Molecular characterisation of two novel 17β-hydroxysteroid dehydrogenase genes from the soybean cyst nematode *Heterodera glycines*

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Summary – *Heterodera glycines Hg-hsd-1* and *Hg-hsd-2* are two homologues of *let-767*, a short-chain dehydrogenase gene from the free-living nematode *Caenorhabditis elegans*. Both genes share similarity to 17β-hydroxysteroid dehydrogenases (17β-HSDs), enzymes known to be involved in the production of steroid hormones in mammals. *Hg-hsd-1* and *Hg-hsd-2* encode distinct protein sequences that share 51% amino acid identity with each other and 48% identity with LET-767. The Hg-HSD-1 protein sequence was most similar to those encoded by ESTs from the lesion nematode, *Pratylenchus penetrans*, and Hg-HSD-2 protein was most similar to sequences from another cyst nematode, *Globodera pallida*. Quantitative real-time PCR revealed the highest expression of both genes in developing females, with relatively low levels in second-stage juveniles, eggs, or males. Transcription levels of both genes declined as females aged, decreasing with increased cuticle pigmentation. Transcription was up-regulated in the cyst bodies and fluid that had been manually separated from the developing embryos, suggesting that *Hg-hsd-1* and *Hg-hsd-2* are most likely involved in gonad development or oogenesis in the female. It is possible that these 17β-hydroxysteroid dehydrogenases are involved in the conversion of sterols, which nematodes must ingest from dietary sources, into necessary steroid hormones.

Keywords – cholesterol, dehydrogenase, embryogenesis, hormone moulting, plant-parasitic nematode, soybean, sterol.

Soybean cyst nematode (SCN) *Heterodera glycines* is a major pathogen of soybean, causing over $700 million in damage annually in the United States (Wrather, 2003). Host resistance is the primary method currently employed to control SCN. However, the existence of an ever changing nematode population dictates that new sources of resistance be developed in order to strengthen the genetic arsenal necessary to minimise damage levels.

The development of controls aimed specifically at disrupting critical nematode target genes is an attractive approach that has been the subject of numerous studies (Urwin *et al*., 2002; Bakhetia *et al*., 2005; Chen *et al*., 2005; Fanelli *et al*., 2005; Rosso *et al*., 2005). While SCN genes encoding proteins secreted from the pharyngeal glands during parasitism have received the most attention (Bekal *et al*., 2003; Gao *et al*., 2003; Davis *et al*., 2004; Vanholme *et al*., 2004), with a few exceptions (Llado *et*

al., 1998; Masler *et al*., 1999; Lilly *et al*., 2005) much of the reproductive biology of the nematode remains unexplored.

Cholesterol is an important precursor of steroid hormones in many organisms and in most insects is converted to 20-hydroxyecdysone, the hormone necessary for moulting and other developmental events (Riddiford, 1993). Nematodes cannot produce cholesterol *de novo* and thus must obtain it or related sterols from dietary sources (Chitwood, 1999; Kurzchalia & Ward, 2003). Although ecdysteroids have been identified in several nematode species, experiments with four species of nematodes in three different laboratories have failed successfully to demonstrate conversion of radiolabelled sterols to ecdysone (reviewed in Chitwood, 1999). A few studies have shown that exogenously applied ecdysteroids can affect developmental or reproductive responses (Barker *et*

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al., 1991; Goudey-Perriere *et al.*, 1992; Soriano *et al.*, 2004) but, as yet, no definitive link between an endogenous ecdysteroid and nematode moulting or development has been demonstrated. Nevertheless, steroid metabolism remains an attractive target for the biorational development of novel nematode controls.

In the free-living nematode *Caenorhabditis elegans*, the gene *let-767* (Kuervers *et al.*, 2003) encodes a protein that is most similar to mammalian steroid enzymes that reduce 17β-hydroxysteroid hormones (Geissler *et al.*, 1994). The null phenotype of *let-767* was shown to be early larval arrest. Non-lethal mutation of the gene results in hypersensitivity to cholesterol limitation and mutants exhibit defects in embryogenesis, female reproductive development and moulting. Because disruption of analogous functions in plant-parasitic nematodes could potentially reduce the damage they cause, our study was undertaken to identify homologues of *let-767* from the soybean cyst nematode and to investigate their expression during nematode development.

Materials and methods

NEMATODE CULTURE AND COLLECTION

Heterodera glycines was cultured on susceptible soybean (*Glycine max* cv. Essex) in pot culture using the moisture wicking system described by Sardanelli and Kenworthy (1997). Cultures were grown at 27°C with exposure to light for 18 h per day. Cysts were collected by washing infected roots into a 20 μm sieve nested over a 60 μm sieve. Second-stage juveniles (J2) were Baermann-extracted from crushed cysts using standard methods. Adult males were isolated by soaking soybean roots that were 12-14 days post inoculation in a beaker of water aerated continuously with an aquarium pump. After 48 h, males were collected from the water by Baermann extraction.

Cysts of different developmental ages were separated by size and colour, and egg masses were removed from cysts under a dissecting microscope. Broken cysts were rinsed with 500 μl tap water in a 500 μm sieve and the filtrate was collected. Cysts and eggs were rinsed in the sieve ten times with 30 ml of tap water. Empty cysts were collected by hand under a dissecting microscope, and the eggs were rinsed into a centrifuge tube. Eggs and cysts were washed separately an additional ten times with 15 ml of tap water to remove any residual cyst fluid. A sample of each cyst fraction was viewed under the dissecting microscope to ensure that eggs and debris were removed.

BIOINFORMATIC ANALYSIS

The amino acid sequence from *Caenorhabditis elegans* gene *let-767* (C56G2.6) was used to search the nematode EST database at EMBL-EBI (http://www.ebi.ac.uk/blast2/parasites.html) for sequences with high similarity in *Heterodera glycines* and other plant-parasitic nematodes. Assembly of the ESTs into contigs (listed in Table 1) was performed using Sequencher 4.5 (Genecodes Corp., Ann Arbor, MI, USA).

PCR AND SEQUENCING AND ANALYSIS

The cDNA sequence was obtained by PCR amplification from a *H. glycines* J2 cDNA library constructed in bacteriophage λ Uni-ZAP XR (Stratagene, La Jolla, CA, USA) that was kindly provided by Eric Davis, North Carolina State University. Bulk phagemid DNA that had been excised from the vector according to the manufacturer's instructions served as template. PCR reactions contained the primers HSD1-1for and HSD1-1rev for *Hg-hsd-1*, or HSD2-1 for and HSD2-1rev for *Hg-hsd-2* (Table 2). Reactions also contained 200 ng template, 250 μM each primer, 0.2 mM dNTPs, 1 unit Eppendorf HotMaster Taq (Brinkmann, Westbury, NY, USA), and manufacturer supplied buffers in 25 μl total volume. Touchdown PCR was performed in an iCycler (BioRad, Hercules, CA, USA) with an initial denaturation step of 95°C for 2 min, followed by 40 cycles of 95°C denaturation for 30 s, 64°C annealing for 30 s, with a decrease of 0.5°C every four cycles, and extension at 72°C for 30 s, and ending with 72°C for 3 min.

Genomic sequences were amplified from *H. glycines* genomic DNA that was prepared from 100 cysts. Cysts were crushed by grinding in liquid nitrogen and prepared with the DNeasy Genomic DNA Kit (Qiagen, Valencia, CA, USA). PCR reactions were assembled as above except 200 ng genomic DNA was included as template. PCR products were separated on 1% agarose in Tris-Acetate-EDTA (TAE), visualised by ethidium bromide staining and UV illumination. PCR products were excised from agarose gels and purified with the QIAquick Gel Extraction Kit (Qiagen). Excised and cleaned PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Plasmid DNA was prepared with the Qiaprep Spin Miniprep Kit (Qiagen) and digested with the restriction enzyme

Table 1. *Putative 17β-hydroxysteroid dehydrogenase genes from nematodes.*

Species	Gene[*]	source	Sequence Type	Sequence ID(s)
Caenorhabditis briggsae		mixed	genomic	CAE72516.1
		mixed	genomic	CAE66202.1
		mixed	genomic	CAE66201.1
Caenorhabditis elegans	*let-767*	mixed	genomic	C56G2.6
	stdh-1	mixed	genomic	C06B3.4
		mixed	genomic	C06B3.5
	stdh-2	mixed	genomic	F11A5.12
	tag-57	mixed	genomic	F25G6.5
Globodera pallida		females	Contig of ESTs	CV577063, CV577904
Heterodera glycines	*Hg-hsd-1*	females	genomic & cDNA	DQ328678
		females	Contig of ESTs	CD48415.1, CB280353.1, CD749061.1, CD749066.1, CB281474.1, CB378273.1, CB824315.1, CB378643.1, CB281598.1, BF013622.1, CB377791.1
	Hg-hsd-2	females	genomic & cDNA	DQ328679
		females	Contig of ESTs	CB825202.1, CB377939.1, CB826240.1, CB375525.1, CB377990.1
Meloidogyne chitwoodi		females	Contig of ESTs	CD682747.1, CF801361.1
Meloidogyne hapla (contig a)		females	Contig of ESTs	CF803689.1, CF803678.1, CF803679.1
Meloidogyne hapla (contig b)		females	Contig of ESTs	CN576709.1, CN576712.1
Meloidogyne hapla (contig c)		egg	Contig of ESTs	BM952123.1, CN575565.1
Meloidogyne incognita (contig a)		females	Contig of ESTs	CF980817.1, CF980444.1, CF980487.1, CK983672.1
Meloidogyne incognita (contig b)		J3/J4	Contig of ESTs	CK984607.1, CK984571.1, CN443018.1, CK984580.1, CK084584.1
Meloidogyne javanica		egg	Contig of ESTs	BG736305, 1.BI745534.1
Pratylenchus penetrans		mixed	Contig of ESTs	BQ626484.1, BQ580421.1
Xiphinema index		mixed	EST	CV5 10544.1
Zeldia punctata		mixed	Contig of ESTs	AW773406.1, AW773512.1, AW773537.1, AW773382.1, AW773383.1

[*] Name given only if known.

*Eco*RI (New England BioLabs, Ipswich, MA, USA) to verify the presence of insert.

Sequencing was performed with plasmid clones and M13F or M13rev primers using the CEQ DTCS Quick Start Kit and analysed with a CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Sequence-specific HSD primers used for sequencing of plasmid inserts or PCR products are shown in Table 2. Additional sequencing was performed at the University of Maryland Center for Biosystems Research. Sequence information was assembled as described above. Homology of predicted proteins was analysed with a tBLASTn search of the nonredundant database at NCBI (Altschul *et al.*, 1997).

Alignments were performed using ClustalW with default options (Thompson *et al.*, 1994). Further processing of alignments was performed with Genedoc (Nicholas

et al., 1997). Sequences have been submitted to GenBank with Accession numbers (DQ328678 for *Hg-hsd-1* and DQ328679 for *Hg-hsd-2*). Neighbour-Joining (NJ) analysis to determine phylogenetic relationships among sequences was performed with PAUP v. 4.0b10 (Swofford, 1998). An NJ phylogram was constructed for a 148 amino acid alignment of nematode HSD sequences, with *Drosophila melanogaster* CG1444-PA (GI:24640442) as the outgroup and bootstrap values based on 1000 replicates.

PRIMER DESIGN AND OPTIMISATION

Primers for real-time PCR were designed for *Hg-hsd-1* or *Hg-hsd-2* sequences using NetPrimer (Premier Biosoft, http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) (Table 2) and synthesised by Sigma-Genosys (St Louis, MO, USA). Primers were selected

Table 2. *Primers used in this study.*

Primer	5′ to 3′ Sequence	Purpose
HSD1-1for	ATGTCTTTTCTTGTCACATTAG	cloning, sequencing
HSD1-1rev	TTCACTCTTCGCCACCGTC	cloning, sequencing
HSD1-for333	GCATCTCATTCGACTTCACCAACGC	sequencing
HSD1-RTfor	ACCAACGCAAATCTGAAGGA	real time PCR
HSD1-RTrev	TCGTAGCTCATCCCGACATT	real time PCR
HSD2-1for	ATGACCTATTATAATGATTCGAATAG	cloning, sequencing
HSD2-1rev	GTCGTGCTGTTTGGATGC	cloning
HSD2-rev673	GTAGAGGAATAGACGGCGTAGTGG	sequencing
HSD2-rev750	TTGGACACACCGTTTGGATG	sequencing
HSD2-RTfor	GCGGACTACGAACGACACAT	real time PCR
HSD2-RTrev	CAAATCGTTCCGGGTAATCA	real time PCR
HgActin-for64	CAATCCCAACCAGAAAAAATGTGC	real time PCR, actin
HgActin-rev667	GCCCACAATGGACGGAAACAC	real time PCR, actin

to span an intron and give rise to products between 80 and 150 bp long. The primers were also selected to have no more than two GC pairs in the last five bases of the 3′ end and to be devoid of secondary structure or primer dimer affinity. Primers HgActin-for64 and HgActin-rev667 (Table 2) were designed from the *H. glycines* actin (AY161282) that was used as the reference gene. Gradient PCR was used to determine the optimum annealing temperature (50°C) for *Hg-actin*, *Hg-hsd-1*, and *Hg-hsd-2*. Standard curves were constructed for five-fold serial dilutions of template, plotting the relative starting amount *vs* threshold cycles with the iCycler software iQ v. 3.1 (BioRad). The correlation coefficient (R) and PCR efficiency (E) were found to be satisfactory for all three primer sets: for Actin, R = 0.996 and E = 103%; for *Hg-hsd-1*, R = 0.895 and E = 80.7%; for *Hg-hsd-2*, R = 0.954 and E = 92.4%.

SYBR GREEN I QUANTITATIVE REAL-TIME PCR

Nematodes were prepared in a solution of RLT buffer (supplied with the RNeasy Micro Kit; Qiagen) supplemented with β-mercaptoethanol and stored at -80°C overnight or until needed. Nematodes were mechanically homogenised in groups of approximately 50 by pulverising in liquid nitrogen. Total RNA was extracted from the various stages using the RNeasy Micro Kit according to the manufacturer's instructions, and treated with DNase I to ensure complete removal of genomic DNA. cDNA was synthesised with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocols, with 1 μl of extracted RNA as template.

Real time PCR reactions consisted of a 25 μl reaction containing a 1 μl aliquot of synthesised first-strand cDNA, 0.4 μM of each gene-specific primer, and 12.5 μl BioRad 1× iQ SYBR Green I Supermix. Each experiment consisted of three replicates per sample/primer pair. No-template controls as well as no-RNA reactions (mock reverse-transcriptions) were included in each run. Real time PCR reactions were performed in 96-well iQ iCycler plates for 4 min at 95°C, followed by 40 cycles of 95°C for 10 s, 59°C for 30 s, and 72°C for 30 s. To ensure that unique products were formed in each sample well, runs ended with a melting curve. The melting curve was determined from data collected during a two-step cycle of 95°C for 1 min and 55°C for 1 min, followed by 80 cycles where the temperature was increased an additional 0.5°C per cycle. Additionally, products were visualised on a 1.5% agarose TAE gel to verify the presence of single bands, as indicated by SYBR Green I dissociation curves. Relative abundance of the hydroxysteroid dehydrogenase transcripts and actin was determined for samples from the different nematode stages using the BioRad iQ software v. 3.1 and Gene Expression Macro v. 1.1, which employ the algorithms described in Vandesompele *et al.* (2002). R-values for each gene were included in calculations of relative expression to account for differences in efficiency for each primer set. Post-run statistical analyses were performed with InStat 3.0 (GraphPad Software, Inc., San Diego, CA, USA). The level of gene expression in each stage or fraction was log_{10} transformed to meet the assumption of equal variance between sample groups and compared by one-way ANOVA, with the mean difference

significant at $P < 0.05$. Multiple comparisons were performed with the Tukey-Kramer Multiple Comparisons Test.

Results

In order to identify homologues from *H. glycines* and other plant-parasitic nematodes, the *C. elegans let-767* amino acid sequence was compared to the nematode EST database (http://www.ebi.ac.uk/blast2/parasites.html). Several EST sequences from *H. glycines* (Table 1) were assembled into two contigs, each comprising an apparent full-length cDNA sequence. To confirm the sequence of the contigs, primers corresponding to the coding region of each gene (Table 2) were used to amplify the two sequences from an excised phagemid library containing J2 cDNA inserts. DNA sequencing confirmed that each amplified insert sequence was identical to the corresponding assembled EST contig. Using the same sets of PCR primers, the genes were also amplified from SCN genomic DNA. Both genes were comprised of six exons and five introns, although the size and position of the introns differed for each (Fig. 1A). The deduced amino acid sequences were most similar to Type 3 17β-hydroxysteroid dehydrogenases, containing the conserved motif TGxxxGxG needed for cofactor binding, and the active site residues YxxS/TK (Mindnich *et al.*, 2004). Based on this similarity, the genes were named *Hg-hsd-1* and *Hg-hsd-2*, for 17β-hydroxysteroid dehydrogenase. *Hg-hsd-1* is a gene of 1672 bp. The open reading frame (ORF) of 954 bp encodes a protein sequence of 317 amino acids, with putative molecular weight of 35.49 kDa. *Hg-hsd-2* is a gene of 2298 bp with a coding region of 1020 bp. This ORF encodes a protein sequence of 339 amino acids with a calculated molecular weight of 37.46 kDa. Protein sequences Hg-HSD-1 and Hg-HSD-2 shared 51% amino acid identity and 69% similarity with each other (Fig. 1B). Comparison to *C. elegans* LET-767 protein revealed that Hg-HSD-1 was 48% identical and 69% similar, and Hg-HSD-2 was 40% identical and 64% similar. Similarity to other *Caenorhabditis* sequences ranged from 40 to 48% for Hg-HSD-1 and from 36 to 40% for Hg-HSD-2.

Comparison of Hg-HSD-1 and Hg-HSD-2 with publicly available EST and genomic DNA sequences from other nematodes (Fig. 2) reflects the expected nematode phylogeny. Hg-HSD-1 was most similar to the deduced amino acid sequence from a contig comprised of two ESTs from mixed-stage *Pratylenchus penetrans*, and Hg-HSD-2 was most similar to the deduced amino acid sequence from a contig of two ESTs from *Globodera pallida* females. Clades containing these sequences appeared as sister groups to a clade comprised entirely of root-knot nematode sequences, and to a clade containing sequences from free-living nematodes, including *C. elegans* LET-767. An amino acid alignment trimmed to compare overlapping regions of partial nematode sequences reveals several residues that are identical or highly conserved in all nematodes (Fig. 3), including part of the dehydrogenase active site signature.

Among known full-length non-nematode sequences, Hg-HSD-1 appeared most closely related to the sequence XP643832.1 from the slime mold, *Dictyostelium discoideum* (43% identical), CG1444-PA from the fruit fly, *Drosophila melanogaster* (41% identical), and BAD-08526.1 from sea squirt, *Ciona intestinalis* (38% identical). For Hg-HSD-2, identities were 37% to *Drosophila*, 36% to slime mould, and 35% to sea squirt. Lower similarity was found in comparison to known 17β-hydroxysteroid dehydrogenases from mammals, including human (unpubl.).

Quantitative real-time PCR was used to compare expression of *Hg-hsd-1* and *Hg-hsd-2* in eggs, juveniles, males, and cysts (females) (Fig. 4). For both genes, the highest level of expression was observed in yellow cysts collected 32 days post-infection (dpi), with low levels in eggs, J2, brown cysts (collected 38 dpi), and males. This initial profile revealed 59-fold higher expression of *Hg-hsd-1* in yellow cysts than in J2 ($P < 0.001$). Mean expression levels for *Hg-hsd-1* were not significantly different among eggs, J2, males, or brown cysts ($P > 0.05$). For *Hg-hsd-2*, mean expression levels were vastly upregulated in yellow cysts compared to J2 (21-fold higher; $P < 0.001$), and there were significant differences among the other stages, except that brown cysts had similar expression to eggs.

To obtain a more detailed profile of transcription in cysts, *Hg-hsd-1* and *Hg-hsd-2* expression was measured in females that were manually separated on the basis of size and cuticle pigmentation. Both genes showed peak expression levels in large white cysts. A dramatic spike in expression of *Hg-hsd-1* was observed in two sizes of young white female nematodes, reaching a maximum of *ca* 600 copies in large white cysts and decreasing as the cysts became darker in colour (Fig. 5A). For *Hg-hsd-2*, transcript levels also peaked in large white cysts and decreased with age, but the maximum copy number in large white cysts (*ca* 156) was not nearly as high as for *Hg-hsd-1* (Fig. 5B).
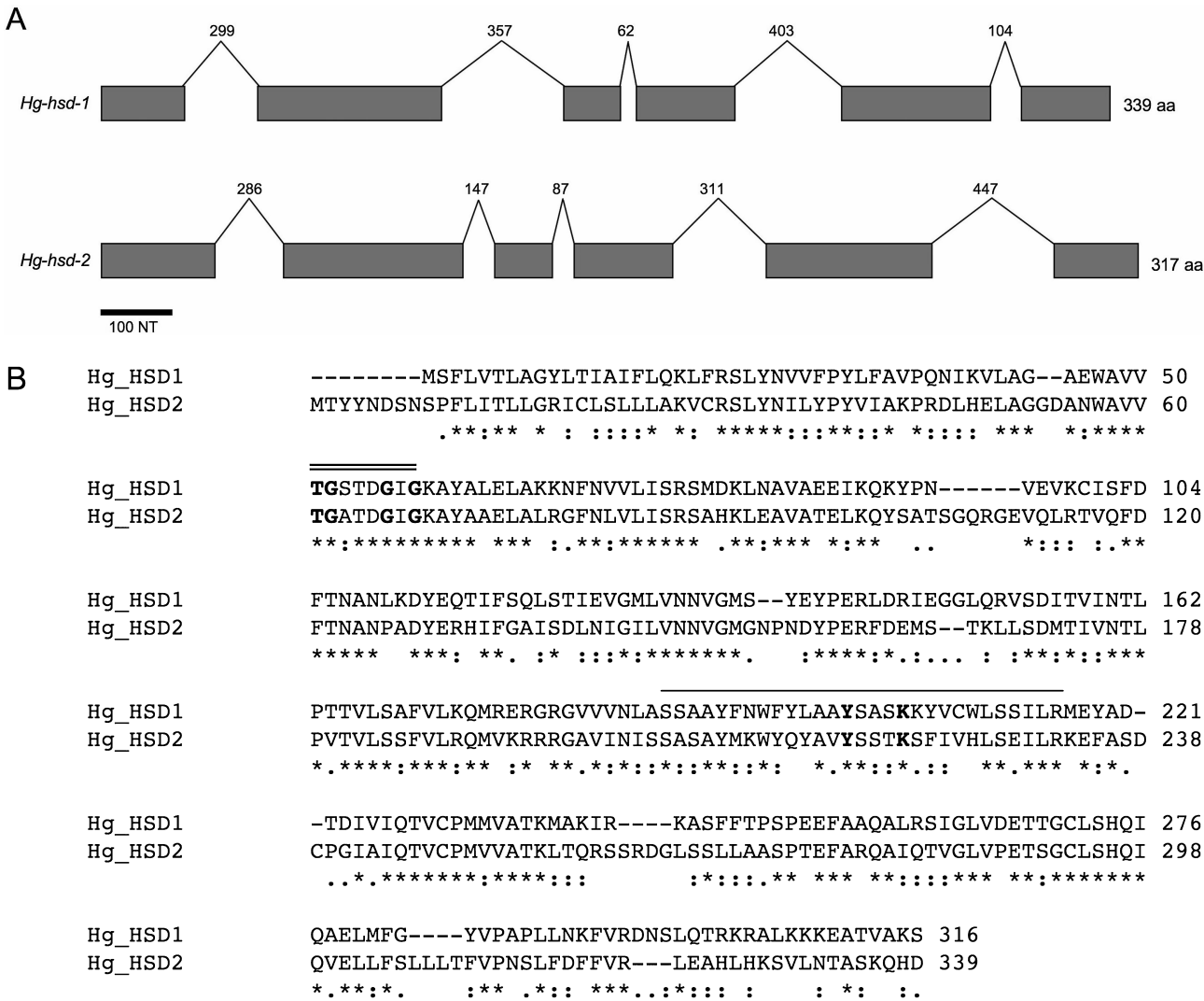
A

B

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Hg_HSD1    --------MSFLVTLAGYLTIAIFLQKLFRSLYNVVFPYLFAVPQNIKVLAG--AEWAVV 50
Hg_HSD2    MTYYNDSNSPFLITLLGRICLSLLLAKVCRSLYNILYPYVIAKPRDLHELAGGDANWAVV 60
                    .**:** * : :::.* *: *****::.**:* *:::: ***  *:****

Hg_HSD1    TGSTDGIGKAYALELAKKNFNVVLISRSMDKLNAVAEEIKQKYPN------VEVKCISFD 104
Hg_HSD2    TGATDGIGKAYAAELALRGFNLVLISRSAHKLEAVATELKQYSATSGQRGEVQLRTVQFD 120
           **:********** *** :.**:****** .**:*** *:**   ..     *::: :.**

Hg_HSD1    FTNANLKDYEQTIFSQLSTIEVGMLVNNVGMS--YEYPERLDRIEGGLQRVSDITVINTL 162
Hg_HSD2    FTNANPADYERHIFGAISDLNIGILVNNVGMGNPNDYPERFDEMS--TKLLSDMTIVNTL 178
           *****   ***: **. :* ::;*:*******.   :****:*.:... : :**:*::***

Hg_HSD1    PTTVLSAFVLKQMRERGRGVVVNLASSAAYFNWFYLAAYSASKKYVCWLSSILRMEYAD- 221
Hg_HSD2    PVTVLSSFVLRQMVKRRRGAVINISSASAYMKWYQYAVYSSTKSFIVHLSEILRKEFASD 238
           *.****:***:** :* **.*:*::*::**::*:  *.**::*.::  **.*** *:*.

Hg_HSD1    -TDIVIQTVCPMMVATKMAKIR----KASFFTPSPEEFAAQALRSIGLVDETTGCLSHQI 276
Hg_HSD2    CPGIAIQTVCPMVVATKLTQRSSRDGLSSLLAASPTEFARQAIQTVGLVPETSGCLSHQI 298
            ..*.*******:****::: :       :*:::.** *** **:::*** **:*******

Hg_HSD1    QAELMFG----YVPAPLLNKFVRDNSLQTRKRALKKKEATVAKS 316
Hg_HSD2    QVELLFSLLLTFVPNSLFDFFVR---LEAHLHKSVLNTASKQHD 339
           *.**:*.    :** .*:: ***..:*::: :    : *: :.
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Fig. 1. *A: Schematic diagram of exons and introns for* Hg-hsd-1 *and* Hg-hsd-2*. Exons are shown as grey boxes and introns are shown as inverted v-shaped lines, with size of introns in base pairs indicated above; B: Alignment of deduced amino acid sequences Hg-HSD-1 and Hg-HSD-2 using CLUSTAL W. An NADPH binding motif that is conserved in all members of the 17β-HSD3 family is designated by the double overline with the consensus glycine residues within the motif TGxxxGxG shown in bold. The short-chain dehydrogenase/reductase protein family signature (PS00061) found in Hg-HSD-1 and Hg-HSD-2 as determined by PROSITE is indicated by the single overline, with active site tyrosine (Y) and lysine (K) in bold. Asterisks, colons, and dots indicate identical residues, conserved substitutions, and semi-conserved substitutions, respectively.*

Although it appeared that expression of *hsd-1* and *hsd-2* was low in eggs, we wanted to confirm this was true for eggs contained within the mother. For this experiment, *ca* 100 cysts were mechanically disrupted by hand, and the eggs and cyst contents were separated from the nematode cuticles. Cysts collected at 18 dpi were chosen for this experiment because this was the point in development when expression was determined to be the highest (unpubl.). Expression of *Hg-hsd-1*, *Hg-hsd-2* and actin in eggs plus cyst fluid, filtered cyst contents, and the empty cyst bodies was determined (Fig. 6). The cuticle fraction showed a higher level of *Hg-hsd-1* than the whole cyst contents, with 693 copies *vs* 505 (average of two data points from each of two different RNA preparations,
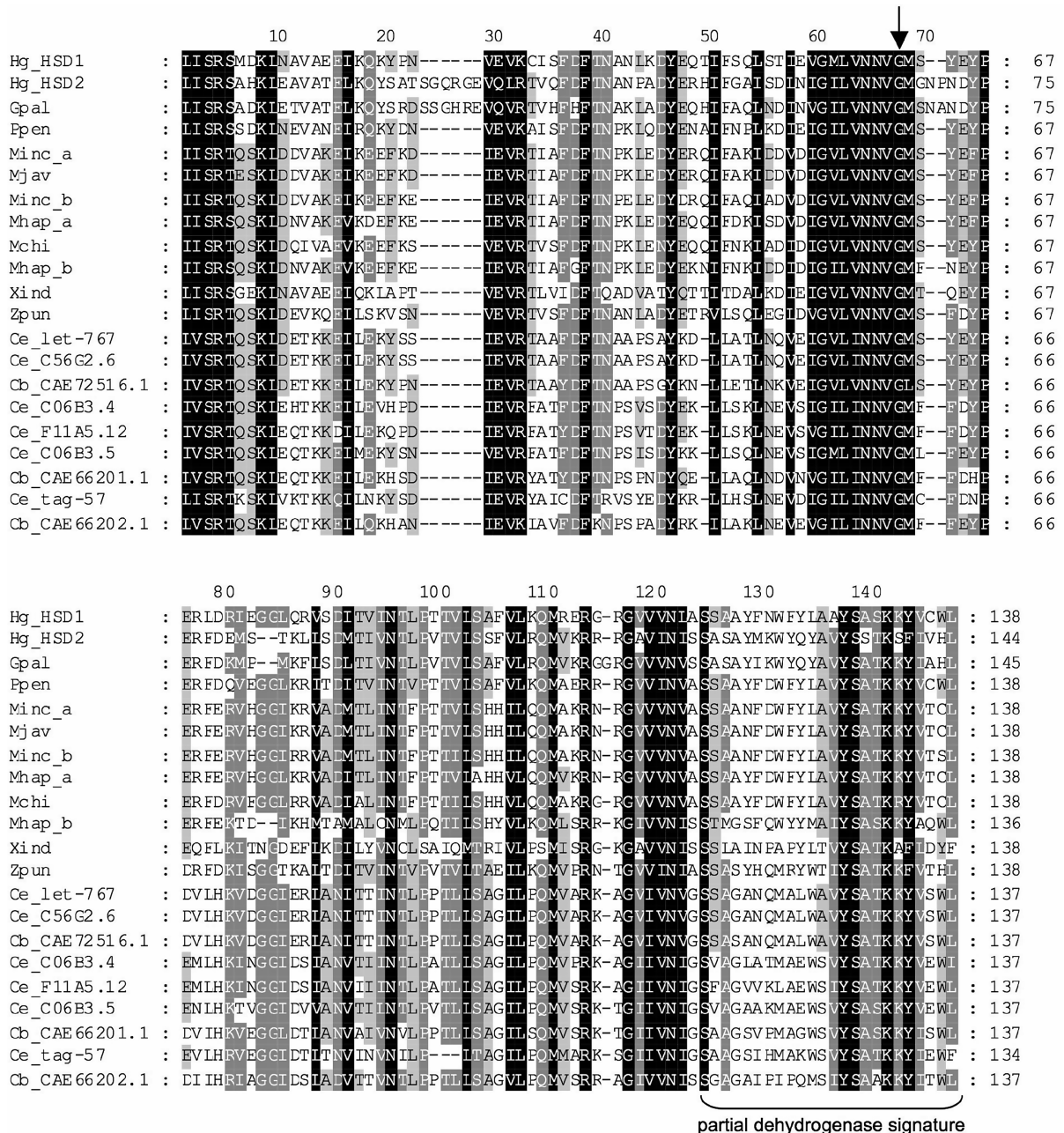
```
                    10        20        30        40        50        60            70
Hg_HSD1       : LISRSMDKINAVAEEIKQKYPN-----VEVKCISFDFINANIKDYEQTIFSQLSTIEVGMLVNNVGMS--YEYP :  67
Hg_HSD2       : LISRSADKIEAVATELKQYSATSGCRGEVQLRTVQFDFINANPADYERHIFGAISDINIGILVNNVGMGNPNDYP :  75
Gpal          : LISRSSDKIETVATELKQYSRDSSGHREVQVRTVHFHFINAKLADYEQHIFAQLNDINVGILVNNVGMSNANDYP :  75
Ppen          : LISRSSDKINEVANEIRQKYDN-----VEVKAISFDFINPKLQDYENAIFNPLKDIEIGILVNNVGMS--YEYP :  67
Minc_a        : IISRTQSKIDDVAKEIKEEFKD-----IEVRTIAFDFINPKLEDYERQIFAKILDVDIGVLVNNVGMS--YEFP :  67
Mjav          : IISRTESKIDDVAKEIKEEFKD-----IEVRTIAFDFINPKLEDYERQIFAKILDVDIGVLVNNVGMS--YEFP :  67
Minc_b        : IISRTQSKIDDVAKEIKEEFKE-----IEVRTIAFDFINPELEDYDRQIFAQIADVDIGVLVNNVGMS--YEFP :  67
Mhap_a        : IISRSQSKIDNVAKEVKDEFKE-----IEVRTIAFDFINPKLEDYEQQIFDKISDVDIGVLVNNVGMS--YEFP :  67
Mchi          : IISRTQSKIDQIVAEVKEEFKS-----VEVRTVSFDFINPKLENYEQQIFNKIADIDIGVLVNNVGMS--YEYP :  67
Mhap_b        : IISRSQSKIDNVAKEVKEEFKE-----IEVRTIAFGFINPKLEDYEKNIFNKIDIDIGILVNNVGMF--NEYP :  67
Xind          : LISRSGEKINAVAEEIQKLAPT-----VEVRTLVIDFIQADVATYQTTITDALKDIEIGVLVNNVGMT--QEYP :  67
Zpun          : LISRTQSKIDEVKQEILSKVSN-----VEVRTVSFDFINANLADYETRVLSQLEGIDVGVLVNNVGMS--FDYP :  67
Ce_let-767    : IVSRTQSKIDETKKEILEKYSS-----IEVRTAAFDFINAAPSAYKD-LIATLNQVEIGVLINNVGMS--YEYP :  66
Ce_C56G2.6    : IVSRTQSKIDETKKEILEKYSS-----IEVRTAAFDFINAAPSAYKD-LIATLNQVEIGVLINNVGMS--YEYP :  66
Cb_CAE72516.1 : IVSRTQSKIDETKKEILEKYPN-----IEVRTAAYDFINAAPSGYKN-LIETLNKVEIGVLVNNVGLS--YEYP :  66
Ce_C06B3.4    : IVSRTQSKIEHTKKEILEVHPD-----IEVRFATFDFINPSVSDYEK-LLSKLNEVSIGILINNVGMF--FDYP :  66
Ce_F11A5.12   : IVSRTQSKIEQTKKDILEKQPD-----IEVRFATYDFINPSVTDYEK-LLSKLNEVSVGILINNVGMF--FDYP :  66
Ce_C06B3.5    : IVSRTQSKIEQTKKEIMEKYSN-----VEVRFATFDFINPSISDYKK-LLSQLNEVSIGILMNNVGML--FEYP :  66
Cb_CAE66201.1 : IVSRTQSKIEQTKKEILEKHSD-----IEVRYATYDFINPSPNDYQE-LLAQLNDVNVGILINNVGMF--FDHP :  66
Ce_tag-57     : LISRTKSKIVKTKKQILNKYSD-----IEVRYAICDFIRVSYEDYKR-LLHSLNEVDIGILVNNVGMC--FDNP :  66
Cb_CAE66202.1 : LVSRTQSKIEQTKKEILQKHAN-----IEVKIAVFDFKNPSPADYRK-IIAKLNEVEVGILINNVGMF--FEYP :  66

                    80        90        100       110       120       130       140
Hg_HSD1       : ERLDRIEGGLQRVSEITVINILETTIVISAFVLKQMRERG-RGVVVNIASSAAYFNWFYIAAYSASKKYVCWL : 138
Hg_HSD2       : ERFDEMS--TKLLSEMTIVNILEVIVISSFVLRQMVKRR-RGAVINISSASAYMKWYQYAVYSSTKSFIVHL : 144
Gpal          : ERFDKMP--MKFISELTIVNILEVIVISAFVLRQMVKRGGRGVVVNVSSASAYIKWYQYAVYSATKKYIAHL : 145
Ppen          : ERFDQVEGGLKRITEITVINIVETTIVISAFVLKQMAERR-RGVVINVASSAAYFDWFYIAVYSATKKYVCWL : 138
Minc_a        : ERFERVHGGIKRVAEMTLINIFETTVISHHILCQMAKRN-RGVVVNVASSAANFDWFYIAVYSATKKYVTCL : 138
Mjav          : ERFERVHGGIKRVAEMTLINIFETTVISHHILCQMAKRN-RGVVVNVASSAANFDWFYIAVYSATKKYVTCL : 138
Minc_b        : ERFERVHGGIRRVAEMTLINIFETTIILSHHILCQMAKRN-RGVVVNVASSAANFDWFYIAVYSATKKYVTSL : 138
Mhap_a        : ERFERVHGGLKRVAEITLINIFETTVIAHHVLCQMVKRN-RGVVVNVASSAAYFDWFYIAVYSATKKYVTCL : 138
Mchi          : ERFDRVEGGLRRVAEIALINIFETTIILSHHVLCQMAKRG-RGVVVNVASSAAYFDWFYIAVYSATKRYVTCL : 138
Mhap_b        : ERFEKTC--IKHMTAMALQNMLEQTILSHYVLKQMLSRR-KGIVVNVASSTMGSFCWYYMAIYSASKKYAQWL : 136
Xind          : EQFLKITNGDEFLKEILYVNCLSAIQMTRIVLPSMISRG-KGAVVNISSSLAINPAPYLTVYSATKAFIDYF : 138
Zpun          : DRFDKISGGTKAITEITVINIVEVIVITAEILSHYVLKQMPRN-TGVVINIASSASYHQMRYWTIYSATKKFVTHL : 138
Ce_let-767    : DVLHKVDGGIERIANITTINILEPTILSAGIILPQMVARK-AGVIVNVGSSAGANQMALWAVYSATKKYVSWL : 137
Ce_C56G2.6    : DVLHKVDGGIERIANITTINILEPTILSAGIILPQMVARK-AGVIVNVGSSAGANQMALWAVYSATKKYVSWL : 137
Cb_CAE72516.1 : DVLHKVDGGIERIANITTINILEPTILSAGIILPQMVARK-AGVIVNVGSSASANQMALWAVYSATKKYVSWL : 137
Ce_C06B3.4    : EMLHKINGGIDSIANVTIINILEATILSAGIILPQMVPRK-AGIIVNIGSVAGIATMAEWSVYSATKKYVEWI : 137
Ce_F11A5.12   : EMLHKINGGIDSIANVIIINILEATILSAGIILPQMVSRK-AGIIVNIGSFAGVVKLAEWSIYSATKKYVEWL : 137
Ce_C06B3.5    : ENLHKTVGGIDVVANVTIINILEVTILSAGIILPQMVSRK-TGIIVNIGSVAGAAKMAEWSVYSASKKYVEWL : 137
Cb_CAE66201.1 : DVIHKVEGGLDTIANVAIVNVLEPTILSAGIILSQMVSRK-TGIIVNIGSAAGSVPMAGWSVYSASKKYISWL : 137
Ce_tag-57     : EVLHRVEGGIDTITNVINVNILE---LTAGIILPQMMARK-SGIIVNIGSAAGSIHMAKWSVYSATKKYIEWF : 134
Cb_CAE66202.1 : DIIHRIAGGIDSIAEVTTVNILEPTILSAGVLPQMVSRR-AGIVVNISSGAGAIPIPQMSIYSAAKKYITWL : 137
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partial dehydrogenase signature

Fig. 2. *Alignment of Hg-HSD-1 and Hg-HSD-2 protein sequences, with conceptual translations from ESTs or cDNAs in plant-parasitic and free-living nematodes. Alignment is trimmed to show only regions present in all sequences. Amino acids present in all are in white text boxed in black; positions conserved in 80% of the sequences are shaded dark grey; positions conserved in 60% of the sequences are shaded light grey. Bracket at amino acids 123-140 corresponds to the short-chain dehydrogenase signature sequence. Only part is shown due to incomplete ESTs. A glycine residue, indicated by the arrow, is the residue mutated in the* C. elegans let-767 s2176 *allele. Abbreviations: Hg_HSD1 and Hg_HSD2 are from* Heterodera glycines; *Gpal* = Globodera pallida; *Ppen* = Pratylenchus penetrans; *Minc_a* = Meloidogyne incognita; *Mjav* = M. javanica; *Minc_b* = M. incognita; *Mhap_a* = M. hapla; *Mchi* = M. chitwoodi; *Mhap_b* = M. incognita; *Xind* = Xiphinema index; *Zpun* = Zeldia punctata. Abbreviations for* Caenorhabditis elegans *sequences are: Ce_let-767, Ce_C56G2.6, Ce_C06B3.4, Ce_F11A5.12, Ce_C06B3.5, and Ce_tag-57; for* C. briggsae: *Cb_CAE72516.1, Cb_CAE66201.1, and Cb_CAE66202.1. Additional details can be found in Table 1.*
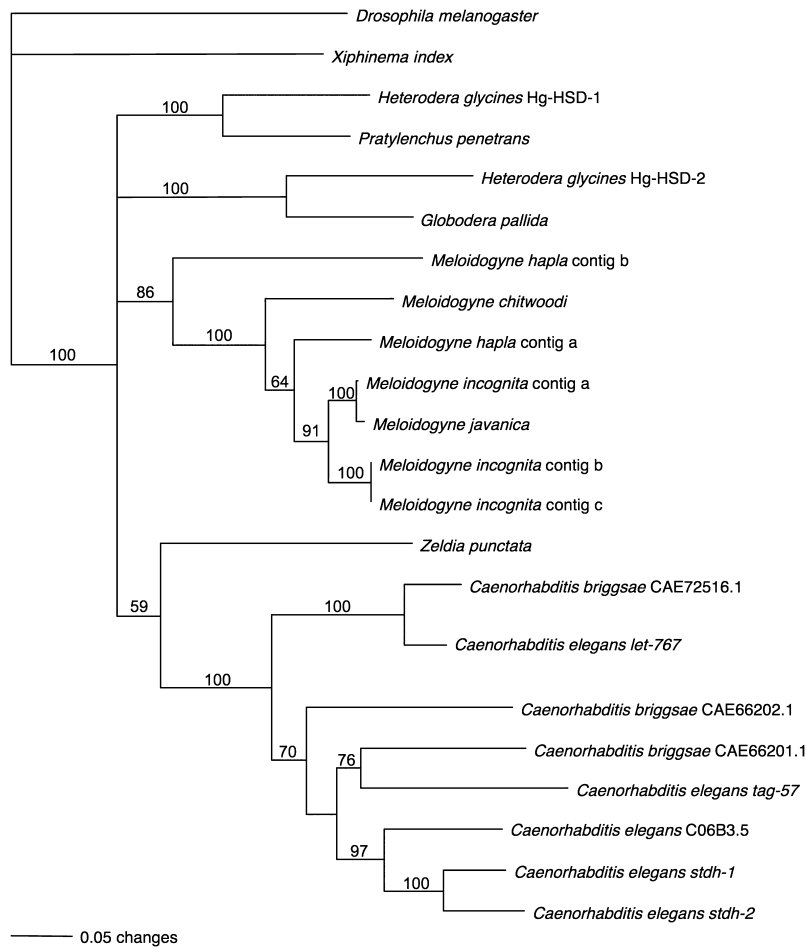
Fig. 3. *Neighbour-Joining 50% majority-rule consensus phylogram of 148 amino acids from* Heterodera glycines *HSD-1, HSD-2 and other plant-parasitic or free-living nematodes, including* Drosophila melanogaster *as the outgroup. Bootstrap values from 1000 replicates as implemented in PAUP 4.0b10 are indicated on branches.*

n = 4). When the eggs were separated from the cyst fluid by filtration, the level of expression in the fluid fraction was much higher, at 973 copies (average of six data points from three RNA preparations, n = 6). For *Hg-hsd-2*, the cuticle fraction showed higher expression (403 copies, average of n = 4) than whole cyst contents (102 copies, average of n = 4). Filtered cyst fluid had higher levels of *Hg-hsd-2* transcript (140 copies, average of n = 6) than the unfiltered cyst contents.

Discussion

Here we report the first two short-chain dehydrogenase genes identified from the plant-parasitic nematode *H. glycines*. Aside from highly conserved functional do-

mains, sequence similarity between the two genes was low and the introns were in different positions, indicating clearly separate genes that possibly have distinct functions. Both Hg-HSD-1 and Hg-HSD-2 were most similar to deduced protein sequences from other plant-parasitic nematodes, with relatively lower similarity to those from free-living nematodes including *C. elegans* LET-767, and the least similarity to HSDs from fruit flies, slime moulds, sea squirt, and other organisms. Hg-HSD-1 was most like sequences from the lesion nematode *P. penetrans*, a migratory endoparasite, but Hg-HSD-2 shared highest similarity to a sequence from another cyst nematode, *G. pallida*. Genetic distances from HSD-1 and HSD-2 were greater for all root-knot nematode sequences examined, all of which grouped together in a single clade (Fig. 3).
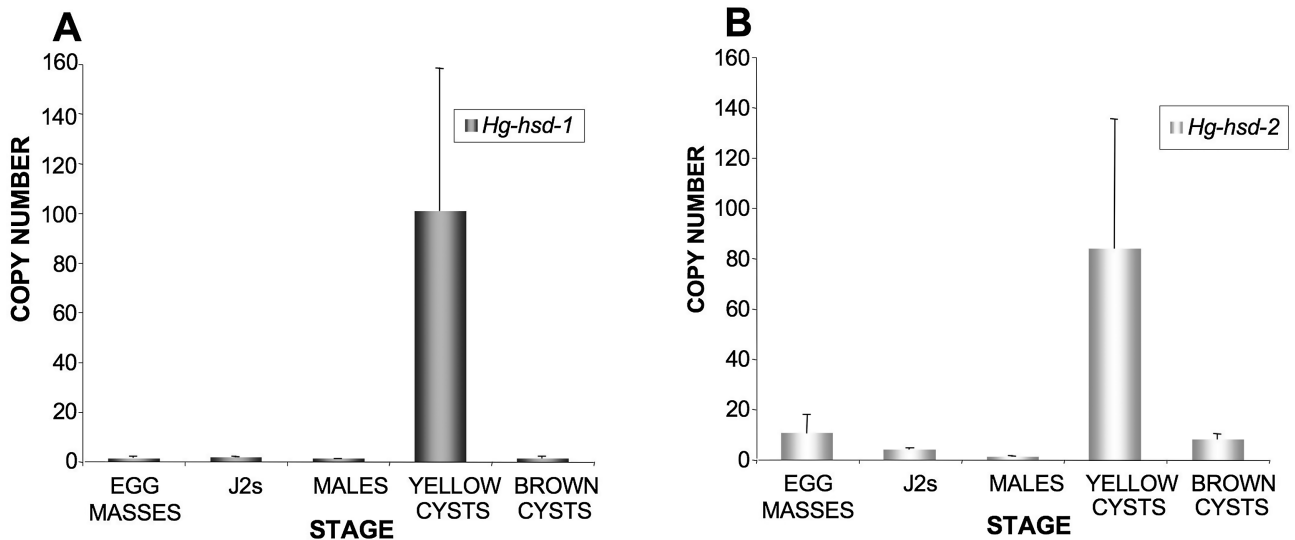
Fig. 4. *Quantitation of gene expression for* Hg-hsd-1 *(A) and* Hg-hsd-2 *(B) in various stages of* Heterodera glycines*, expressed as copy number. C_t values were determined for each gene in triplicate and normalised to C_t for actin by the method of Pfaffl (2001). Values expressed as mean $\pm$ SD.*
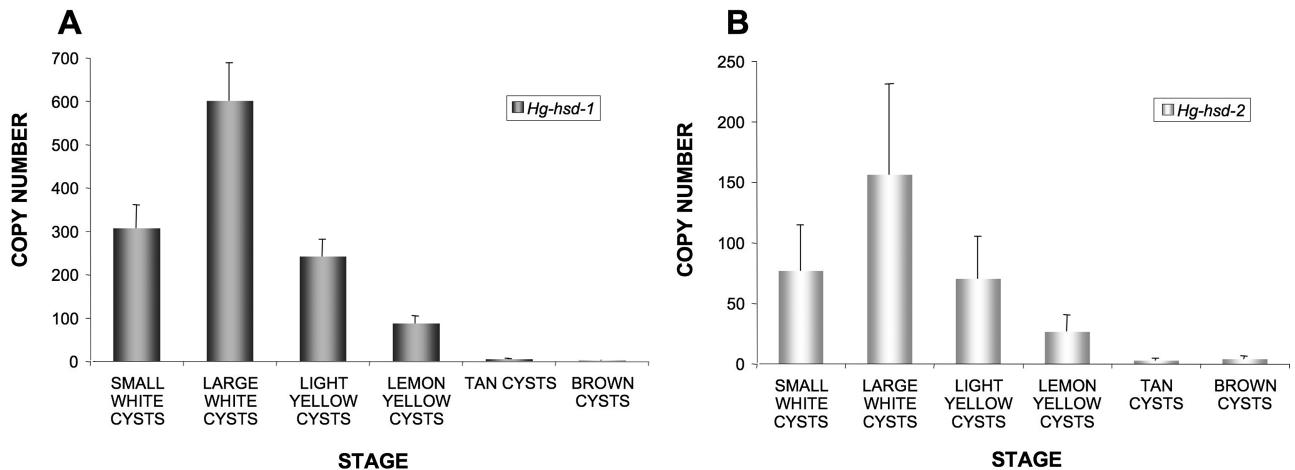
Fig. 5. *Quantitation of gene expression for* Hg-hsd-1 *(A) and* Hg-hsd-2 *(B) in developing* Heterodera glycines *females separated according to size and pigmentation. Values expressed as mean values $\pm$ SD.*

Hg-HSD-2 and the *G. pallida* contig were set apart from other sequences by a number of uniquely shared amino acids, including a stretch of six amino acids (SxxxxE) not found in any other sequence (Fig. 2, positions 23-28). The function of this motif is not known, but it could provide enzyme specificity unique to cyst nematodes. More likely, this insertion may be unrelated to function, having arisen by chance in an ancestral cyst nematode. Not surprisingly, the majority of ESTs encoding putative 17β-HSDs from the sedentary endoparasites came from female cDNA li-

braries, with the exception of ESTs from *Meloidogyne hapla* and *M. javanica* eggs. It is possible that these libraries were constructed in a way that enriched rare transcripts. Alternatively, the presence of substantial levels of 17β-HSD mRNA as reflected in the ESTs may indicate different regulation of these genes in the root-knot nematodes. For the free-living nematodes or migratory plant-parasitic species, ESTs came from mixed-stage cDNA libraries that would undoubtedly include messages present at high levels in hermaphrodites or females.

A

B
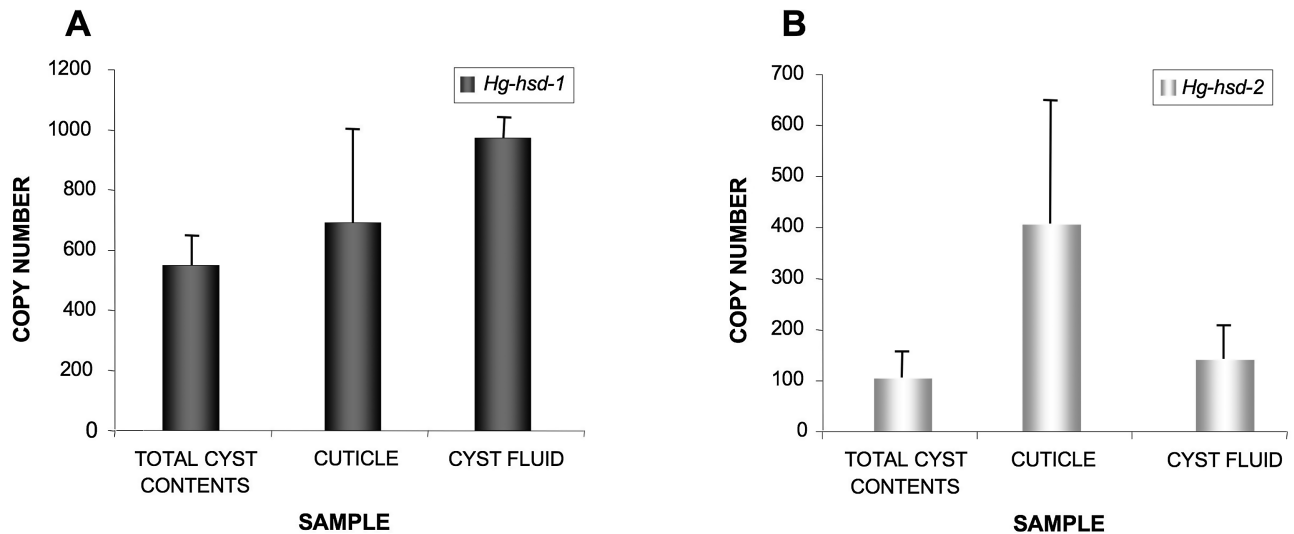
Fig. 6. *Quantitation of gene expression for* Hg-hsd-1 *(A) and* Hg-hsd-2 *(B) in* Heterodera glycines *cysts collected 18 dpi, with the female body contents manually separated from the eggs. Values expressed as mean values ± SD.*

Quantitative real-time PCR revealed significant up-regulation of *Hg-hsd-1* and *Hg-hsd-2* in developing females, with both genes showing highest expression in plump, white cysts. No effort was made to separate developmental ages in the initial experiment, so the relatively large variation in transcription data from pooled yellow cysts (Fig. 4) probably reflects the presence of females at a mixture of developmental ages. However, when females were aged more precisely (Fig. 5), the data appeared more consistent within each group of cysts. In contrast to the up-regulation observed in cysts, transcription of both genes was very low in embryos, regardless of whether they were mechanically extracted from mature females, dead cysts, or a gelatinous matrix (not shown). Similarly, transgenic *C. elegans* containing a *let-767::gfp* (green fluorescent protein) reporter showed an absence of *gfp* expression during embryogenesis (Kuervers *et al.*, 2003). The relative absence of 17β-HSD mRNAs in *H. glycines* eggs indicates that *Hg-hsd-1* and *Hg-hsd-2* are not required for embryogenesis. However, it is possible that if HSD-1 and HSD-2 functions are necessary for embryo development, they may be maternally provided as proteins. Although measurements were not quantitative, *C. elegans let-767* expression was observed during all other developing stages, including males (Kuervers *et al.*, 2003). This appears in contrast to the apparently tighter regulation observed for *Hg-hsd-1* and *Hg-hsd-2* expression, which was limited to a narrower window in fertile females and was nearly absent in J2 or males.

In *C. elegans*, mutation of the *let-767* gene led to severe developmental abnormalities (Kuervers *et al.*, 2003). Reproductive defects were observed in nematodes carrying defects in this gene, including an extruding vulva phenotype, delayed germline formation, or primitive gonads lacking oocytes or embryos. In some mutants, defects in oogenesis led to reduced fecundity, with 30% of the offspring appearing disorganised and undifferentiated. Thus, the hydroxysteroid dehydrogenase encoded by *let-767* has been implicated in these processes. Similarly, peak expression of *H. glycines Hg-hsd-1* and *Hg-hsd-2* corresponded to the time when females are actively producing embryos (at 18 dpi), so it is possible that both genes play an important role in reproductive development or oogenesis in this species.

Mutant *let-767* phenotypes were enhanced by cholesterol deprivation, with larval arrest occurring at an earlier stage in *let-767 (mel)* than was observed with cholesterol-supplemented media (Kuervers *et al.*, 2003). Also, homozygotes of three *C. elegans let-767* alleles exhibited moulting defects, including failure to detach the old cuticle or a pinching of the cuticle in the tail region. While the obligate parasitism of *H. glycines* does not allow for similar sterol deprivation experiments, disruption of *Hg-hsd-1* and *Hg-hsd-2* by RNAi could shed some light on the necessity of these genes for development of the gonad, embryogenesis, and moulting.

Both Hg-HSD-1 and Hg-HSD-2 had hallmarks of 17β-hydroxysteroid short-chain dehydrogenases, includ-

ing NADP(H) binding domain and dehydrogenase signature sequences (Fig. 1). The 17β-HSDs define enzymes typically involved in the interconversion of 17-ketosteroids with 17-hydroxysteroids (Mindnich *et al.*, 2004), i.e., reactions in which the side chain present in ecdysteroids or membrane sterols has been replaced with an oxygen-containing substituent. Delineation of the 12 currently identified 17β-HSD subtypes is based on homology and catalytic information largely from the vertebrate enzymes. However, there appears to be a great deal of overlap in substrate specificity and substantial complexity in the phylogenetic relationships among the classes. For instance, the type 12 17β-HSDs typically function in fatty acid elongation and yet they share significant sequence homology with the type 3 enzymes that act upon sterols. Based upon sequence similarity, Mindnich *et al.* (2004) suggested that LET-767 is a type 12 17β-HSD but the *in vivo* evidence in *C. elegans* (Kuervers *et al.*, 2002) overwhelmingly points to a role in sterol metabolism, more consistent with type 3 enzymes. While multi-functionality in 17β-HSDs has been previously noted (Mindnich *et al.*, 2004), it remains unclear if that is the case for LET-767. Within this framework, Hg-HSD-1 and Hg-HSD-2 both appear to be most homologous to type 3 17β-HSDs, with minimal similarity to type 12 sequences (E values $\sim 10^{-9}$).

While it is clear that the *H. glycines* sequences share some features with *C. elegans let-767*, it is worth noting some of the differences in sterol metabolism that exist between plant-parasitic and free-living nematodes. The greater similarity of the *H. glycines* 17β-HSD protein sequences to counterparts in *P. penetrans* and *G. pallida* is consistent with the fact that sterol metabolism in *C. elegans* is quite different from the cyst and lesion nematodes studied thus far, reflecting the closer relationships of the plant parasites to each other than to *C. elegans*. In *C. elegans*, the primary metabolic transformation upon the sterol nucleus is desaturation at C-7, whereas the major nuclear modification in *Heterodera zeae*, *Pratylenchus agilis* and *Globodera solanacearum* is saturation of the C-5 double bond (Orcutt *et al.*, 1978; Chitwood *et al.*, 1999). Dissimilarity in the adult expression patterns of *Hg-hsd-1* and *Hg-hsd-2* relative to *let-767* seems to reflect yet another unique aspect of sterol metabolism in the plant parasites.

Because nematodes must feed upon soybean roots to develop, the roots likely contain compounds that modulate the maturation of *H. glycines.* The nutritional dependence of nematodes upon their plant hosts for sterols indicates that these some of these modulators could be steroids or could affect nematode steroid function. The small-to-moderate difference in sterol composition between nematode-infected and uninfected plant roots (Zinovieva *et al.*, 1990; Hedin *et al.*, 1995) may or may not indicate that sterols are such modulators. However, soybean flavonoids, which occur in roots as well as beans, are important dietary phytoestrogens for humans and are potent modulators of human 17β-HSDs (Le Bail *et al.*, 1998). Additionally, numerous studies have revealed various biological effects of exogenously applied plant, insect or mammalian steroids upon nematodes (Chitwood, 1999; Soriano *et al.*, 2004; Udalova *et al.*, 2004). Consequently, an exploration of soybean phytoestrogens or other mediators of steroid function could lead to the discovery of natural inhibitors of SCN development. The discovery of Hg-HSD-1 and Hg-HSD-2 from *H. glycines* opens the door to further exploration of steroid metabolism and possible inhibitors in this species.

Acknowledgements

References

ALTSCHUL, S.F., MADDEN, T.L., SCHÄFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W. & LIPMAN, D.J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* 25, 3389-3402.

BAKHETIA, M., CHARLTON, W., ATKINSON, H.J. & MCPHERSON, M.J. (2005). RNA interference of dual oxidase in the plant nematode *Meloidogyne incognita*. *Molecular Plant-Microbe Interactions* 18, 1099-1106.

BARKER, G.C., MERCER, J.G., REES, H.H. & HOWELLS, R.E. (1991). The effects of ecdysteroids on the microfilarial production of *Brugia pahangi* and the control of meiotic reinitiation in the oocytes of *Dirofilaria immitis*. *Parasitology Research* 77, 65-71.

BEKAL, S., NIBLACK, T.N. & LAMBERT, K.N. (2003). A chorismate mutase from the soybean cyst nematode *Heterodera glycines* shows polymorphisms that correlate with virulence. *Molecular Plant-Microbe Interactions* 16, 439-446.

CHEN, Q., REHMAN, S., SMANT, G. & JONES, J.T. (2005). Functional analysis of pathogenicity proteins of the potato cyst nematode *Globodera rostochiensis* using RNAi. *Molecular Plant-Microbe Interactions* 18, 621-625.

CHITWOOD, D.J. (1999). Biochemistry and function of nematode steroids. *Critical Reviews in Biochemistry and Molecular Biology* 34, 273-284.

DAVIS, E.L., HUSSEY, R.S. & BAUM, T.J. (2004). Getting to the roots of parasitism by nematodes. *Trends in Parasitology* 20, 134-141.

FANELLI, E., DI VITO, M., JONES, J.T. & DE GIORGI, C. (2005). Analysis of chitin synthase function in a plant parasitic nematode, *Meloidogyne artiellia*, using RNAi. *Gene* 349, 87-95.

GAO, B., ALLEN, R., MAIER, T., DAVIS, E.L., BAUM, T.J. & HUSSEY, R.S. (2003). The parasitome of the phytonematode *Heterodera glycines*. *Molecular Plant-Microbe Interactions* 16, 720-726.

GEISSLER, W.M., DAVIS, D.L., WU, L., BRADSHAW, K.D., PATEL, S., MENDONCA, B.B., ELLISTON, K.O., WILSON, J.D., RUSSELL, D.W. & ANDERSON, S. (1994). Male pseudohermaphroditism caused by mutations of testicular 17beta-hydroxysteroid dehydrogenase 3. *Nature Genetics* 7, 34-39.

GOUDEY-PERRIERE, F., SIMO, B.F., MACCARIO, J., PERRIERE, C. & GAYRAL, P. (1992). Effects of ecdysteroids on reproductive physiology of *Nippostrongylus brasiliensi*s (Nematoda) in vivo. *Comparative Biochemistry and Physiology* 103, 105-109.

HEDIN, P.A., CALLAHAN, F.E., DOLLAR, D.A. & CREECH, R.G. (1995). Total sterols in root-knot nematode *Meloidogyne incognita* infected cotton *Gossypium hirsutum* (L.) plant roots. *Comparative Biochemistry and Physiology* 111, 447-452.

KUERVERS, L.M., JONES, C.L., O'NEIL, N.J. & BAILLIE, D.L. (2003). The sterol modifying enzyme LET-767 is essential for growth, reproduction and development in *Caenorhabditis elegans. Molecular Genetics and Genomics* 270, 121-131.

KURZCHALIA, T.V. & WARD, S. (2003). Why do worms need cholesterol? *Nature Cell Biology* 5, 684-688.

LE BAIL, J.C., LAROCHE, T., MARRE-FOURNIER, F. & HABRIOUX, G. (1998). Aromatase and 17beta-hydroxysteroid dehydrogenase inhibition by flavonoids. *Cancer Letters* 133, 101-106.

LILLY, C.J., GOODCHILD, S.A., ATKINSON, H.J. & URWIN, P.E. (2005). Cloning and characterisation of a *Heterodera glycines* aminopeptidase cDNA. *International Journal for Parasitology* 35, 1577-1585.

LLADO, R.R., URWIN, P., ATKINSON, H. & GILMARTIN, P.M. (1998). Hgm1, a novel male-specific gene from *Heterodera glycines* identified by differential mRNA display. *Molecular and Biochemical Parasitology* 95, 45-52.

MASLER, E.P., KOVALEVA, E.S. & SARDANELLI, S. (1999). Comparison of FaRP immunoreactivity in free-living nematodes and in the plant-parasitic nematode *Heterodera glycines*. *Annals of the New York Academy of Sciences* 97, 253-263.

MINDNICH, R., MÖLLER, G. & ADAMSKI, J. (2004). The role of 17beta-hydroxysteroid dehydrogenases. *Molecular and Cellular Endocrinology* 218, 7-20.

NICHOLAS, K.B., NICHOLAS, JR, H.B. & DEERFIELD, II, D.W. (1997). GeneDoc: Analysis and visualization of genetic variation. *Embnew. News* 4,14.

ORCUTT, D.M., FOX, J.A. & JAKE, C.A. (1978). The sterol, fatty acid, and hydrocarbon composition of *Globodera solanacearum*. *Journal of Nematology* 10, 264-269.

PFAFFL, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 29, 2002-2007.

RIDDIFORD, L.M. (1993). Hormone receptors and the regulation of insect metamorphosis. *Receptor* 3, 203-209.

ROSSO, M.N., DUBRANA, M.P., CIMBOLINI, N., JAUBERT, S. & ABAD, P. (2005). Application of RNA interference to root-knot nematode genes encoding oesophageal gland proteins. *Molecular Plant-Microbe Interactions* 18, 615-620.

SARDANELLI, S. & KENWORTHY, W.J. (1997). Soil moisture control and direct seeding for bioassay of *Heterodera glycines* on soybean. *Journal of Nematology* (Supplement) 29, 625-634.

SORIANO, I.R., RILEY, I.T., POTTER, M.J. & BOWERS, W.S. (2004). Phytoecdysteroids: a novel defense against plant-parasitic nematodes. *Journal of Chemical Ecology* 30, 1885-1899.

SWOFFORD, D.L. (1998). *PAUP* Phylogenetic Analysis Using Parsimony (*and other methods), version 4.0.* Sunderland, MA, USA, Sinauer Associates.

THOMPSON, J.D., HIGGINS, D.G. & GIBSON, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673-4680.

UDALOVA, Z.V., ZINOV'EVA, S.V., VASIL'EVA, I.S. & PASESHNICHENKO, V.A. (2004). Correlation between the structure of plant steroids and their effects on phytoparasitic nematodes. *Applied Biochemistry and Microbiology* 40, 93-97.

URWIN, P.E., LILLEY, C.J. & ATKINSON, H.J. (2002). Ingestion of double-stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. *Molecular Plant-Microbe Interactions* 15, 747-752.

VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE PAEPE, A. & SPELEMAN, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* 3, R0034.1-R0034.11.

VANHOLME, B., DE MEUTTER, J., TYTGAT, T., VAN MONTAGU, M., COOMANS, A. & GHEYSEN, G. (2004). Secre-

tions of plant-parasitic nematodes: a molecular update. *Gene* 12, 13-27.

WRATHER, J.A., KOENNING, S.R. & ANDERSON, T.R. (2003). Effect of diseases on soybean yields in the United States and Ontario (1999-2002). *Plant Health Progress* doi:10.1094/PHP-2003-325-01-RV.

ZINOVIEVA, S.V., VASYUKOVA, N.I. & OZERETSKOVSKAYA, I.L. (1990). Involvement of plant sterols in the system tomatoes–nematode *Meloidogyne incognita*. *Helminthologia* 27, 211-216.