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Transcriptome analysis of a parasitic clade V nematode: Comparative analysis of potential molecular anthelmintic targets in *Cylicostephanus goldi* ☆

Krystyna Cwiklinski [a,*], J. Yvette Merga [b], Sarah L. Lake [c], Catherine Hartley [a], Jacqui B. Matthews [d], Steve Paterson [e], Jane E. Hodgkinson [a]

[a] Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool, Liverpool L3 5RF, UK
[b] Department of Epidemiology and Population Health, Institute of Infection and Global Health, University of Liverpool, Liverpool CH64 7TE, UK
[c] Department of Molecular and Clinical Cancer Medicine, Institute of Translational Medicine, University of Liverpool, Liverpool L69 3BX, UK
[d] Moredun Research Institute, Edinburgh EH26 0PZ, UK
[e] Centre for Genomic Research, University of Liverpool, Liverpool L69 7ZB, UK

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Clade V nematodes comprise several parasitic species that include the cyathostomins, primary helminth pathogens of horses. Next generation transcriptome datasets are available for eight parasitic clade V nematodes, although no equine parasites are included in this group. Here, we report next generation transcriptome sequencing analysis for the common cyathostomin species, *Cylicostephanus goldi.* A cDNA library was generated from RNA extracted from 17 *C. goldi* male and female adult parasites. Following sequencing using a 454 GS FLX pyrosequencer, a total of 475,215 sequencing reads were generated, which were assembled into 26,910 contigs. Using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases, 27% of the transcriptome was annotated. Further in-depth analysis was carried out by comparing the *C. goldi* dataset with the next generation transcriptomes and genomes of other clade V nematodes, with the *Oesophagostomum dentatum* transcriptome and the *Haemonchus contortus* genome showing the highest levels of sequence identity with the cyathostomin dataset (45%). The *C. goldi* transcriptome was mined for genes associated with anthelmintic mode of action and/or resistance. Sequences encoding proteins previously associated with the three major anthelmintic classes used in horses were identified, with the exception of the P-glycoprotein group. Targeted resequencing of the glutamate gated chloride channel α4 subunit (*glc-3*), one of the primary targets of the macrocyclic lactone anthelmintics, was performed for several cyathostomin species. We believe this study reports the first transcriptome dataset for an equine helminth parasite, providing the opportunity for in-depth analysis of these important parasites at the molecular level. Sequences encoding enzymes involved in key processes and genes associated with levamisole/pyrantel and macrocyclic lactone resistance, in particular the glutamate gated chloride channels, were identified. This novel data will inform cyathostomin biology and anthelmintic resistance studies in future.

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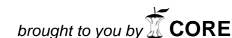
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1. Introduction

Clade V parasites of the phylum Nematoda represent some of the most economically important gastrointestinal nematodes of domestic animals (Blaxter et al., 1998). In the absence of commercial vaccines, management of these parasites is based on anthelmintic treatment, historically using three classes of anthelmintic: benzimidazoles (BZ), tetrahydropyrimidines/imidothiazoles (TETR/IMID) and macrocyclic lactones (ML). Recently three new classes of anthelmintic have been licensed for control of parasitic nematodes in small ruminants: the cyclodepsipeptide, emodepside, the aminoacetonitrile (AAD) derivative, monepantel and the

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers: **AM887978**–**AM887979**, **KC249936**–**KC249942** and the transcriptome dataset is available at the European Nucleotide Archive http://www.ebi.ac.uk/ena/data/view/PRJEB3962 and AfterParty http://afterparty.bio.ed.ac.uk/study/show/4014626.

* Corresponding author. Address: Veterinary Parasitology, Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool, Liverpool L3 5RF, UK. Tel.: +44 151 7950234; fax: +44 151 7950236.
E-mail address: k.cwiklinski@liverpool.ac.uk (K. Cwiklinski).

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spiroindole, derquantel (Epe and Kaminsky, 2013). Extensive and inappropriate use of anthelmintics in livestock has led to the development and spread of anthelmintic resistance in gastrointestinal parasites (Kaplan, 2004). Resistance to the three original anthelmintic classes is a widespread problem and many studies have reported multi-class resistant populations (Kaplan, 2002, 2004; Sutherland and Leathwick, 2011; Martínez-Valladares et al., 2012b; Papadopoulos et al., 2012; Torres-Acosta et al., 2012; Verissimo et al., 2012). Over two decades of exploring the link between resistance phenotype and candidate gene polymorphisms has yet to reveal the genetic mechanisms of resistance for the majority of anthelmintic drugs (Gilleard, 2006; James et al., 2009; Beech et al., 2011). Understanding this genetic basis of resistance and developing biomarkers for monitoring the impact of drug selection pressure on parasite populations remains a priority for effective helminth control in future. With few exceptions (Schougaard and Nielsen, 2007; Bourguinat et al., 2011; Bowman, 2012; Reinemeyer, 2012), parasitic nematodes that display anthelmintic resistance are represented by the clade V Nematoda. Amongst the parasites displaying resistance are several nematode species infecting cattle, such as *Ostertagia ostertagi* and *Cooperia oncophora* (Sutherland and Leathwick, 2011), and sheep, such as *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Papadopoulos et al., 2012). Other relevant clade V nematodes include *Oesophagostomum* spp. of cattle and pigs (Gerwert et al., 2002; Condi et al., 2009) and cyathostomins of horses (Kaplan, 2002).

For many years, studies of anthelmintic resistance mechanisms have focused on a candidate gene approach targeting key proteins presumed to be involved in anthelmintic action; more than 30 studies have explored these targets to assess genetic differences between phenotypically susceptible and resistant parasites (Beech et al., 1994; Elard et al., 1996; Pape et al., 2003; Drogemuller et al., 2004; Cudekova et al., 2010; Martínez-Valladares et al., 2012a). The MLs ivermectin (IVM) and moxidectin (MOX) are the anthelmintics of choice for the control of the majority of parasites, resulting in extensive study of potential targets, in particular the glutamate gated chloride channels (GluCl, Wolstenholme, 2011, 2012), the ABC transporters (James and Davey, 2009) and β-tubulin molecules (de Lourdes Mottier and Prichard, 2008). Polymorphisms or gene expression levels for many of these genes have been extensively studied in *H. contortus* and the non-parasitic model organism *Caenorhabditis elegans*, yet an understanding of the gene, or genes, involved in IVM resistance remains unresolved at the molecular level. Since resistance to IVM appears to be multigenic (McCavera et al., 2007), more systematic global studies of genes or proteins involved in IVM resistance need to be undertaken in order to reveal new molecular candidates and in the process provide larger sequence datasets for genome resource-poor organisms. The gastrointestinal parasites of ruminants remain the primary focus for these novel approaches and recently both the detection of transcriptomic responses of *T. circumcincta* adult worms exposed to IVM in vitro and genome wide analyses of controlled genetic crosses of *H. contortus* have provided preliminary evidence of new genetic markers for IVM resistance-conferring genes (Dicker et al., 2011a,b; Redman et al., 2012).

Comparative analysis with *C. elegans* and other parasitic nematodes offers a complementary approach to extensive analysis of the individual parasite. Next generation transcriptomes are publically available for *Caenorhabditis* spp. (http://www.wormbase.org) and nine clade V nematodes (http://nematode.net/NN3_frontpage.cgi), eight of which are derived from parasitic nematodes; *Ancylostoma caninum*, *C. oncophora*, *Necator americanus*, *Oesophagostomum dentatum*, *O. ostertagi*, *T. circumcincta*, *T. colubriformis* and *Dictyocaulus viviparus* (which has been alternatively classified as either clade IV or clade V; http://nematode.net/NN3_frontpage.cgi; Martin et al., 2012). To date, no extensive sequencing project has been carried out for any equine parasitic nematode, despite the cyathostomins being ubiquitous, important pathogens of equids worldwide (Love et al., 1999). These nematodes comprise a complex group of parasites with 50 species that are morphologically difficult to distinguish (Hodgkinson et al., 2001, 2003; Cwiklinski et al., 2012), although 10 common species predominate in natural infections (Lichtenfels et al., 1998). These parasites have developed resistance to all of the anthelmintic classes currently available for their control, with widespread resistance to BZ and areas of high levels of resistance to pyrantel (Stratford et al., 2011; Molento et al., 2012) whilst resistance to IVM and MOX is emerging (Molento et al., 2012). To date, sequences encoding β tubulin isotype 1 and 2 proteins (Pape et al., 1999; Clark et al., 2005), two P-glycoproteins (*Pgps*), (Drogemuller et al., 2004) and the aforementioned *GluCl* α and β subunits sequences (Tandon et al., 2006) have been amplified from cyathostomins. Here, we present the first known large scale transcriptome dataset generated from *Cyclicostephanus goldi* parasites; a common cyathostomin species. This resource will allow in-depth analysis to aid the characterisation of existing and novel anthelmintic targets, support detailed understanding of the biology of cyathostomins and facilitate investigation of the population genetic structure of these important parasites. To highlight the utility of this resource this study reports the targeted resequencing of *GluCl* genes that are thought to be a primary mechanism of ML resistance.

2. Materials and methods

2.1. Collection of parasites

Individual adult worms were collected from the dorsal colon, ventral colon and caecum of horses at a northwestern UK abattoir and/or a population of horses maintained in Kentucky, USA (Hodgkinson et al., 2008), washed in 1× PBS (pH 7.4), the parasite heads removed for morphological identification and the remainder of the parasites stored in liquid nitrogen as described previously (Hodgkinson et al., 2001). Identification to genus and species was according to Lichtenfels et al. (1998).

2.2. 454 transcriptome sequencing

Seventeen *C. goldi* adult worms collected from a population of horses maintained in USA were used for RNA extraction. Total RNA was extracted using Trizol reagent (Life Technologies, UK) and purified using a PureLink™ RNA Mini Kit (Life Technologies) according to the manufacturer's instructions. RNA integrity and concentration were confirmed using the Bioanalyzer 2100 (Agilent Technologies, UK). Double stranded (ds) cDNA synthesis was carried out using the SMART approach (Evrogen, Russia; Zhu et al., 2001). Part of the ds cDNA sample was normalised using the duplex-specific nuclease (DSN) normalisation method (Evrogen; Zhulidov et al., 2004). Normalisation included cDNA denaturation/reassociation, treatment by DSN (Shagin et al., 2002) and amplification of the normalised fraction by PCR (26 cycles). The remaining ds cDNA sample was retained for synthesis of a cDNA library (see Section 2.4). The yield was determined using the NanoDrop 1000. Sequencing was carried out on a 454 GS FLX instrument (Roche 454) by the Centre of Genomic Research, University of Liverpool, UK.

2.3. Bioinformatic analysis

Raw data sequences were aligned and assembled using the Roche 454 GS-Assembler software (v2.0.01.14). The resulting

contiguous sequences (contigs) were subjected to analysis by the Basic Local Alignment Search Tool (BLASTx; Altschul et al., 1990) against the Swissprot database (UNIPROT; www.uniprot.org). Protein prediction and functional annotation of the contig sequences was performed using the PartiGene pipeline, specifically prot4EST (Wasmuth and Blaxter, 2004) and annot8r (Schmid and Blaxter, 2008) software. Prot4EST, the protein prediction tool that uses BLAST, ESTScan, DECODER and longest open reading frame (Longest ORF) to generate the protein translation, was run using the default parameters. The predicted proteins were also subjected to analysis using the programs SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/; Petersen et al., 2011) and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) to predict signal peptides and transmembrane domains, respectively. Functional annotation of the conceptually translated proteins was carried out using annot8r at default settings that assigned Gene Ontology (GO), Enzyme Commission (EC) number function and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms based on BLASTp E-values at a cut off of $1e^{-8}$. Further KEGG annotation was carried out using the KEGG Automated Annotation Server (KAAS; http://www.genome.jp/tools/kaas/) web tool using *C. elegans* as the template sequence (Moriya et al., 2007). Analysis of the metabolic pathways within the clade V nematodes was carried out using the Pathway function in NEMBASE4 (Elsworth et al., 2011). The predicted proteins were also classified using the InterProScan program with default settings through BLAST2GO (Conesa et al., 2005). Homologues/orthologues in other nematodes were identified using BLASTp (version 2.2.23; bit score cut off of >50; Parkinson et al., 2004) against clade V nematode transcriptomes (accessed from http://www.nematode.org), the *C. elegans* transcriptome (release WS233; http://www.wormbase.org) and phylum Nematoda genomes (*C. elegans*: release WS238, http://www.wormbase.org; *Ascaris suum*: **PRJNA62057**, Wang et al., 2012 and **PRJNA80881**, Jex et al., 2011; *H. contortus*: FTP directory/pub/pathogens/Haemonchus/contortus/genome/ at ftp.sanger.ac.uk; *Trichinella spiralis*: **PRJNA12603**, Mitreva et al., 2011).

2.4. cDNA library construction

A cDNA library was constructed using the SMART™ cDNA Library Construction Kit (Clontech, Takara Bio Europe, France) according to the manufacturer's instructions. The ds cDNA not used as part of the normalisation protocol prior to transcriptome sequencing was used here, following additional amplification (according to manufacturer's instructions). The λ phage packaging reaction was carried out using the Gigapack III Gold Packaging Extract (Agilent Technologies, UK), according to the manufacturer's instructions.

2.5. Individual RNA extractions and Rapid Amplification of cDNA Ends (RACE) cDNA amplification

Total RNA was extracted from individual adult cyathostomin worms collected from horses at a northwestern UK abattoir, using Trizol reagent (Life Technologies) according to the manufacturer's instructions. RACE cDNA was amplified using the SMARTer RACE cDNA Amplification Kit (Clontech, Takara Bio Europe, France) according to the manufacturer's instructions.

2.6. PCR amplification and cloning

PCRs were carried out in 50 µl reaction volumes using either the Advantage® 2 PCR Enzyme System (Clontech, Takara Bio Europe) or the Expand High Fidelity PCR System (Roche, UK) according to the manufacturers' instructions. A total of 1–2 µl of template was used per reaction (*Cylicocyclus nassatus*, *Cyathostomum catinatum*, *Cyathostomum tetracanthum* RACE cDNA or *C. goldi* cDNA library). Standard splice leader 1-based PCR and RACE protocols with primers designed from *GluCl* sequences from *C. nassatus*, *C. elegans* and *H. contortus*, as well as the *C. goldi* sequence data from the single contig identified as being GluCl-specific in the transcriptome were used to amplify the GluCls. The primers used for the different reactions are shown in Supplementary Table S1. The cycling conditions were an initial 2 min at 94 °C, followed by 35 cycles at 94 °C for 15 s, annealing temperature (Tm) for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 7 min. Negative and positive control samples were included where appropriate. PCR products were analysed by agarose gel electrophoresis using SYBR® Safe DNA stain (Life Technologies). PCR products were cloned into the pGEM-T Easy vector system (Promega, UK), transformed into JM109 *Escherichia coli* competent cells (Promega) according to the manufacturer's instructions and sequenced by GATC-Biotech (Germany).

2.7. Sequence analysis of the cyathostomin GluCl channels

Analyses by BLASTn and BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were carried out on the cloned and sequenced GluCl PCR products to confirm the assigned identity of the sequences. Analysis of potential N-terminal signal peptide sequences was carried out using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/; Petersen et al., 2011). Alignments of the nucleotide and amino acid sequences were carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3. Results

3.1. 454 sequencing and assembly of the C. goldi transcriptome

Over 450,000 high quality reads were generated following 454 sequencing of a cDNA library constructed from RNA pooled from 17 adult mixed sex *C. goldi* worms. The sequencing reads were assembled using GS-Assembler (v2.0.01.14) into 26,910 contigs (Table 1). The size of the cyathostomin genome is currently not known. Of the limited number of cyathostomin genes previously published, four were identified within the transcriptome analysed here: the LIM domain-containing gene (**EU014896**; Matthews et al., 2008), *GluCl α4* subunit gene (**AY727925**; Tandon et al., 2006), β tubulin gene (**AF181093**; Pape et al., 1999) and the mitochondrial cytochrome oxidase c subunit 1 (*cox-1*) gene (**AF263472**–**AF263489**; McDonnell et al., 2000). Due to the normalisation process, sequences corresponding to the ribosomal genes were not identified (Kaye et al., 1998; McDonnell et al., 2000; Cwiklinski et al., 2012).

3.2. Putative functional classification using GO and KEGG classifications

ORFs were predicted using Prot4EST for the 26,910 contigs resulting in a total of 26,928 putative protein sequences (18 contigs had proteins predicted by both the BLAST algorithm and Longest ORF during Prot4EST analysis). Functional annotation was carried out using annot8r, resulting in putative functional classifications for 7,521 (27.9%) of the proteins inferred from the cyathostomin transcriptome based on GO, EC number function and KEGG terms. In particular, GO terms were assigned to 7,359 (27%) contigs, represented by three main categories: biological processes, cellular component and molecular function (Table 1). Over 50% of the number of occurrences for specific GO terms fell into the binding component and the intracellular component for the molecular function and cellular component, respectively (Fig. 1). Further functional classification was carried out using the KAAS web tool,

Table 1
Results of *Cylicostephanus goldi* transcriptome sequencing and bioinformatic analysis in this study.

Description of transcriptome assembly and bioinformatic analysis	Result
No. unassembled Expressed Sequence Tags (average length ± S.D.)	475,215 (291.4 ± 178.3)
Contigs (average length ± S.D.)	26,910 (471.9 ± 325.4)
Proteins predicted by Prot4EST	26,928
Signal peptides (SignalP)	859 (3.1%)
Containing transmembrane domains (TMHMM)	4,262 (15.8%)
Predicted Gene Ontology (GO) terms	7,359 (27%)
Biological process	6,861 (4780 terms)
Cellular component	6,234 (905 terms)
Molecular function	6,440 (2051 terms)
No. of biological pathways predicted (KAAS)	237
Returning InterPro Scan results	9,021 (33.5%) 3,981 InterPro terms
Annotated sequences	14,038 (52.1%)

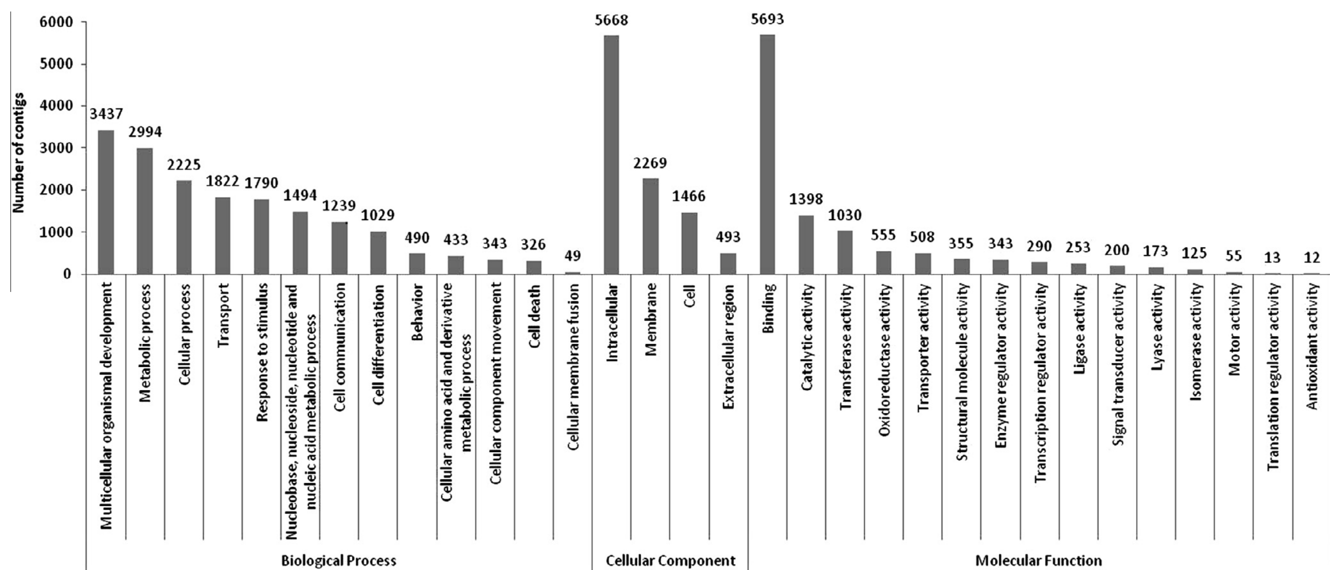

Fig. 1. Graphical representation of Gene Ontology classification of the *Cylicostephanus goldi* proteins predicted from sequencing of the transcriptome. The analysis is summarised in the three main categories: biological process, cellular component and molecular function.

assigning the predicted proteins to metabolic pathways (KEGG) based on homology to *C. elegans* proteins. A total of 4,683 (17.4%) of the 26,928 putative proteins were predicted to be involved in 237 metabolic pathways, representing 1,994 different KEGG Orthology (KO) terms. The number of contigs represented by a particular KO term ranged from 1 to 55. The KO terms represented by more than 10 contigs included high representation of cathepsins, astacins, histones and glutathione S-transferase (GST) proteins (Table 2; Supplementary Table S2). The proteins predicted from the transcriptome dataset were screened for signal peptides and transmembrane domains, resulting in signal peptides being predicted for 859 (3.1%) of the predicted proteins and 4,262 (15.8%) protein sequences found to contain transmembrane domains (Table 1).

3.3. Comparison of the C. goldi transcriptome with expressed sequence tag (EST) datasets and next generation transcriptomes from clade V nematodes

Following initial analysis and putative functional classification of the *C. goldi* transcriptome dataset based on homology to the general GO and KEGG databases using annot8r and KAAS software, 7,943 (29.5%) predicted proteins could be annotated. Further analysis was therefore carried out to increase the level of annotation by comparing the *C. goldi* dataset with more closely related clade V nematode transcriptome datasets (Martin et al., 2012), as well as the currently available phylum Nematoda genomes (Jex et al., 2011; Mitreva et al., 2011; Wang et al., 2012). Comparison of the cyathostomin KAAS analysis with that of the 19 clade V nematode EST datasets present in NEMBASE4 showed that the cyathostomin KEGG dataset is very similar to that observed amongst other clade V nematodes. In total, enzymes of 107 different pathways were identified, suggesting that these pathways are involved in core processes (Fig. 2). The analysis carried out using the *C. elegans* transcriptome dataset (http://www.wormbase.org) and eight parasitic clade V next generation transcriptomes (Martin et al., 2012), showed that a similar number of contigs within the datasets to that of the *C. goldi* transcriptome, which allowed significant comparisons to be made (bit score cut off of >50; Parkinson et al., 2004). The number of *C. goldi* contigs identified as having a significant hit with transcripts in each heterologous species (*A. caninum*, *C. oncophora*, *D. viviparus*, *N. americanus*, *O. dentatum*, *O. ostertagi*, *T. circumcincta*, *T. colubriformis* and *C. elegans*, each analysed separately) was calculated as the percentage of the total contig number in the cyathostomin transcriptome (Table 3). Datasets of three species (*O. dentatum*, *C. elegans* and *D. viviparus*) were found to match 40–45% of the contigs within the *C. goldi* transcriptome; the five remaining datasets ranged from 24% to 38%. Interestingly comparing the *C. goldi* dataset to all of the clade V nematode transcriptome datasets as one group resulted in a higher level of homology (51.5%), representing 13,865 of the predicted protein sequences. This result indicates that there are several sequences that may not be shared across all the clade V nematodes and emphasises

Table 2
The most represented proteins within the *Cylicostephanus goldi* dataset inferred from KEGG Automatic Annotation Server (KAAS) analysis, shown by Kyoto Encyclopedia of Genes and Genomes (KEGG) values and descriptions.

No. of *C. goldi* contigs	KEGG value	Description
55	K01363	CTSB; cathepsin B [EC:3.4.22.1]
41	K08076	astacin [EC:3.4.24.21]
30	K00799	gst; glutathione S-transferase [EC:2.5.1.18]
27	K11251	H2A; histone H2A
24	K07976	RAB; Rab family, other
23	K11252	H2B; histone H2B
20	K00699	UGT; glucuronosyltransferase [EC:2.4.1.17]
17	K01090	protein phosphatase [EC:3.1.3.16]
17	K05692	ACTB_G1; actin beta/gamma 1
17	K08568	CTSZ; cathepsin X [EC:3.4.18.1]
16	K01104	protein-tyrosine phosphatase [EC:3.1.3.48]
16	K01529	E3.6.1. – unclassified
15	K08708	Nuclear hormone receptor family member (BLAST)
14	K00029	maeB; malate dehydrogenase (oxaloacetate-decarboxylating)(NADP+) [EC:1.1.1.40]
14	K01915	glnA; glutamine synthetase [EC:6.3.1.2]
13	K00600	glyA, SHMT; glycine hydroxymethyltransferase [EC:2.1.2.1]
13	K01679	fumC; fumarate hydratase, class II [EC:4.2.1.2B]
13	K10352	MYH; myosin heavy chain
12	K00164	OGDH, sucA; 2-oxoglutarate dehydrogenase E1 component [EC:1.2.4.2]
12	K05312	CHRNN; nicotinic acetylcholine receptor, invertebrate
12	K11253	H3; histone H3
11	K13289	CTSA; cathepsin A (carboxypeptidase C) [EC:3.4.16.5]
11	K01897	ACSL, fadD; long-chain acyl-CoA synthetase [EC:6.2.1.3]
11	K06269	PPP1C; protein phosphatase 1, catalytic subunit [EC:3.1.3.16]
11	K07977	ARF; Arf/Sar family, other
10	K11275	H1_5; histone H1/5
10	K00939	adk; adenylate kinase [EC:2.7.4.3]
10	K01251	ahcY; adenosylhomocysteinase [EC:3.3.1.1]
10	K01681	ACO, acnA; aconitate hydratase 1 [EC:4.2.1.3]
10	K02930	RP-L4e, RPL4; large subunit ribosomal protein L4e
10	K06147	ABCB-BAC; ATP-binding cassette, subfamily B, bacterial

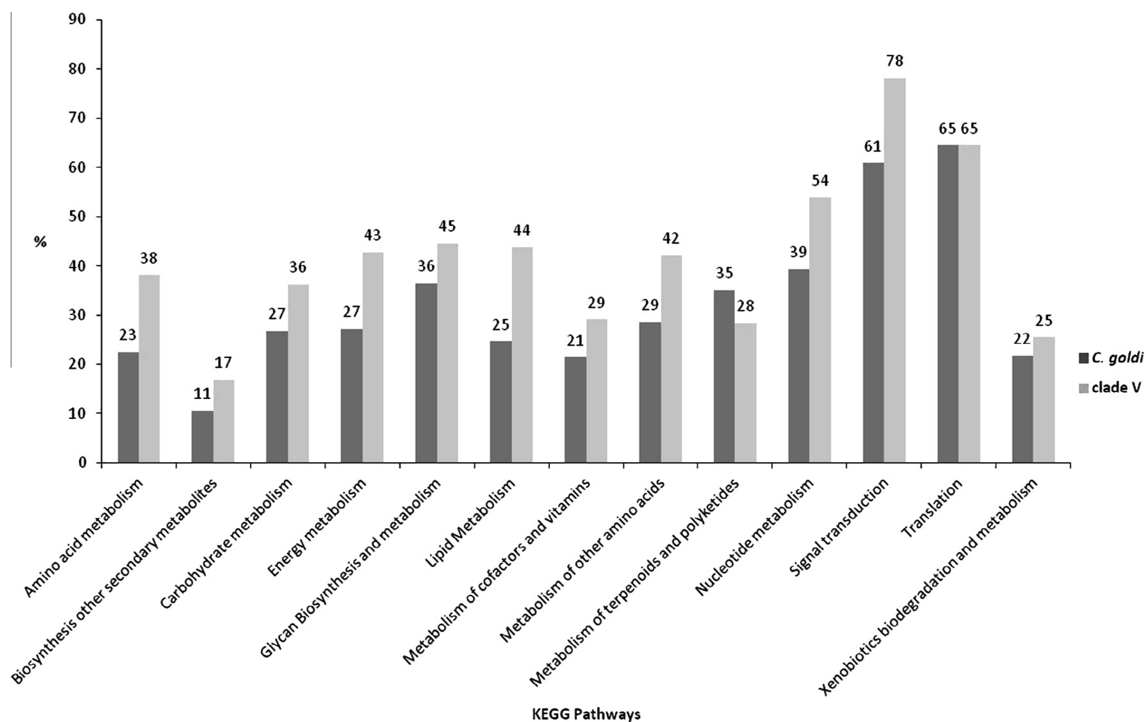

Fig. 2. Graphical representation of the *Cylicostephanus goldi* KEGG Automatic Annotation Server analysis. The *C. goldi* data was compared with metabolic pathway analysis of the clade V nematodes represented in NEMBASE 4. The number of enzymes matched is shown as a percentage of the total number of enzymes present within the pathway.

the requirement of using all available datasets to improve the annotation statistics of newly sequenced species. Although several clade V nematode transcriptome datasets are now publicly available, it is not the case for genome datasets for these species. The predicted cyathostomin proteins were compared with the available genome datasets from the phylum Nematoda, specifically *C. elegans*, *A. suum*, *H. contortus* and *T. spiralis* (Table 3). This analysis showed that the *C. goldi* predicted proteins shared the greatest

Table 3
Comparative BLASTp analysis between the *Cylicostephanus goldi* transcriptome, clade V nematode transcriptomes and phylum Nematoda genomes.

Species (no. of predicted proteins)	Total no. of hits	*C. goldi* contig hits	
		No. of contig hits	%
Haemonchus contortus[a] (24,781)	108,118	12,384	45.9
Oesophagostomum dentatum (30,024)	122,818	12,056	44.7
Dictyocaulus viviparus (36,626)	69,782	10,985	40.7
Caenorhabditis elegans (WS233; 44,803)	137,368	10,769	40.0
Caenorhabditis elegans[a] (WS238; 26,248)	91,095	10,689	39.7
Cooperia oncophora (29,903)	85,161	10,322	38.3
Ostertagia ostertagi (34,792)	84,342	9,867	36.6
Ascaris suum			
PRJNA62057[a]	50,454	9,792	36.3
PRJNA80881[a]	49,687	9,728	36.1
Trichostrongylus colubriformis (27,593)	50,495	9,286	34.5
Teladorsagia circumcincta (33,124)	34,479	8,574	31.8
Trichinella spiralis[a] (16,380)	31,833	6,543	24.3
Necator americanus (9,681)	18,877	6,481	24.0

Unless otherwise indicated transcriptome datasets were used.
[a] Genome datasets.

homology to *C. elegans* and *H. contortus*, 39.7% and 45.9%, respectively, with the lowest level of homology being found between *C. goldi* and *T. spiralis* (24.3%). Additional analysis with the available nematode genome datasets increased the percentage of annotation in the cyathostomin transcriptome dataset to 52.1%.

3.4. Analysis of potential anthelmintic targets within the cyathostomin transcriptome

We identified all anthelminitic resistance candidate genes previously described (Clark et al., 2005; Wolstenholme, 2011, 2012), with the exception of the *Pgp* genes. *Cylicostephanus goldi* contigs identified as containing candidate gene sequences matched those in the *C. oncophora*, *D. viviparus*, *O. dentatum*, *O. ostertagi* and *T. colubriformis* transcriptomes, with representation of β tubulin gene sequences within the *N. americanus* and *T. circumcincta* datasets.

3.4.1. β tubulin genes

β tubulin sequence isotypes have been identified previously, with *isotype 1* and *isotype 2* well characterised for cyathostomins (Pape et al., 1999; Clark et al., 2005; Lake et al., 2009). Single nucleotide polymorphisms (SNP) associated with BZ resistance have been consistently observed in the β tubulin *isotype 1* rather than *isotype 2* (Hodgkinson et al., 2008). Here, seven contigs were found to span the entire length of the β tubulin protein, with two contigs (contig02200 and contig02193) representing an isotype 1 profile and the remaining five (contig00354, contig00355, contig17580, contig19769 and contig24974) representing an isotype 2 profile, based on SNP profiles described by Clark et al. (2005).

3.4.2. Nicotinic acetylcholine receptor (nAChR) genes

Sequences relating to the *nAChR* genes were initially identified as part of the KAAS analysis with 12 contigs matching nAChR receptor sequences. Further analysis revealed that seven of these contigs showed high sequence identity to UNC-29, UNC-38, LEV-1 and ACR-8 sequences (*C. elegans* nomenclature; Fleming et al., 1997; Richmond and Jorgensen, 1999; Culetto et al., 2004; Towers et al., 2005; Boulin et al., 2011). Three of these genes (*unc-29*, *unc-38* and *lev-1*) have been identified in *T. circumcincta*, *T. colubriformis* and *H. contortus* (Neveu et al., 2010).

3.4.3. GluCl genes

A single contig from the *C. goldi* transcriptome was identified as a GluCl sequence, corresponding to the 3′ end of the published cyathostomin gene *GluCl* α (Tandon et al., 2006), a gene originally not assigned as a specific subunit, although phylogenetic analysis grouped the sequence with *C. elegans glc-3* (Tandon et al., 2006). Targeted resequencing of the *GluCl* α subunit was carried out for four common cyathosomin species: *C. catinatum*, *C. tetracanthum*, *C. nassatus* and *C. goldi*. We consistently amplified only one *GluCl* α sequence, corresponding to the α4 (*glc-3*) subunit. Consensus sequences were compiled for each species and compared against all known parasitic GluCl sequences (Supplementary Fig. S1). Based on ClustalW2 analysis, the cyathostomin sequences show higher sequence identity to the available GLC-3 sequences, consistent with analysis by Tandon et al. (2006) (Supplementary Fig. S1); we subsequently designated this gene, as GluCl *glc-3*, corresponding to the α4 subunit. For all four cyathostomin species the GluCl sequence shows the highest sequence identity to the GLC-3 sequence from *O. dentatum* (85–90%) rather than the *H. contortus* GLC-3 sequence (37–81%), consistent with the transcriptome analysis (Fig. 3). Clustal W2 alignment analysis revealed that the GluCl α4 consensus sequences derived from the four cyathostomin species (including the previously published *C. nassatus* sequence, (Tandon et al., 2006) showed 88-96% amino acid similarity with each other. The two sequences from *C. nassatus* showed 4% intra-specific variation.

Although extensive analysis of the GluCls has been carried out for *H. contortus*, only a few sequences have been identified in other parasitic species. Using the cyathostomin GluCl sequences identified within this study, the next generation transcriptomes for *C. oncophora*, *D. viviparus*, *N. americanus*, *O. dentatum*, *O. ostertagi*, *T. circumcincta* and *T. colubriformis* were mined for potential GluCl sequences. Several contigs were identified as ligand gated channels (11–36 contigs), including GluCls (1–9 contigs). Based on the available parasitic GluCl data, including the cyathostomin sequences, contigs were identified as corresponding to several GluCl α subunits (data not shown).

4. Discussion

Presented here is, to our knowledge, the first high throughput transcriptome dataset for an equine nematode parasite. Over 26,000 contigs were generated from RNA extracted from 17 *C. goldi* worms. The number of worms used was kept to a minimum to avoid complications of intra-specific sequence heterogeneity, as reported in previous studies (Blouin et al., 1995). Prior to sequencing this transcriptome, very little cyathostomin sequence data was publically available, with only the genes involved in anthelmintic mode of action/resistance being extensively studied (Drogemuller et al., 2004; Tandon et al., 2006; Hodgkinson et al., 2008). Of the cyathostomin genes characterised previously, only two gene families were not identified here: the *Pgp* genes, consistent with other clade V transcriptome analyses, particularly *T. circumcincta* (Dicker et al., 2011a); and the immunodiagnostic antigen, gut-associated larval antigen (*Cy-GALA)*, whose absence in this adult stage dataset confirms it is primarily transcribed in mucosal larval stages (McWilliam et al., 2010). It is interesting that *Pgp* transcripts were not identified here and it may be that *Pgp*s are expressed at higher levels within other life cycle stages. The *T. circumcincta* study highlighted developmental transcriptional differences for *Pgp-9* mRNA amongst life cycle stages (Dicker et al., 2011b). A lower level of transcription within the adult stage, as well as insufficient coverage may be responsible for the lack of *Pgp* sequences identified here.

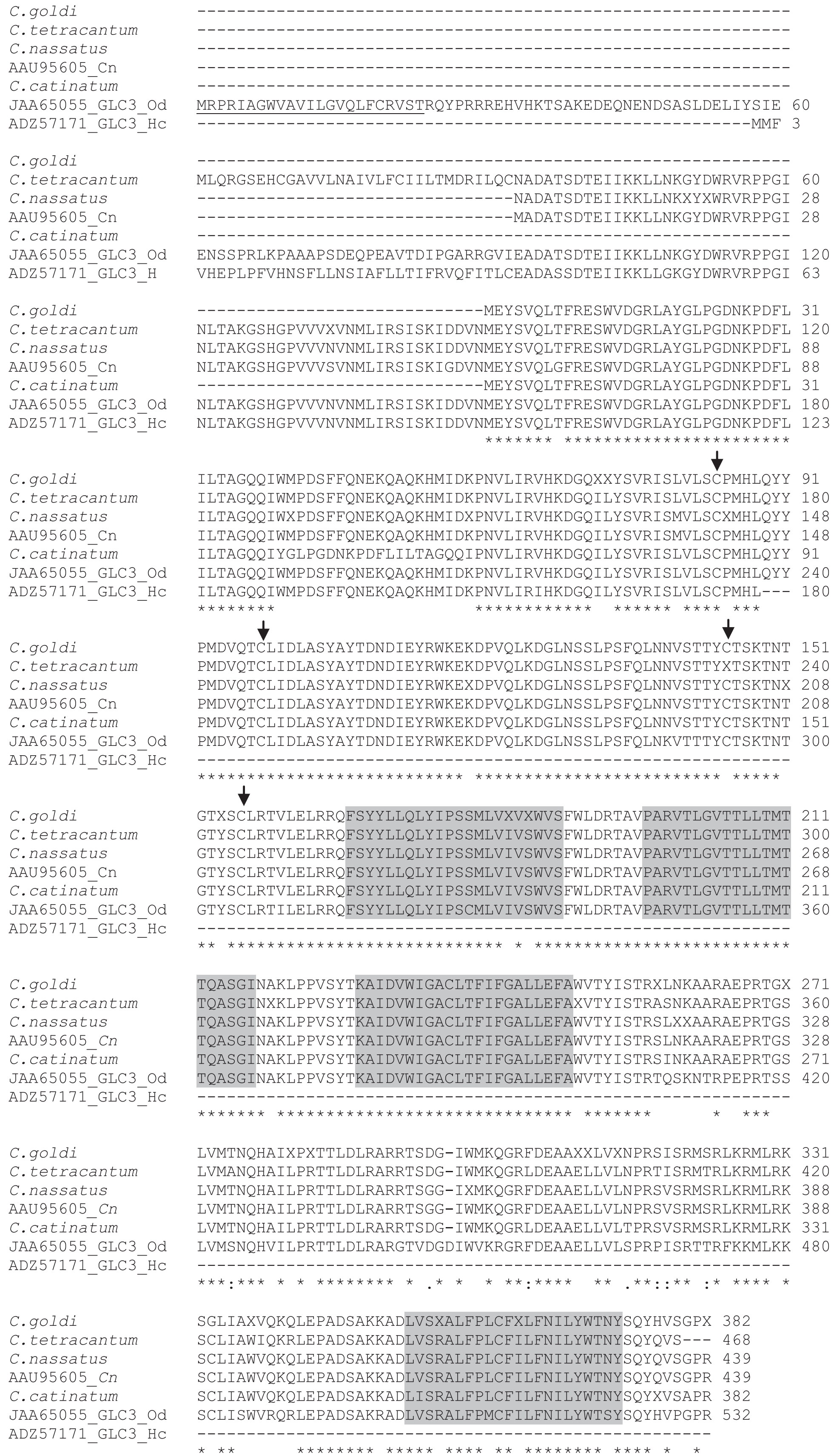

Fig. 3. GLC-3 amino acid alignment by ClustalW2. Consensus sequences from four cyathostomin species were compared with the glutamate gated chloride channel (GluCl) GLC-3 sequence from *Oesophagostomum dentatum* (**JAA65055**) and *Haemonchus contortus* (**ADZ57171**). **AAU95605** represents the *Cylicocyclus nassatus* GluCl α4 sequence (Tandon et al., 2006). Only one species, *O. dentatum*, was predicted to have an N-terminal signal sequence (underlined: SignalP). The arrows represent the conserved cysteine residues and the four transmembrane domains are highlighted in grey.

Based on general GO and KEGG database analysis, only 27% of the 26,928 putative protein sequences could be annotated. Due to the small proportion of nematode-specific sequences within the GO and KEGG databases, most of which relate to *C. elegans* and *Brugia malayi*, our results were not unexpected and are similar to other nematode studies (Cantacessi et al., 2010). Further analysis was carried out focussing on nematode-specific datasets, specifically the clade V nematodes (Blaxter et al., 1998), using the sequences in NEMBASE4 and the next generation transcriptomes currently publicly available for these nematodes (Martin et al., 2012). Comparing next generation transcriptomes of other clade V nematodes with that of *C. goldi* showed that three species in particular showed higher identity: *O. dentatum, C. elegans* and *D. viviparus*. The high sequence identity of the *C. elegans* dataset is most likely due to the *C. elegans* dataset being comprised of a greater number of contig sequences (44,803), reflecting the extensive sequence data available for this model nematode compared with other parasitic nematodes (Table 3). As this is the first published cyathostomin transcriptome, comparisons against other cyathostomin genera could not be made. Outwith the cyathostomin genera (Lichtenfels et al., 1998), the result that *O. dentatum* showed the greatest sequence identity to the cyathostomin transcriptome is consistent with taxonomical classifications of these nematode species, with cyathostomin species and *Oesophagostomum* spp. belonging to the Strongyloidea superfamily (Blaxter et al., 1998; Holterman et al., 2006; Kumar et al., 2012). The number of annotated proteins increased when all of the clade V transcriptome datasets were compared together against the *C. goldi* predicted proteins (51.5%). The percentage of annotation was further increased by comparing the *C. goldi* dataset against the available phylum Nematoda genomes. As would have been expected the nematodes from clade V showed the greatest level of homology to the *C. goldi* dataset, compared with the lower levels identified for the clade I nematode, *T. spiralis* and clade III nematode, *A. suum*. It is likely that a large proportion of the genes identified across all of the datasets analysed are involved in core processes rather than species-specific functions. Consequently, these are important genes to study when considering the design of future anthelmintics that can be used for many parasitic nematode species.

Using the available annotation tools, only approximately 50% of the cyathostomin transcriptome has been annotated. Several expected target genes, such as the *Pgp* transcripts, are also absent. The low proportion of annotated proteins derived from the cyathostomin transcriptome sequences is likely due to technical reasons, resulting from limitations in assembly and the subsequent generation of partial ORFs. A breakdown of the 26,910 contigs based on nucleotide bp length indicated that approximately 50% of this transcriptome dataset is comprised of short nucleotide reads. The normalisation protocol used on the cDNA library used for sequencing led to a 3′ end bias, which was evident from the low proportion of signal peptides and therefore potentially full-length proteins being identified. Using the protein prediction tool, prot4EST, all of the contigs were conceptually translated using BLAST, ESTScan and Longest ORF, with the majority of the shorter proteins being derived from contigs using Longest ORF. Despite this, the large number of small contig sequences, together with the 3′ end bias, could have affected the algorithms used for protein prediction and subsequent annotation. Comparisons of contig sequence length versus sequence annotation showed that, as would be expected, the longer the peptide sequence, the higher the probability of annotation (Supplementary Table S3). As more genome datasets become available for nematodes, especially those more closely related to the cyathostomins, a higher proportion of gene homologues/orthologues are likely to be identified, improving the annotation statistics of this *C. goldi* transcriptome dataset.

The mechanisms of resistance are not fully understood for the majority of anthelmintics (Kohler, 2001; Gilleard, 2006). Differences in the speed at which different species or genera develop resistance indicate that different selection pressures may apply or that underlying genetic mechanisms may vary (Sutherland and Leathwick, 2011). Therefore responses to anthelmintics may be species-specific and the development of resistance may involve various combinations of genes (Gilleard and Beech, 2007). In the face of such complexity, transcriptome and genome datasets offer a more comprehensive approach to studying resistance mechanisms with the potential to reveal a greater number of putative anthelmintic targets and/or species-specific differences. This is illustrated here for the cyathostomins by a series of findings highlighting the utility of the transcriptome dataset for the identification of existing and new drug targets.

Multiple *H. contortus*-specific GluCl α subunits have been identified (Table 4; Yates et al., 2003; Glendinning et al., 2011), however despite a candidate gene approach and mining of the *C. goldi* transcriptome, only one GluCl α subunit, α4 (*glc-3*), could be isolated for cyathostomins. This may indicate that the other subunits are transcribed at a lower level, transcription may be developmentally regulated as with *Hco_glc-2* and *Hco_avr-14* (Delany et al., 1998; Jagannathan et al., 1999) or the GluCl α4 subunit may be the dominant subunit in cyathostomins. Alternatively, this may be a feature of the partial nature and limitations of assembly of the transcriptome dataset. Further analysis is required to fully determine the number of GluCl subunits present for cyathostomins, however targeted resequencing of the GluCl α4 (*glc-3*) sequence for the four different species of cyathostomin (*C. catinatum*, *C. tetracanthum*, *C. nassatus* and *C. goldi)* showed that there is up to 12% inter-specific variation for this GluCl, with a reported 4% intra-specific variation for the *C. nassatus* sequences when comparing our protein sequence with that of Tandon et al. (2006). Very few GluCl sequences have been identified for other parasitic nematodes. Using the GluCl sequences identified in this study for the cyathostomins, the clade V next generation transcriptomes were mined for GluCl sequences. Analysis confirmed subunit types for *O. dentatum* and identified novel sequences in *C. oncophora*, *D. viviparus*, *O. ostertagi* and *T. colubriformis*. Despite the fact that these datasets may not be complete, several different profiles of GluCl sequences were identified for the different clade V nematodes (data not shown). This analysis has shown that although there are taxonomical similarities between clade V parasitic species, there is also variation in *GluCl* transcription across the clade, which may impact anthelmintic resistance in these different nematode species.

Recently analysis by Gilleard and colleagues (Gilleard, 2006; Saunders et al., 2013) of the *H. contortus* genome revealed two additional β tubulin isotypes expressed at extremely low levels relative to the isotypes 1 and 2, already known to be associated with BZ resistance (Kwa et al., 1993, 1995; Silvestre and Cabaret, 2002; Hodgkinson et al., 2008). Given this low level of expression, the lack of evidence for these additional isotypes in the transcriptome of adult cyathostomins does not exclude their presence within the cyathostomin genome and may indicate that BZ resistance involves a greater cohort of β tubulin isotypes. This study has identified sequences corresponding to the nAChR (*unc-29*, *unc-38* and *lev-1*) in cyathostomins, facilitating the investigation of the mode of TETR/IMID resistance. In *A. caninum*, low levels of transcription of three *nAChR* subunits are thought to be responsible for the resistant phenotype (Kopp et al., 2009), whilst increased expression of a truncated nAChR subunit, UNC-63, was observed in three trichostrongylid nematode species (Neveu et al., 2010). Analysis at the single channel level in *O. dentatum* revealed that resistant isolates have fewer active receptors than susceptible isolates and

Table 4
Available glutamate gated chloride channel sequence data for clade V nematodes.

Caenorhabditis elegans	*Haemonchus contortus*	*Cooperia oncophora*	*Cylicocyclus nassatus*	*Oesophagostomum dentatum*	*Ostertagia ostertagi*	*Teladorsagia circumcincta*
avr-14	avr-14	avr-14		avr-14	avr-14	avr-14
avr-15				avr-15		
glc-1						
glc-2	glc-2	glc-2	glc-2	glc-2		
glc-3	glc-3		glc-3	glc-3		
glc-4	glc-4			glc-4		
	glc-5					
	glc-6					

References: Cully et al. (1994), Etter et al. (1996), Delany et al. (1998), Jagannathan et al. (1999), Yates et al. (2003), Liu et al. (2004), Njue and Prichard, (2004), Njue et al. (2004), Cook et al. (2006), Tandon et al. (2006), El-Abdellati et al. (2011), Glendinning et al. (2011), Williamson et al. (2011), Martínez-Valladares et al. (2012a,b) and Wolstenholme (2012).

that one of the nAChR subtypes was missing in the resistant isolate (Robertson et al., 1999; Kohler, 2001).

Use of the transcriptome for identification of drug targets is not exclusive to the anthelmintics historically used for cyathostomin control but extends to the newer drugs. The cyclodepsipeptides have been shown to be active against a variety of parasitic nematodes, including cyathostomins (von Samson-Himmelstjerna et al., 2000). They are thought to act on calcium activated potassium channels, in particular SLO-1 and adhesion G-protein coupled receptors such as the latrophilins, LAT-1 and LAT-2 (Saeger et al., 2001; Krucken et al., 2012). No sequences comparable with the latrophilins were identified here, however this does not mean they are not present within the cyathostomin genome for reasons previously discussed. Investigations by Kaminsky et al. (2008) into the mode of action of AAD have shown that monepantel also acts on a distinct group of nAChRs, the DEG-3 group. Analyses of AAD-resistant *C. elegans* and *H. contortus* mutants indicate that ACR-23 and DEG-3 type (DES-2) nAChR are involved (Kaminsky et al., 2008). A comparative study amongst *C. elegans*, *B. malayi* and *T. spiralis*, from clade V, III and I, respectively, showed that *des-2* is conserved across all three clades (Williamson et al., 2007). Mining of the *C. goldi* transcriptome for potential gene targets of these two classes demonstrated the presence of a short contig (contig01298; data not shown) displaying sequence similarity (95% across 42 amino acids) to DES-2, indicating potential utility for AAD anthelmintics against cyathostomins in future.

Presented here is, to our knowledge, the first published large-scale transcriptome analysis of *C. goldi*, a common cyathostomin species, a major pathogen of horses. The cyathostomins are a complex group of parasites, comprised of several species, which can make molecular analyses difficult. High levels of genetic variation exist in helminth parasites and knowledge of the population genetic structure of cyathostomins is important to understand evolutionary processes such as adaptation to the host and drug resistance. Identifying the genes associated with anthelmintic resistance is the first step to developing markers to monitor resistance genes, which is important for the anthelmintics currently available and for the few new anthelmintics in development. To date the molecular genetic analysis of this complex group of parasites has been restricted to specific loci in non-genic regions of DNA (Kaye et al., 1998; McDonnell et al., 2000; Cwiklinski et al., 2012). The availability of this transcriptome dataset forms the basis for analysis of the populations of cyathostomins and the complexity of infections, by exploring variation in gene sequences at multiple loci. Until further cyathostomin transcriptomes and annotated genomes are available, the *C. goldi* transcriptome will provide the primary source of sequence data for investigation of these parasites at the molecular level.

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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.2013.06.010.

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