develop to L4, but the L4s are unable to escape from the L3 cuticle, indicating that these proteases are required for the degradation and escape from the third-stage cuticle, namely the ecdysis step of the moulting process (Rhoads *et al.* 1998; Guiliano *et al.* 2004; Lustigman *et al.* 2004). This moult also relates to an increased expression of developmentally essential cysteine and metalloproteases, suggesting a crucial requirement for these enzymes in this process (Richer *et al.* 1992; Hong *et al.* 1993; Gamble *et al.* 1996; Lustigman *et al.* 1996; Rhoads *et al.* 1997, 1998).

There are several families of metalloproteases in nematodes, with the astacin metalloproteases belonging to the M12A family (Möhrlen *et al.* 2003). Astacin metalloproteases are structurally distinct zinc metallo-endopeptidases, characterized by 2 conserved motifs in the catalytically-active astacin domain: the catalytic active-site (HExxHxxGFxHExxRxDRD)

for binding the essential Zn<sup>2+</sup> and the methionine-turn (SxMHY) which maintains the enzyme conformation. The active-site zinc is penta-coordinated and is bound by the 3 histidine residues and the glutamic acid residue in the active-site and the tyrosine residue in the methionine-turn (Stöcker *et al.* 1993). There are 39 Nematode Astacin (NAS) metalloproteases in the free-living nematode, *C. elegans*, divided into 6 subgroups (I to VI) that correlate with the functionally significant C-terminal domain structure (Möhrlen *et al.* 2003). The subgroup V enzymes (NAS-33 to NAS-38) have been identified to be essential for the correct development of *C. elegans*, in that worms mutant in *nas-34* display a delayed hatching phenotype (Hishida *et al.* 1996), those mutant in *nas-35* (*a.k.a.* *dpy-31*) have a dumpy appearance, indicating a specific role in the proper formation of the cuticle (Novelli *et al.* 2004), and worms lacking *nas-36* and *nas-37*, but particularly *nas-37*, show defects in the shedding of the cuticle, indicating a specific role for these enzymes in cuticle ecdysis (Davis *et al.* 2004; Suzuki *et al.* 2004). It is significant to note that the subgroup V enzymes share many domain characteristics to BMP-1, the vertebrate procollagen C-peptidase (PCP) (Novelli *et al.* 2004).

Earlier studies by Gamble *et al.* (1989, 1996) identified an inhibitable protease activity from the infective larvae of *H. contortus*, which was involved in the moulting process. This unidentified enzyme was found in the L3 exsheathing fluid and formed a distinctive refractile ring ~20 µm from the anterior tip, a prerequisite to cap removal and exsheathment. Davis *et al.* (2004) demonstrated that recombinant NAS-37 from *C. elegans* was effective in inducing refractile ring formation on isolated L3 cuticles of *H. contortus*. This information suggests that the protease in *H. contortus* exsheathing fluid and its substrate are functionally conserved to NAS-37 from *C. elegans*. The subgroup V astacin metalloproteases, which have essential roles in the hatching and moulting of *C. elegans*, may therefore represent potential vaccine and drug targets for the control of parasitic nematodes.

The aim of this study was to search the genome databases for *H. contortus* and *B. malayi* to identify homologues of *C. elegans* NAS-36 and NAS-37, and then clone and characterize these homologues. The results, particularly with regards to the NAS-36 homologues, are described in this paper and the implications discussed.

## MATERIALS AND METHODS

### *Nematode strains*

The *Caenorhabditis elegans nas-36* deletion allele, *tm1636*, and the *nas-37* deletion allele, *tm410*, were obtained from Shohei Mitani of the National

Bioresource genome deletion project (Japan). These mutant strains were backcrossed 4 times against wild-type nematodes to provide the following strains: TP87 (*nas-36*, *tm1636*) and TP99 (*nas-37*, *tm410*). The TP87 and TP99 strains were combined to produce the double mutant TP93 (*tm1636/tm410*). *Haemonchus contortus* and *Brugia malayi* were provided by Dr Frank Jackson, Moredun Research Institute and Professor Rick Maizels, University of Edinburgh, respectively.

#### Preparation of genomic DNA, RNA and cDNA from *H. contortus* and *B. malayi*

Genomic DNA was isolated from adult nematodes using a standard protocol involving homogenization in proteinase K, followed by repeated phenol:chloroform extraction (Stepek *et al.* 2010b). Total RNA was isolated from adult nematodes following Trizol (Invitrogen) extraction (Stepek *et al.* 2010b), and cDNA was prepared using the AffinityScript Multiple Temperature cDNA synthesis kit (Stratagene), following the manufacturer's recommendations.

#### Isolation of the cDNA and genomic DNA of *H. contortus nas-36*

The *C. elegans* NAS-36 protein sequence was obtained from Wormbase and a BLAST search was performed in the *H. contortus* database ([http://www.sanger.ac.uk/Projects/H\\_contortus/](http://www.sanger.ac.uk/Projects/H_contortus/)), initially using the options labelled 'assembled contigs (27/01/06)' and 'sequence reads (01/08/05)'. The sequence read haem-1027e22.q1k, and contigs 058685 and 007354, had the highest homology scores, and were entered into the Scaffold program, designed by Dr Robin Beech, McGill University, which assembles a group of physically linked sequences using the *H. contortus* genome project databases. From the Scaffold results, 2 further sequence reads were identified, haem-227i23.p1k and haem-227i23.q1k, although the opposite end of the initial read, haem-1027e22.q1k, was not identified, such that cloning of this sequence was not feasible. The full 7419 bp product between sequence reads haem-227i23.p1k and haem-227i23.q1k was obtained by PCR and fully sequenced. Potential intron-exon splice sites were predicted using the GeneWise tool on ExpASy (<http://us.expasy.org/>), and then the coding sequence was translated using the Translate tool (ExpASy) and aligned with the *C. elegans* protein sequence using ClustalX and BoxShade. This alignment highlighted that the sequence was incomplete, and amplification of the 5' and 3' ends of the *H. contortus nas-36* cDNA was performed using the Invitrogen 5'-RACE and 3'-RACE systems with the following primers: Hc nas-36 RT (5'-caaattgcatgtgctgaac-3'), Hc nas-36 R1

(5'-gcatcgatctgaacagtaagc-3'), Hc nas-36 R1a (5'-gcaagttgatgtgcac-3'), Hc nas-36 R2 (5'-cgattgacacac-ctgtc-3'), Hc nas-36 5' R3 (5'-cgaaagaaagatgtctcttc-3'), Hc nas-36 3' F1 (5'-cgtggctgcagtgctc-3') and Hc nas-36 3' F2 (5'-cactgcgctaaactac-3'). The complete coding sequence (1839 bp) was assembled in ContigExpress from the 5'- and 3'-RACE products and the original partial sequence, prior to full-length cloning and sequence confirmation from genomic DNA. The primers, Hc nas-36 F (5'-atgctactcttag-tattactc-3') and Hc nas-36 R (5'-ttacttagccaattc-gaagtc-3'), were used to PCR the genomic sequence of *H. contortus nas-36*, and the 5' and 3' unknown genomic regions were sequenced using the primers Hc36 g F2 (5'-ccagtattactgcacttc-3') and Hc36 g R2 (5'-cagcgggtgccataatc-3'). ExpASy was used to determine intron-exon splice sites (GeneWise) and the presence of a signal peptide (SignalP), whereas ClustalX and BoxShade were used for the alignment of inferred protein sequences. Gene Structure Draw (<http://warta.uni-muenster.de/cgi-bin/Tools/StrDraw.pl>) was used to produce a scaled schematic depicting the positions of the introns and exons in the gene. Primer sequences for the initial cloning are available from the authors on request.

#### Isolation of the cDNA and genomic DNA of *B. malayi nas-36*

A similar BLAST search was performed using the *C. elegans* NAS-36 protein in the *B. malayi* genome TIGR database (<http://www.tigr.org/tdb/e2k1/bma1/>), and a homologous gene was found in TIGR assembly 13313 (locus identifier: Bm1\_07340). The protein was aligned to the *C. elegans* protein sequence using ClustalX and BoxShade. Amplification of the 5'- and 3'-ends of the available coding sequence using the Invitrogen 5'- and 3'-RACE systems and the primers Bm nas-36 RT (5'-cacgttgaccaattgtc-3'), Bm nas-36 R1 (5'-catgttcgcttcgtgtc-3'), Bm nas-36 R2 (5'-ccaattgatattcctgtctac-3'), Bm36 R3 (5'-caaatcgatattttattgg-3'), Bm nas-36 F1 (5'-gttccagtactgtgttg-3') and Bm nas-36 F2 (5'-gatatgctgctacgataagc-3') confirmed the *in silico* prediction of 1746 bp. To generate 3-dimensional structure predictions for the nematode astacins, the complete amino acid sequence of each protein was submitted to SWISS-MODEL (<http://swissmodel.expasy.org/SWISS-MODEL.html>) in First Approach mode. The closest matched template for each protein represented the structure of astacin from *Astacus astacus* (pdb accession number: 1qjjA). The resultant structures were downloaded and viewed using Swiss Pdb-Viewer (Schwede *et al.* 2003).

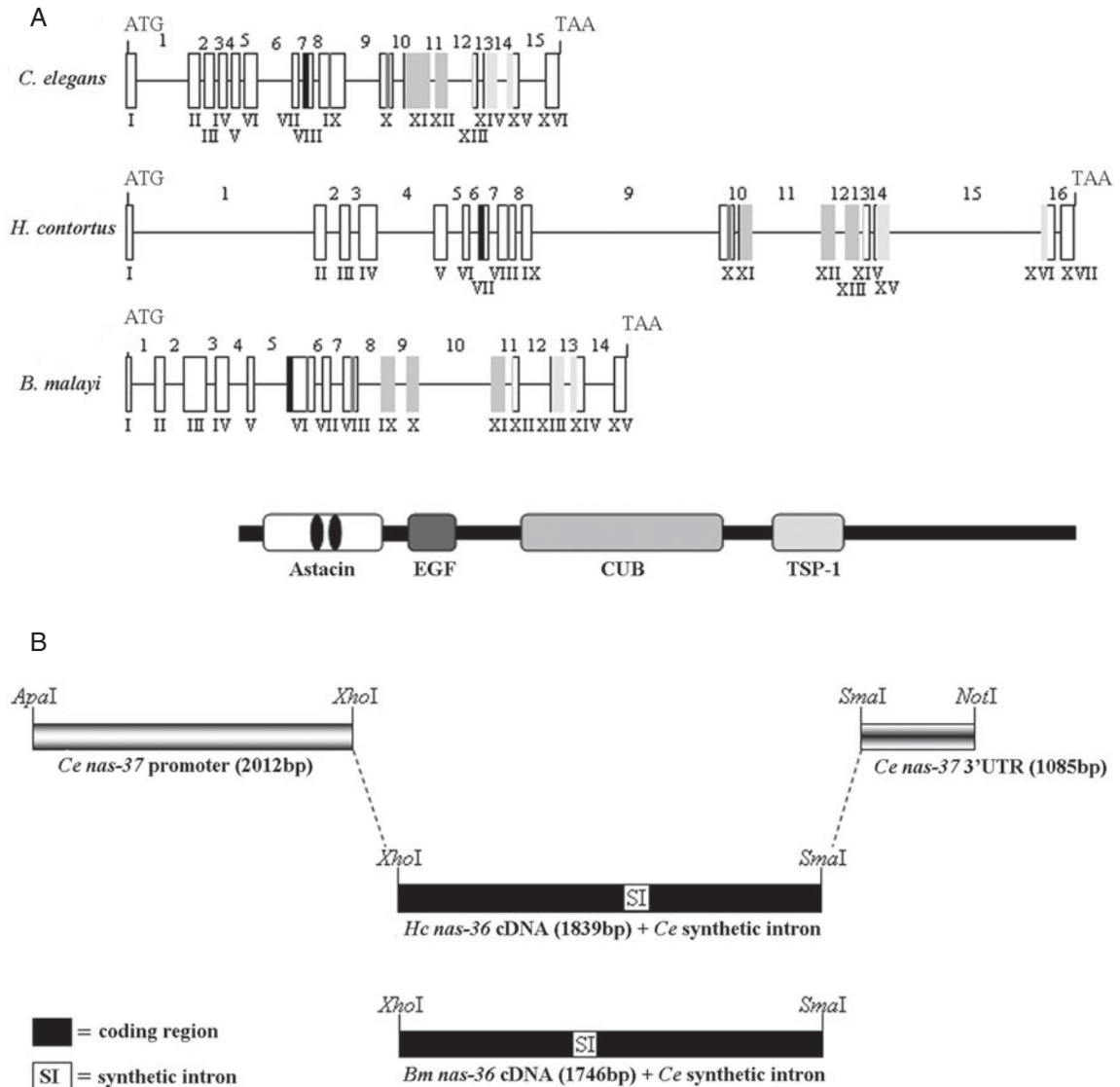


Fig. 1. (A) Gene structures of *C. elegans*, *H. contortus* and *B. malayi nas-36*. The introns are indicated by lines, while the exons are open boxes. The exons are numbered below with Roman numerals, with the introns numbered above the lines. The translational start and stop codons are indicated by the ATG and TAA, respectively. The catalytic zinc-binding site in the astacin domain is shaded black, and the conserved methionine-turn, also in the astacin domain, is shaded dark grey. The EGF (epidermal growth factor) domain is shaded medium grey, the CUB (Clr/C1s, embryonic sea urchin *Uegf*, BMP-1) domain is light grey and the TSP-1 (thrombospondin type 1) domain is the lightest shade of grey. A schematic of the NAS-36 protein, showing each identified domain, is below the gene structures. The catalytic zinc-binding site and methionine-turn are represented by black oval shapes. (B) Schematic representation of *H. contortus* and *B. malayi nas-36* rescue constructs. The rescue vector (*pnas-37*) involved was composed of the *C. elegans nas-37* promoter and 3'-UTR sequences, inserted into pBlueScript SK-. The *nas-36* rescue constructs contained the *C. elegans nas-37* promoter and 3'-UTR sequences, and either the full-length *nas-36* coding sequence from *H. contortus* or *B. malayi*, with a single *C. elegans* synthetic intron inserted into both genes. The black boxes indicate coding regions, with the white boxes representing the synthetic intron (SI).

#### Generation of *H. contortus* and *B. malayi nas-36* rescue constructs

A 2021 bp *ApaI-XhoI C. elegans nas-37* promoter was generated by PCR from *C. elegans* N2 genomic DNA using the primers, Ce37p F (5'-gcggggcccaatttcaagtctagctctg-3') and Ce37p R (5'-cgcctcagtgctgcaaatagaacatc-3'), and cloned into pBlueScript SK-. A 1085 bp *SmaI-NotI C. elegans nas-37* 3'-UTR was generated by PCR from

*C. elegans* N2 genomic DNA using the primers, Ce37u F (5'-gcgcccgggaaccgatatttttg-3') and Ce37u R (5'-cgcgcccggcgcgatagacctttggaagttagac-3'), and cloned into the *Ce nas-37* promoter-pBlueScript construct to create the plasmid *pnas-37* (Fig. 1B).

The coding sequences of the *H. contortus* and *B. malayi nas-36* genes were isolated by PCR using the primers Hc36 *XhoI* F (5'-gcgctcgcgatagctactcttagtattactc-3') and Hc36 *SmaI* R (5'-cgcccgggttacttagccaattcgaagtc-3'), and Bm36 *XhoI*

F (5'-gcgctcgagatgaaagaattgcacacagtc-3') and Bm36 SmaI R (5'-cgccccgggttattcgagtggaatcaatagag-3'), and *PfuUltra* polymerase to generate products of 1839 bp and 1746 bp in length, respectively. These PCR products were cloned into pCR-TOPO2.1, and a synthetic intron (5'-gtaagttaaactattcgttactaacttaaacatttaattttcag-3') was inserted by ligation of a double-stranded oligo into a *HincII* (*H. contortus*) or *StuI* (*B. malayi*) blunt-ended restriction site. These products were then cloned into the *XhoI*-*SmaI* digested *pnas-37* vector to create either an *H. contortus nas-36-* or a *B. malayi nas-36-rescue* construct (Fig. 1B). The sequences of these plasmids were verified across the cloning sites using the primers Ce37p inF (5'-gatatcgcaaatctcaaag-3') and Ce37u inR (5'-gttattgttggtgacatac-3'), and over the synthetic intron insertion using the primers Hc36 SI F (5'-caacgtgacaaggatc-3') and Bm36 SI F (5'-gtggtagacaaggaatc-3') for the *H. contortus* and *B. malayi* constructs, respectively.

These *nas-36* rescue constructs were micro-injected into the syncytial gonad of *C. elegans nas-36*(TP87), *nas-37*(TP99) and *nas-36/-37*(TP93) mutant nematodes at a concentration of either 1 ng/ $\mu$ l (*H. contortus*) or 25 ng/ $\mu$ l (*B. malayi*), together with 5 ng/ $\mu$ l of a *dpy-7::GFP* fusion construct and pBlueScript SK- to make the final concentration up to 150 ng/ $\mu$ l. Nine, 3 and 4 transgenic lines, respectively, were obtained for each of the worm strains following injection with the *B. malayi* rescue construct. Transformants were selected based on green fluorescent protein (GFP) fluorescence. Nematodes were transferred to 2% agarose:0.1% azide pads and viewed under differential interference contrast (DIC) or fluorescence (GFP) lenses using a Axioskop2 microscope (Zeiss), and images were taken using an AxioCam camera and Axiovision software. In addition, PCR was performed on single worms using 3 GFP-positive and 3 GFP-negative worms per line, and for 3 N2 worms and 3 *nas-36*(TP87), *nas-37*(TP99) and *nas-36/-37*(TP93) mutant worms, using the rescue primers Bm36 *XhoI* F and Bm36 *SmaI* R.

#### Recombinant protein expression of *H. contortus* and *B. malayi* NAS-36

The coding sequences of *H. contortus* and *B. malayi* NAS-36 were isolated by PCR using the primers rHc36matF (5'-gcggggtacctcattcgatgcaagc-3') and rHc36matR (5'-cgcaagcttttacttagccaattcgaagtc-3') or Bm36matF (5'-gcggggtaccatgaaagaattgcacacag-3') and Bm36matR (5'-cgccctgcagttcgagtggaatcaatagag-3'), and *PfuUltra* polymerase to generate products of 1472 bp and 1743 bp in size, respectively. These PCR products were cloned into pCR-TOPO2.1 and sequenced with M13 forward and M13 reverse primers. These products were then cloned into the *KpnI*-*HindIII* digested pQE30 vector for

Table 1. Comparison of the *nas-36* genes between *C. elegans*, *H. contortus* and *B. malayi*

	<i>C. elegans</i>	<i>H. contortus</i>	<i>B. malayi</i>
Intron number	15	16	14
Mean intron size (bp)	147	441	210
Intron size range (bp)	40–486	52–1781	72–688
Exon number	16	17	15
Mean exon size (bp)	117	109	117
Exon size range (bp)	52–245	52–169	52–246
Gene size (genomic)	4082 bp	8916 bp	4702 bp
Gene size (coding)	1854 bp	1839 bp	1746 bp

*H. contortus nas-36* or the *KpnI*-*PstI* digested pQE30 vector for *B. malayi nas-36* to create protein expression constructs. These plasmids were used to transform *E. coli* M15 (pREP4) cells, and expression of the encoded proteins was induced by an adapted auto-induction method (Studier, 2005), as described previously (Steppek *et al.* 2010b).

#### Astacin activity assay

An astacin assay was performed to determine the activity of recombinant NAS-36 from both *H. contortus* and *B. malayi* using a method adapted from previous studies (Gamble *et al.* (1989, 1996; Stöcker and Zwilling, 1995). Briefly, recombinant enzyme (final concentration of 5  $\mu$ g/ml) was incubated with ZnCl<sub>2</sub> at a final concentration of 300  $\mu$ M and either 0, 1, 2, 5 or 10 mM (final concentrations) 1, 10-phenanthroline, and the volume was made up to 100  $\mu$ l (inclusive of substrate) with 50 mM HEPES, pH 8.0 buffer. Incubation occurred for 1 h at 25 °C prior to the addition of Suc-Ala-Ala-Ala-pNA to a final concentration of 250  $\mu$ M and subsequent incubation at 25 °C for a further 2 h. Absorbance was measured using a plate reader at 450 nm. Each sample was assayed in triplicate.

## RESULTS

### Identification of *H. contortus* and *B. malayi* *nas-36* orthologues

Orthologues of *nas-36* were identified in the genomes of both *H. contortus* and *B. malayi*, with genomic sequences of 8916 bp and 4702 bp, respectively, compared with the genomic sequence of 4082 bp in *C. elegans* (Table 1 and Fig. 1A). Figure 2 illustrates an alignment incorporating the astacin and C-terminal domains of the NAS-36 sequences from *C. elegans*, *H. contortus* and *B. malayi*. This sequence has 66.5% identity between *C. elegans* and *H. contortus*, 54.2% between *C. elegans* and *B. malayi*, and 53.5% between *H. contortus* and *B. malayi*. The

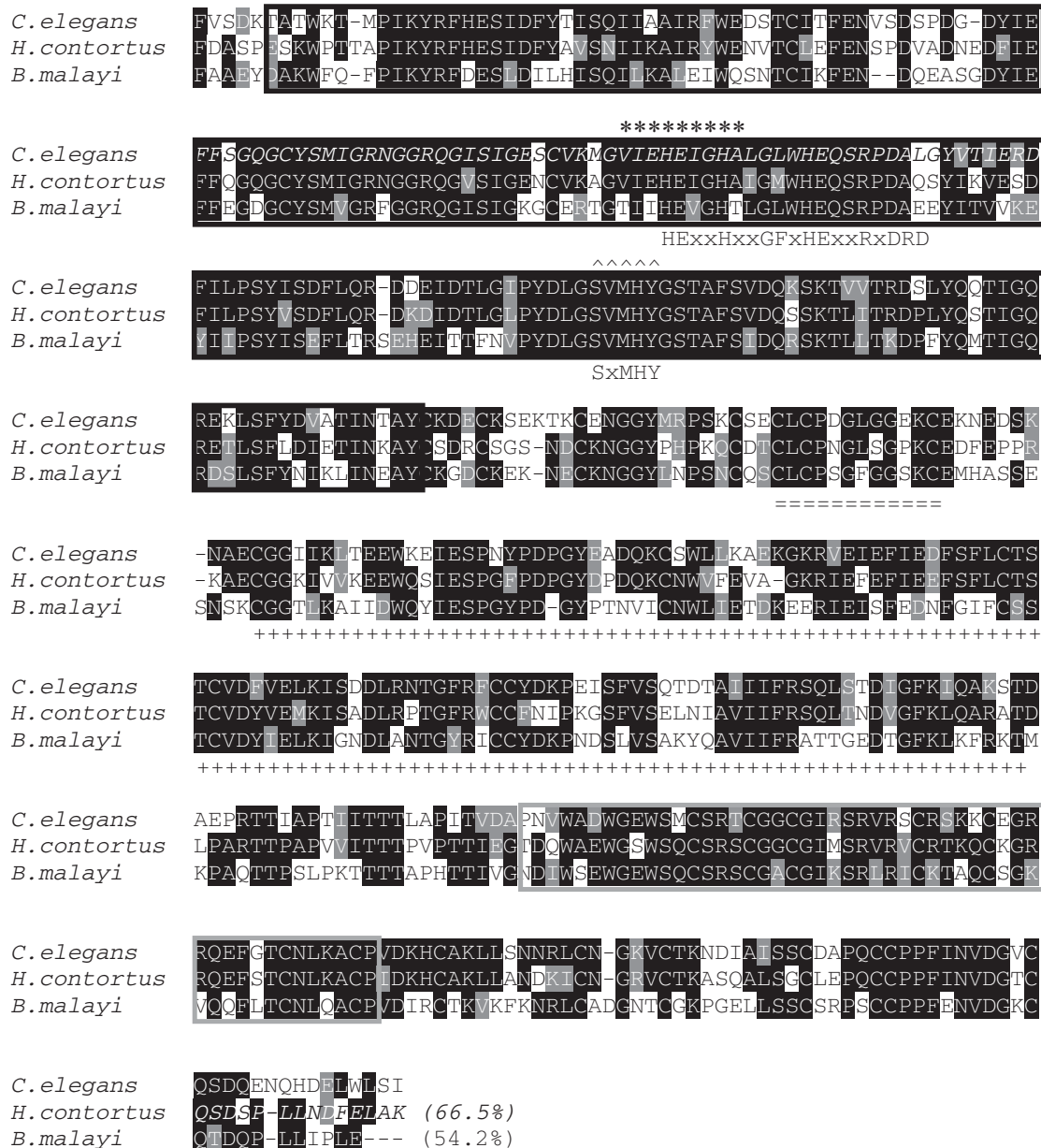


Fig. 2. Alignment of the nematode NAS-36 homologues. Amino acid sequences were aligned using ClustalX and prepared for display using BoxShade. Identical amino acids are shaded black and similar amino acids are shaded grey. The astacin domain is indicated in a black box, with the catalytic zinc-binding site (HExxHxxGFxHExxRxDRD) marked by \* and the conserved methionine-turn (SxMHY) by ^. The EGF domain is double-underlined, the CUB domain is marked by + and the TSP-1 domain by a grey box. The percentage sequence identity between *C. elegans* and the parasitic nematodes is shown at the end of the alignment.

NAS-36 proteins from *C. elegans*, *H. contortus* and *B. malayi* range from 55 to 65 kDa, with pIs between 5.00 and 6.58.

The catalytic astacin domain (Pfam PF01400), which includes the crucial zinc-binding site (HExxHxxGFxHExxRxDRD) and the conserved methionine-turn (SxMHY), displays significant homology between *C. elegans* and the parasitic nematodes. The NAS-36 proteins from these three nematodes also contain C-terminal EGF (epidermal growth factor) (Pfam PF00008), CUB (Clr/C1 s, embryonic sea urchin *Uegf*, BMP-1) (Pfam PF00431) and TSP-1 (thrombospondin type 1)

(Pfam PF00090) domains (Fig. 2). SwissModel analysis of the predicted structural models of the astacin domain of NAS-36 from *C. elegans*, *H. contortus* and *B. malayi* indicate a similarity in structure between the proteins from the 3 nematodes and the crayfish astacin template. Figure 3 demonstrates that NAS-36 from *H. contortus* and *B. malayi* appears to be more structurally similar to *C. elegans* NAS-37a (alternative transcript a) than NAS-36, although the sequence identities are much greater to *C. elegans* NAS-36 (27% and 25% between *C. elegans* NAS-37a and *H. contortus* NAS-36 or *B. malayi* NAS-36, respectively).

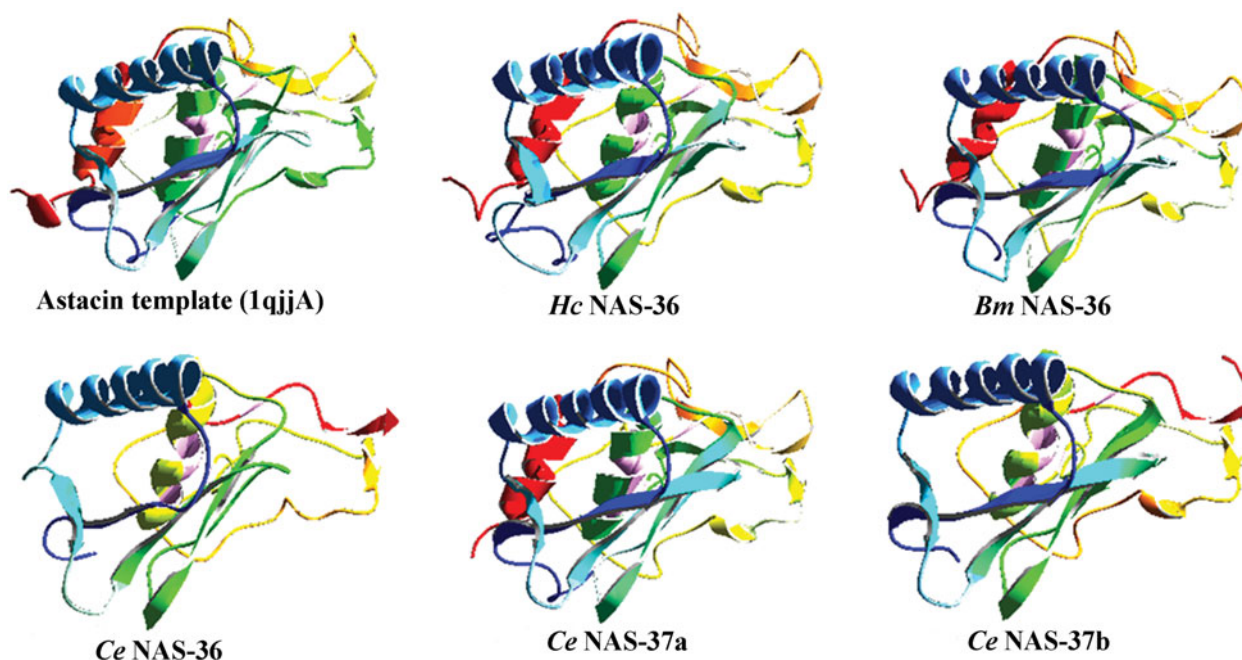


Fig. 3. Predicted structural models of the astacin domain of the NAS-36 proteins from *C. elegans*, *H. contortus* and *B. malayi*. The three-dimensional structure predictions for the complete amino acid sequences were generated using SWISS-MODEL in First Approach mode. The closest matched template for each protein represented the structure of astacin from *Astacus astacus* (pdb accession number: 1qjjA). The resulting structures were downloaded and viewed using Swiss Pdb-Viewer. The amino acids are coloured violet to red from the N-terminal to the C-terminal. The amino acids that are crucial for the catalytic active site are coloured pink.

#### Complementation of *nas-36*, *nas-37* and *nas-36/-37* double mutant nematodes with a *B. malayi nas-36* construct

The *C. elegans nas-36(tm1636)*, *nas-37(tm410)* and *nas-36/-37* deletion mutant strains were transformed, via microinjection, with a *B. malayi nas-36* rescue construct, resulting in 9, 3 and 4 independent transgenic lines, respectively. These lines were examined microscopically for the absence of the characteristic cuticle constriction of the mutant strain and the appearance of the wild-type phenotype (Fig. 4). The *B. malayi nas-36* construct was able to successfully rescue the *C. elegans nas-36* (Fig. 4B and C), *nas-37* (Fig. 4E and F) and the combined *nas-36* and *nas-37* mutant phenotypes (Fig. 4H and I). PCR from individual worms indicated that only the GFP-positive worms for each transgenic line amplified a product of 1746 bp (Fig. 4J–L), confirming that rescue of *C. elegans nas-36* single, *nas-37* single and the *nas-36/-37* double mutant worms correlated with the presence of the *B. malayi nas-36* transgenic construct.

Complementation experiments were also performed using an *H. contortus nas-36* rescue construct and the *C. elegans* single and double mutant strains. A construct concentration of 25 ng/ $\mu$ l proved to be too toxic for the *nas-36* and *nas-37* mutant strains. Concentrations of 10 ng/ $\mu$ l and 1 ng/ $\mu$ l of the *H. contortus nas-36* construct also proved to be too toxic, with no rescue being observed in any of the aforementioned *C. elegans* deletion strains. All

of the transgenic strains complemented with the *H. contortus* construct displayed severe morphological defects, in addition to the MI1 defects associated with the *nas-36* and *nas-37* mutant phenotypes.

#### Expression of *H. contortus* and *B. malayi active NAS-36*

The NAS-36 proteins from *H. contortus* and *B. malayi* were cloned and inserted into the pQE30 His-tag vector and the recombinant NAS-36 proteins from *H. contortus* and *B. malayi* were expressed in *E. coli* following an auto-induction method then affinity purified on Ni-NTA columns. These recombinant nematode NAS-36 proteins were then analysed in the astacin activity assay in the presence of increasing concentrations of the metalloprotease-specific inhibitor, 1,10-phenanthroline, and using the substrate, Suc-Ala-Ala-Ala-pNA, to determine their activities. Fig. 5 demonstrates that the activity of the recombinant NAS-36 proteins from both nematode species is inhibited by increasing concentrations of inhibitor, thereby suggesting that NAS-36 from the parasitic nematodes, *H. contortus* and *B. malayi*, is indeed a functional zinc metalloprotease.

#### DISCUSSION

Astacin metalloproteases exist in a diverse range of organisms, from bacteria and invertebrates to



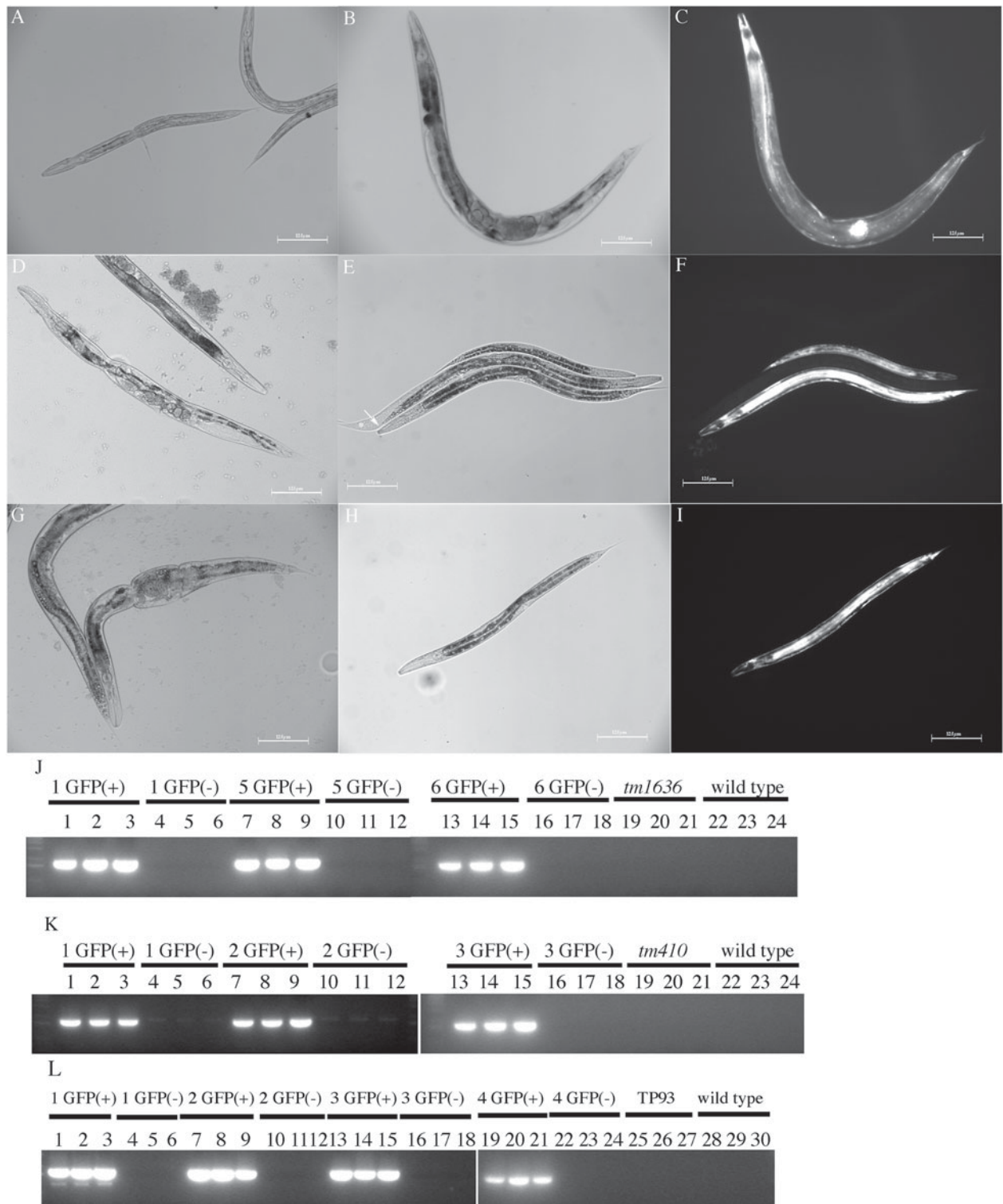


Fig. 4. A *B. malayi nas-36* homologue complements the *C. elegans nas-36(tm1636)*, *nas-37(tm410)* and *nas-36/-37* double mutant strains. (A) DIC image of a *nas-36(tm1636)* mutant worm with cuticle constrictions. (B) DIC image of a *nas-36(tm1636)* worm rescued with the *B. malayi nas-36* cDNA construct. (D) DIC image of a rescued *nas-37(tm410)* mutant worm. (E) DIC image of *nas-37(tm410)* worms rescued with the *B. malayi nas-36* cDNA construct. The arrow indicates the unshed cuticle still attached to a non-rescued worm. (G) DIC image of a *nas-36/-37* double mutant worm with severe cuticle constriction. (H) DIC image of a *nas-36/-37* double mutant worm rescued with the *B. malayi nas-36* cDNA construct. (C, F, I) GFP images of (B, E and H), respectively, depicting the fluorescent transgene marker. (J) Single worm PCR was performed for 3 GFP positive and 3 GFP negative worms for each of 3 *nas-36(tm1636)* lines (1, 5, 6), and for 3 N2 worms and 3 *nas-36(tm1636)* mutant worms, to confirm expression of the transgene. (K) Single worm PCR was performed for each of the *nas-37(tm410)* lines (1–3), as for *nas-36(tm1636)* above. (L) Single worm PCR was performed for each of the *nas-36/-37* lines (1–4), as for *nas-36(tm1636)* above. Only the GFP positive worms for each strain amplified the correct product of 1746 bp for the *B. malayi nas-36* gene.

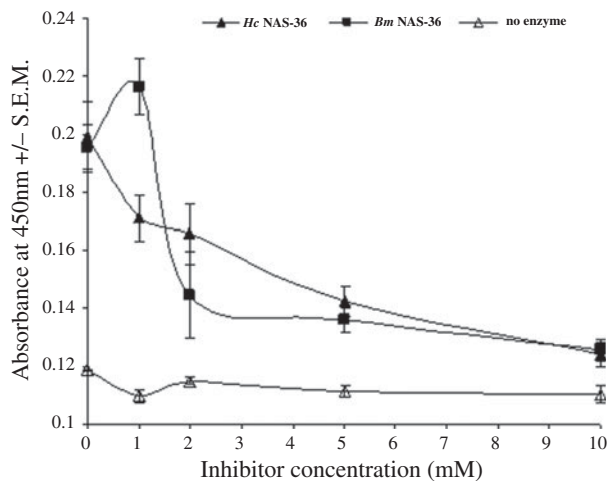


Fig. 5. Astacin activity of recombinant NAS-36 from *H. contortus* and *B. malayi*. Five  $\mu\text{g/ml}$  of each of the recombinant enzymes was incubated with 0.3 mM  $\text{ZnCl}_2$ , 0, 1, 2, 5 or 10 mM 1, 10-phenanthroline and 250  $\mu\text{M}$  Suc-Ala-Ala-Ala-pNA. Each sample was performed in triplicate and the mean and standard errors of the mean were plotted.

vertebrates (Möhrlen *et al.* 2006), many of which demonstrate essential developmental functions including digestion, hatching, embryogenesis, processing of the extracellular matrix, skeleton formation and morphogenesis (Bond and Beynon, 1995). The astacin metalloproteases have also been found to have crucial developmental roles in a wide range of free-living and parasitic nematodes, including *C. elegans* (Hishida *et al.* 1996; Möhrlen *et al.* 2003; Davis *et al.* 2004; Novelli *et al.* 2004; Suzuki *et al.* 2004; Park *et al.* 2010), *O. volvulus* (Borchert *et al.* 2007), *H. contortus* (Gamble *et al.* 1989; Stepek *et al.* 2010b), *A. caninum* (Williamson *et al.* 2006) and *S. stercoralis* (Gallego *et al.* 2005). In general, the *C. elegans* astacins are associated with tissues exposed to the external environment and have diverse functions which, in part, relate to unique nematode-specific extracellular structures, such as the eggshell, the cuticular exoskeleton and the cuticular pharyngeal grinder (Park *et al.* 2010).

Many of the developmentally essential nematode proteases represent potential drug targets; in addition to the astacins, this includes members of the cathepsin cysteine proteases. The cathepsin L and Z-like cysteine proteases from *B. malayi* are essential for embryogenesis, larval moulting and cuticle and eggshell remodelling (Ford *et al.* 2009), whereas those from *O. volvulus* are essential during the ecdysis step of the L3 to L4 moult (Lustigman *et al.* 2004) and the *C. elegans* cathepsin Z-like cysteine protease is likewise involved in the ecdysis steps of moulting (Hashmi *et al.* 2004).

The spatial expression of the *C. elegans* astacins relates to their functions; for example, NAS-34 has a specific role in embryo hatching, reflected in its

expression in the oocytes and embryos (Park *et al.* 2010). Enzymes that play important roles in collagen biogenesis, cuticle formation and the moulting process are predominantly expressed in the underlying hypodermal syncytium (Page and Winter, 2003). In addition, some of these enzymes are found to be secreted from the glandular system that surrounds the oesophagus and may be particularly important in the latter stages of cuticle removal or ecdysis, with many representing important components of the moulting exsheathment fluid (Albertson and Thomson, 1976; Nelson *et al.* 1983; Bird, 1987). Many of the subgroup V nematode astacin metalloproteases share these cuticle-related expression patterns, i.e., they are found in the excretory duct, pharynx and hypodermal cells (Davis *et al.* 2004; Novelli *et al.* 2004; Suzuki *et al.* 2004; Stepek *et al.* 2010b). *C. elegans* DPY-31 was found to be expressed throughout the life-cycle in the hypodermal cells (Novelli *et al.* 2004) and in the oesophageal glands (Stepek *et al.* 2010b), whereas the heterologous expression of the *B. malayi* promoter/reporter gene revealed predominant expression in the oesophageal gland cells and gut of *C. elegans*, leading to the suggestion that DPY-31 plays a role in the construction of the nematode cuticle (Stepek *et al.* 2010b). Although the current study does not examine the expression pattern of NAS-36 from *C. elegans*, previous studies have described this expression also to be in the hypodermal cells (Suzuki *et al.* 2004). Davis *et al.* (2004) showed that NAS-37 from *C. elegans* was abundantly expressed in the excretory duct and in the oesophagus and also in the hypodermal cells of the cuticle 4 h prior to each moult. This expression pattern is consistent with the enzyme's role in nematode cuticle ecdysis and the moulting process.

This current study has identified and characterized the homologues of NAS-36 from the parasitic nematodes *H. contortus* and *B. malayi*. There is greater homology to NAS-36 between *C. elegans* and *H. contortus* than between either *C. elegans* and *B. malayi* or *H. contortus* and *B. malayi*, which is consistent with the nematode class V astacin metalloprotease, DPY-31 (Stepek *et al.* 2010b). This level of homology would be expected based on the phylogenetic relationship between these three nematodes (Blaxter *et al.* 1998). Our previous study indicated that the *dpy-31* gene found in both *H. contortus* and *B. malayi* contained larger introns than in *C. elegans* (Stepek *et al.* 2010b). A similar feature has now also been described for the *nas-36* gene, suggesting that genes from the parasitic nematodes, in general, have larger introns than their *C. elegans* orthologues.

To date, no NAS-37 homologues have been found in either *H. contortus* or *B. malayi*, although Ghedin *et al.* (2007) identified the locus Bm1\_49145 from the *B. malayi* genome database as being a putative NAS-37 homologue. Subsequent BLAST-searching

in the *C. elegans* database revealed this locus to have greatest homology to *C. elegans* NAS-30, and further searching of the *B. malayi* TIGR database with *C. elegans* NAS-37 confirmed this homology with NAS-30. A potential NAS-37 homologue was initially identified in the *H. contortus* genome, although subsequent experimental analysis only extended the initial sequence to include a signal peptide, astacin domain and a C-terminal EGF domain (data not shown). Thus, subsequent analysis indicated that the potential NAS-37 homologues identified in *H. contortus* and *B. malayi* were not in fact true orthologues of *C. elegans* NAS-37.

Three-dimensional homology modelling predicted the astacin domain structures of NAS-36 from *H. contortus* and *B. malayi* to be more similar to the predicted structure of NAS-37 (alternative transcript a) from *C. elegans*. However, the percentage amino acid identity between these protein domains is much greater to NAS-36 from *C. elegans* than to *C. elegans* NAS-37. Therefore, the proteins from the parasitic nematodes encode NAS-36 homologues that share the specific protein folding pattern of *C. elegans* NAS-37. Three-dimensional homology modelling also predicted the structure of *C. elegans* NAS-30 to be more similar to the paralogous proteins NAS-36 and NAS-37b than to NAS-37a (data not shown).

NAS-36 shares a similar function in cuticle ecdysis to NAS-37 in *C. elegans*, although the latter protein appears to be more important, having a stronger phenotype in the absence of the *nas-37* gene. Therefore, NAS-36 may be essential for cuticle ecdysis in parasitic nematodes and suggests a degree of redundancy in the *C. elegans* astacin family. Williamson *et al.* (2007) indicated potential gene redundancy in proteins that belong to the Cys-loop ligand gated ion channels family, with a larger gene family in *C. elegans* in comparison to *B. malayi* and *Trichinella spiralis*. Another nematode astacin metalloprotease of subgroup V, NAS-34, is necessary for normal hatching of the embryo of *C. elegans* (Hishida *et al.* 1996). Our genome database screens, to date, have been unable to identify homologues of this gene in either *H. contortus* or *B. malayi* (Stepek and Page, unpublished data). Homologous proteins to the *C. elegans* clade V astacin, NAS-38, were however isolated from the genomes of *H. contortus* and *B. malayi* (Stepek and Page, unpublished data). NAS-34 is expressed in the early embryo, largely in the hypodermal cells (Hishida *et al.* 1996), whilst WormBase ([www.wormbase.org](http://www.wormbase.org)) describes the expression of NAS-38 to be in the reproductive system, vulva, hypodermis and seam cells throughout the *C. elegans* life cycle. Therefore, NAS-38 may perform a key role in the hatching of the parasitic nematode embryos, superseding the requirement for NAS-34 in these nematodes. Möhrlein *et al.* (2003) suggested that the *C. elegans* astacins possess overlapping functions, particularly those genes belonging

to the same subgroups. There are 39 individual astacin metalloproteases in the genome of *C. elegans* (Möhrlein *et al.* 2003), approximately 13 in the *B. malayi* genome (Ghedini *et al.* 2007) and an, as yet, unknown number in the genome of *H. contortus*, although this current study may suggest that there are fewer members than in the *C. elegans* genome. The presence of larger, redundant gene families in *C. elegans* compared to the parasitic nematodes, which have comparably sized genomes, is becoming a well-established feature (Ghedini *et al.* 2007; Williamson *et al.* 2007) and may either be due to gene expansion in *C. elegans* or indeed gene loss in the parasitic nematodes.

The conservation of function between NAS-36 and NAS-37 is evident from the rescue experiments using *B. malayi nas-36* to complement the *C. elegans* mutant strains of *nas-36* (TP87), *nas-37* (TP99) and *nas-36/nas-37* (TP93), as *B. malayi nas-36* was able to rescue all three mutant strains, returning a wild-type phenotype. This further supports the hypothesis that NAS-36 is the key molecule involved in cuticle ecdysis of parasitic nematodes, and suggests a conservation of function for the developmentally essential NAS-36 metalloprotease between parasitic and free-living nematodes. Although the *B. malayi nas-36* was able to rescue the *C. elegans* mutant strains at 25 ng/ $\mu$ l, the *H. contortus nas-36* rescue construct proved to be too toxic for these mutants even at very low concentrations. Such toxicity was not, however, reported in a parallel study in which a *H. contortus dpy-31* rescue construct was used to successfully rescue the corresponding *C. elegans* mutant (Stepek *et al.* 2010b). We hypothesize that the toxicity observed in this case may be due to an overexpression effect of the highly homologous *H. contortus* NAS-36 enzyme in *C. elegans*. In support of this contention, it is significant to note that overexpression, albeit at a higher concentration, of the *H. contortus* cuticle protease BLI-5 also led to severe morphological defects in wild type *C. elegans* (Stepek *et al.* 2010a).

In conclusion, NAS-36 and NAS-37 play an important function in cuticle ecdysis during the development of *C. elegans* (Davis *et al.* 2004), and NAS-36 has also now been shown to have a crucial role in the development of *H. contortus* and *B. malayi*, and may therefore represent an ideal candidate as a target for developing a new control strategy against parasitic nematodes.

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