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Several Grassland Soil Nematode Species Are Insensitive to RNA-Mediated Interference

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Abstract: Phenotypic analysis of defects caused by RNA mediated interference (RNAi) in *Caenorhabditis elegans* has proven to be a powerful tool for determining gene function. In this study we investigated the effectiveness of RNAi in four non-model grassland soil nematodes, *Oscheius* sp FVV-2., *Rhabditis* sp, *Mesorhabditis* sp., and *Acrobeloides* sp. In contrast to reference experiments performed using *C. elegans* and *Caenorhabditis briggsae*, feeding bacteria expressing dsRNA and injecting dsRNA into the gonad did not produce the expected RNAi knockdown phenotypes in any of the grassland nematodes. Quantitative reverse-transcribed PCR (qRT-PCR) assays did not detect a statistically significant reduction in the mRNA levels of endogenous genes targeted by RNAi in *Oscheius* sp., and *Mesorhabditis* sp. From these studies we conclude that due to low effectiveness and inconsistent reproducibility, RNAi knockdown phenotypes in non-*Caenorhabditis* nematodes should be interpreted cautiously.

Key words: RNAi, Konza prairie, soil nematode, molecular biology.

The development of high-throughput sequencing technologies now facilitates the acquisition of genome or transcriptome information for even poorly characterized species. Although data obtained from these genome projects is typically annotated by comparison to evolutionary related model species, increasingly emphasis is being placed on characterizing gene function directly in the organisms of interest (Sommer, 2009). However, the development of genetic and transgenic approaches in non-model systems has often proven to be technically challenging (Schlager et al., 2009). Therefore, transient knockdown approaches such as RNAi are attractive alternatives to study biological processes in non-model organisms (Dong and Friedrich, 2005; Ohnishi et al., 2006; Mutti et al., 2008; Tomoyasu et al., 2008; Ford et al., 2009).

RNA mediated interference (RNAi) occurs by a widely conserved mechanism that leads to the specific degradation of mRNA that is complementary to an exogenously introduced dsRNA sequence. In *C. elegans,* RNAi assays are typically carried out by injecting double stranded RNA into the body cavity or distal gonad; with the resulting phenotype observed in the progeny of the injected nematode (Fire et al., 1998). Remarkably, robust phenotypic effects of RNAi are also observed when *C. elegans* is soaked in a solution of dsRNA, or even feed on bacteria expressing dsRNA from a plasmid (Timmons et al., 2001). A systemic response (Winston et al., 2002), where the silencing signal spreads from the site of introduction throughout the organism, is observed in plants, *C. elegans*, and several arthropod species (Dong and Friedrich, 2005; Ohnishi et al., 2006; Tomoyasu et al., 2008). The systemic nature of the RNAi silencing in *C. elegans* requires the membrane protein SID-1, which is thought to act as a channel through which dsRNA enters cells (Winston et al., 2002; Feinberg and Hunter, 2003). The absence of *sid-1* from the genome of *Drosophila* has been suggested as a possible explanation for the lack of a robust systemic RNAi response in this species (Dong and Friedrich, 2005). However, based on evidence from molecular and phylogenetic analyses of arthropod *sid*-like genes, Tomoyasu and colleges (2008) have recently raised doubts about this association between the presence of SID-1 in the genome and the occurrence of a systemic RNAi response. Several other genes central to the RNAi pathway in *C. elegans* are functionally conserved across all metazoans, most likely due to the important role this and related pathways play in gene regulation, and the silencing of parasitic elements such as transposons (Hamilton and Baulcombe, 1999; Wang et al., 2006).

The effectiveness of RNAi as a genetic tool in *C. elegans* has generated much interest in establishing RNAi systems in other nematodes. Winston et al., (2007) showed that, amongst nine *Caenorhabditis* species, only *C. brenneri* was conclusively deficient in RNAi by microinjection. Despite the conservation of injection RNAi in *Caenorhabditis*, only *C. elegans* and the uncharacterized species *C. n. sp1* were sensitive to feeding RNAi (Winston et al., 2007), suggesting that significant differences in the RNAi mechanism exist even amongst closely related species (Lilley et al., 2012; Nuez and Felix 2012). Outside of the *Caenorhabditis* genus, the RNAi response in several parasitic and free-living nematodes varies by species, specific gene targeted or method used to introduce the dsRNA. For example, the plant parasitic nematodes *Globodera pallida* and *Heterodera glycines*, are sensitive to RNAi when high concentrations of dsRNA and octoprolline (to induce pharyngeal pumping) are included in the soaking media (Urwin et al., 2002; Sukno et al., 2007). The human filarial parasite *Brugia malayi*, rodent parasite *Nippostrongylus brasiliensis,* and the insect parasite *Heterorhabditis bacteriophora*, appear to be

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susceptable to RNAi (Hussein et al., 2002; Aboobaker and Blaxter, 2003; Ciche and Sternburg, 2007; Ford et al., 2009). However, in the gastrointestinal nematodes *Haemonchus contortus* and *Ostertagia ostertagi*, RNAi was effective against only 2 of 11, and 5 of 8 genes, respectively (Geldhof et al., 2006; Visser et al., 2006). Similarly, Lendner (2008) and colleagues were unable to detect any RNAi response in the parasitic nematode *Heligmosomoides polygyrus*. Amongst the free-living species *Panagrolaimus superbus* is sensitive to RNAi (Shannon et al., 2008), but several studies in the two satellite model systems *Oscheius tipulae* and *Pristionchus pacificus* have failed to identify a robust RNAi response (Felix, 2006). The evolutionary implication of this patchy occurrence of RNAi within Nematoda remains a mystery, especially given the inferred importance of this mechanism in protecting the genome from parasitic genetic elements (Viney and Thompson, 2008).

In this study we investigate the feasibility of using RNAi as a tool to study gene function in *Oscheius* sp. FVV-2, *Rhabditis* sp., *Mesorhabditis* sp. and *Acrobloides* sp., nematodes found in soil sampled from the Konza Prairie Biological Station near Manhattan, Kansas. These nematodes represent bacterial feeding species that are important for nutrient-cycling and the regulation of microbial populations in grassland soils (Griffiths, 1994; Jones et al, 2006). We failed to observe robust RNAi phenotypes in any of the species tested using standard *C. elegans* feeding and injection RNAi techniques targeted to endogenous genes. We outline the technical challenges in working with non-model nematodes and emphasize that appropriate interpretation of the phenotypic effects of RNAi knockdown for many nematodes may remain a significant challenge.

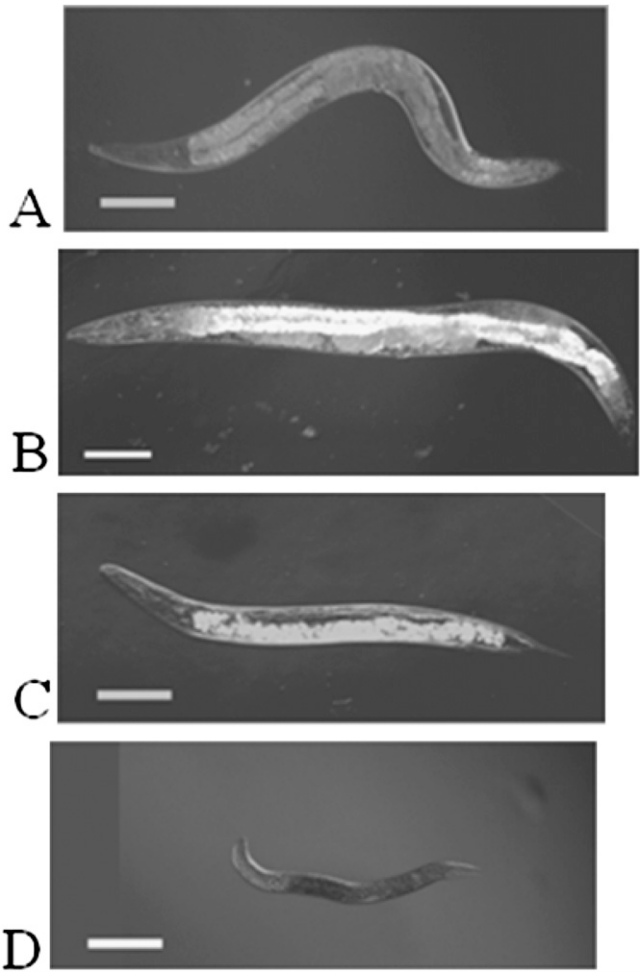

FIG. 1. Bright field images of (A) *Oscheius* sp., (B) *Rhabditis* sp., (C) *Mesorhabditis* sp., (D) *Acrobeloides* sp., at 10x magnification. Bar= 100μM.

MATERIALS AND METHODS

Nematode isolation and identification: The nematodes used in this study were isolated from soil samples collected at the Konza Prairie Biological Station located 15 km from Manhattan, Kansas, U.S.A: *Oscheius sp.* FVV-2 (isolate KS555), *Mesorhabditis sp.* (isolate KS601), *Rhabditis* sp. (isolate KS594), *Acrobeloides sp.* (isolate KS586) (Fig. 1). The *Oscheius* sp., *Rhabditis* sp, *Mesorhabditis* sp belong to the same family (Rhabditidae) as *C. elegans*, while *Acrobeloides* is a member of the Cephalobidae family (Fig. 2) (De Ley, 2006). Nematode isolates were maintained at 20 °C on NGM plates seeded with the standard *Escherichia coli* strain OP50. Molecular identification to the genus level was made based on consensus matches to 18s rRNA sequences in the NCBI database (accession numbers HQ130502-HQ130507). *Oscheius tipulae* has been shown to be insensitive to RNAi (Louvet-Vallée et al., 2003), however, we included *Oscheius sp.* FVV-2 in our analysis as even closely related nematode species show differential response to RNAi (Winston et al., 2007). To our knowledge RNAi methods have not been attempted on these ecologically important grassland soil nematodes.

Genes targeted by RNAi: Three genes, *dpy-5*, *unc-54*, *sqt-1* were used to assay RNAi sensitivity in the grassland nematodes as they produce easily recognized Dumpy (Dpy, short/fat), Uncoordinated (Unc, paralyzed), and cuticle knockdown phenotypes in *C. elegans*, respectively (Brenner, 1974; Park and Kramer, 1994). Degenerate primers designed to bind highly to conserved regions of homologs of the *C. elegans unc-54*, *ama-1* (Sanford et al., 1983), and *dpy-5* and *sqt-1* genes were used to isolate the DNA sequences targeted by RNAi in this study (Table 1). *C. elegans dpy-5* was the highest BLASTX match in the NCBI non-redundant database to *dpy-5* PCR products from *Oscheius* sp., and *Rhabditis* sp gDNA, confirming that these primers successfully amplified a portion of the *dpy-5* gene in these species (Table 2). In *Oscheius* sp., *Rhabditis* sp and *Mesorhabditis* sp., the *unc-54* primers amplified a product with sequence homology to members of the Myosin II heavy chain family, which includes the closely related *C. elegans* genes *unc-54*, *let-75*, and *myo-2*, therefore we labeled

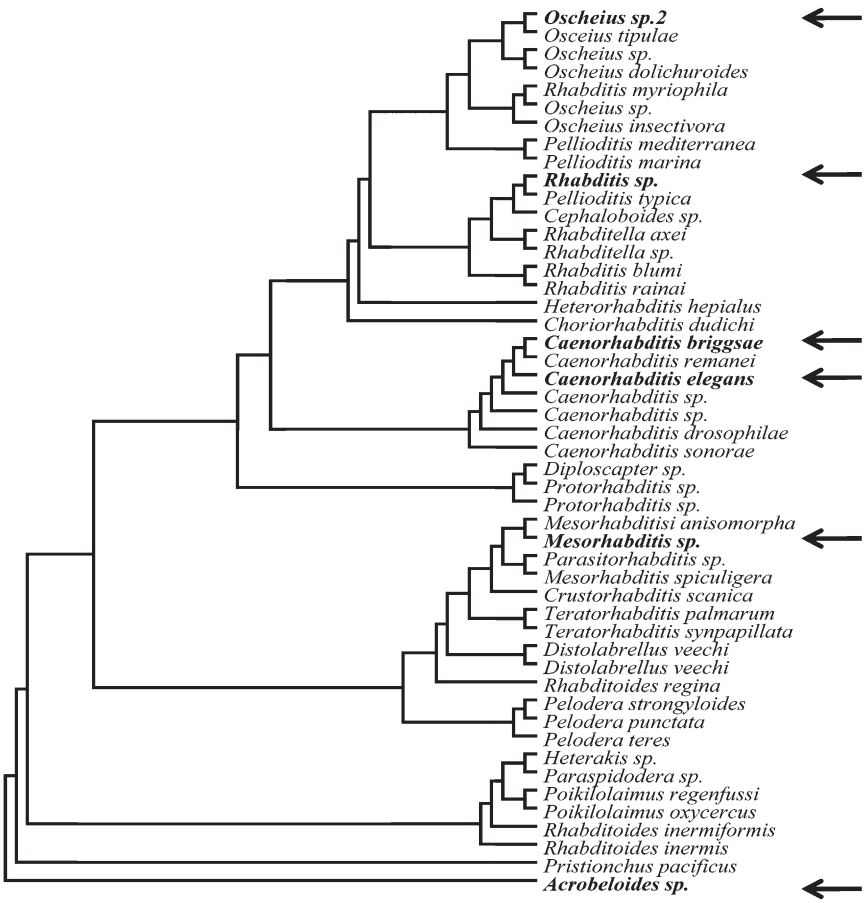

FIG. 2. Summarized phylogeny showing the relationships between the nematodes used in this study (arrow and bold). Tree arrangement based on the phylogeny of (De Ley, 2006).

these sequences as *unc-54* (Table 2). As neither the *dpy-5* nor *unc-54* primer combinations amplified a PCR product from *Acrobeloides* gDNA, we made use of a *sqt-1* cDNA sequence available for the related species *Zeldia punctia* (accession #: AW773473), to design a set of PCR primers specific for this gene. The *sqt-1* primers amplified two products that sequencing and BLASTX searches showed were most similar to the *Z. punctia sqt-1* cDNA clone (Table 2), therefore we called these sequences *Acrobeloides sqt-1A* and *sqt-1B*. Sequences amplified in this study have been submitted to the NCBI database under accessions: *Oscheius* (JQ713945, JQ713946, JQ713947), *Mesorhabditis* (JQ713948, JQ713949), *Rhabditis* (JQ713952, JQ713953, JQ713954), *Acrobeloides* (JQ713950, JQ713951).

RNAi methods: Gene sequences were cloned into pGEM-T (Promega, Madison WI) and amplified with the primers that added T7 promoter binding sites to the 5' and 3' end of the resulting PCR product (Table 1). The T7 tagged PCR product was used to generate double stranded RNA by *in vitro* transcription with the Megascript T7 polymerase kit (Ambion, Austin TX) according to the manufacturer's instructions. Approximately 1μg/μl of dsRNA was injected into the distal region (rachis) of both gonad arms (if didelphic) using the standard *C. elegans* microinjection procedure (Fire et al., 1998). Initially DIC microscopy was used to identify possible gonad injection sites in the distal gonad. *C. elegans* has a didelphic gonad with each arm characterized by a relatively large and well defined rachis and containing many oocytes (Fig. 3A). The gonad morphology of each grassland soil nematode species had some similarities with that of *C. elegans*, but also some significant differences. The didelphic gonad of *Oscheius* sp. FVV-2 is most similar, but with a smaller rachis and fewer oocytes (Fig. 3A, B). The didelphic *Rhabditis* sp., and monodelphic *Mesorhabditis* sp. gonads appear to have a rachis that is even more reduced in size and contains even fewer oocytes compared to *Oscheius* sp. FVV and *C. elegans* (Fig. 3C, D). *Acrobeloides* lacked a well-defined rachis, with the distal gonad terminating with a string of single celled oocytes (Fig. 3E). Based on the atypical gonad morphology and small size (Fig. 3), microinjection experiments were not performed with *Acrobeloides*. *Mesorhabditis* sp. requires a con-specific male to be added to the plate after injection. Feeding RNAi experiments were performed with the IPTG inducible L4440 plasmid and *E. coli* strain HT115 using standard *C. elegans* techniques (Timmons et al., 2001), with a male added to the feeding plate for *Mesorhabditis* sp. Control plates were seeded with HT115 containing empty L4440 vector.

TABLE 1. PCR primers used in this study.

Target	Sequence (5'-3')	Species
dpy-5	MH991: GAYATGTAYGAYGAYGTNATGGG MH993: CC/ideoxyI/GSNGGRCAYTTDATRCA	*Oscheius sp. Rhabditis sp.*
unc-54	MH1030: GTGCGTTACAACTGCTTGAA MH1032: GCGTAACGTTGGACGAAGTC	*Oscheius* sp., *C. elegans*
sqt-1	MH1028: AATTGTGGCGTGATATCATG MH1029: GGGAAACCTTTGGGTCC	*Acrobeloides* sp.
unc-54	MH1085:AAR TTY GAR AAR AC/ideoxyI/ GAR GAY ATG MH1086: AAY TTC ATR TTN CCC ATR TGC AT	*Rhabditis/ Mesorhabditis.*
ama1	1108: GTA TCN ATH TTY TAY GAR ATG CC 1109: ARR AAN ACR TCR TCY TCC AT	*Rhabditis/ Mesorhabditis.*
unc-54	MH1116: CCC AAG TCG AGG TCT CCC MH1117: AGA GTG GCA CGC TTC TCG	*C. briggsae*
ama-1	MH1118: CGA GCT CGC CGA CGT ACA CC MH1119: GTT GGC GAC GTC GGA GAG TAC TG	*C. briggsae*
dpy-5	MH1120: GGG CTC CGC GCT TTC CAG MH1121: GAG AGC TCG GAG GAT TCA GAG A	*C. briggsae*
Sid-1	MH1122: GAY ACN CCN TGY ATH CAR GTN AT MH1123: GTC CAR TCN GTR TCR TCY TG	N/A
dpy-5	MH987: CTGGGGCTCCTGGGTTTCCTG MH977: CGTCGTCGGATTCGGCGC	*C. elegans*
Ama-1	MH1147: GTY AAG AAG YTN ACN ATG GAR CA MH1148: TCGAANGADATNACRTTRTTCAT	*Oscheius* sp.
vector	MH1041: TAATACGACTCACTATAGGG pGEMT7R : TAATACGACTCACTATAGGGATATGGTCGACCTGC	N/A

Real Time Quantitative Reverse transcribed PCR: Three biological replicates, each consisting of 25 progeny (young adults) collected from an independently injected individual, were performed for each qRT-PCR experiment. RNA was extracted using Trizol (Invitrogen, Carlsbad CA) as described by the manufacturer. Resulting RNA was treated with 1 unit/μg of DN*ase* I (Promega, Madison WI) before dT-primed reverse transcription using the Superscript kit (Invitrogen, Carlsbad CA). All RT experiments included control reactions containing no reverse transcriptase to identify contamination from genomic DNA. The resulting cDNA was diluted 1/5 in water and 1 μl was used as template in a 20 μl qRT-PCR reaction with amplification detected by SYBR® florescence. Optimization experiments using three technical replicates resulted in very little technical variation (SD between replicates < 0.38), therefore all subsequent experiments were performed with two technical replicates (Pfaffl, 2001). All primer sequences used in the qRT-PCR are available on request. Real-time PCR was carried out on a Bio-Rad iCycler (Bio-Rad, Hercules CA) with the cycling conditions: 1 cycle 95°C × 5 min, 40 cycles of 95°C × 10 sec, 57°C × 45 sec. Melt curve analysis was also performed to verify primer specificity. Ct values were determined using the iCycler iQ software version 3.1 (Bio-Rad, Hercules CA). Normalized relative expression ratios and statistics (Based on a Pair Wise Fixed Reallocation Randomization Test) were determined by the REST software package, version 2.0.7 (Pfaffl et al., 2002), with expression normalized to the housekeeping gene *ama-1* (large subunit of RNA polymerase II) of each species tested.

TABLE 2. Genes targeted by RNAi.

Gene	Grassland nematode	Top database hit[a]	e-value
dpy-5	*Oscheius sp* FVV-2	*C. elegans dpy-5*	8e-16
	Rhabditis sp	*C. elegans dpy-5*	5e-40
unc-54	*Oscheius sp* FVV-2	*C. briggsae let-75*	1e-33
	Rhabditis sp	*C. briggsae unc-54*	9e-107
	Mesorhabditis sp	*C. elegans unc-54*	2e-108
sqt-1A	*Acrobeloides* sp	*Z. punctata sqt-1*	9e-20
sqt-1B	*Acrobeloides* sp	*Z. punctata sqt-1*	8e-18
ama-1	*Oscheius sp* FVV-2	*Oscheius tipulae ama-1*	4e-38
	Rhabditis sp	*Rhabditis* sp *ama-1*	2e-16
	Mesorhabditis sp	*Mesorhabditis* sp *ama-1*	1e-23

[a] BLASTX hit to the non-redundant database.

Bioinformatics: To identify potential orthologs of *C. elegans sid-1* from mammals, arthropods, and nematodes, we performed TBLASTN searches of the NCBI non-redundant and EST databases. Sequences with a BLASTX e-value smaller than $1e^{-5}$ were included in the phylogenetic analysis. In the non-*Caenorhabditis* nematodes full-length sequences for *Brugia malayi*, and partial cDNA sequences for *Xiphinema index* (genbank #AW773473) and *H. bacteriophora* (Sandhu et al., 2006) were found in the NCBI database. A *sid-1* ortholog could not be identified in the recently completed genome of the satellite model species *P. pacificus*. For the phylogenetic analysis a protein alignment of the SID-1 sequences generated by *CLUSTLW2* (http://www.ebi.ac.uk/Tools/clustalw2/) was used to create a corresponding biologically relevant DNA alignment before the removal of third codon positions to reduce homoplasy. As has been reported previously the *C. elegans* SID-1 N-terminal

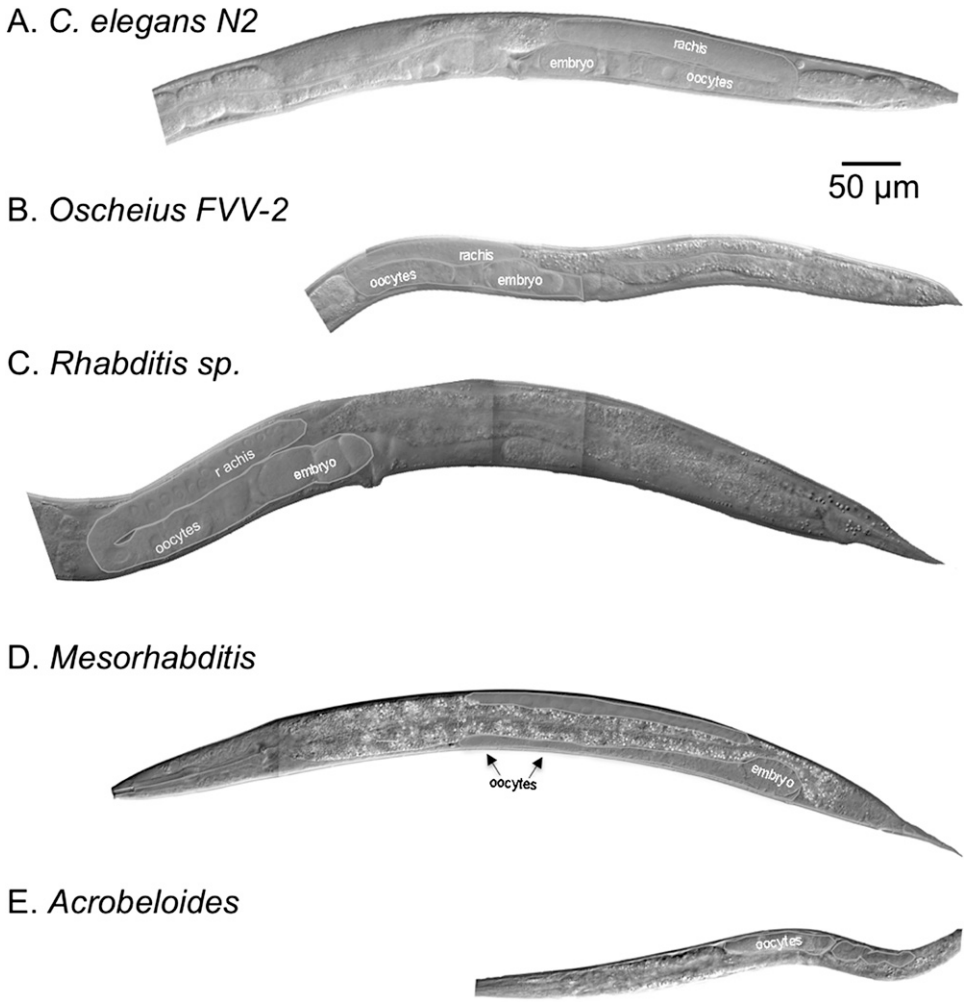

FIG. 3. DIC composite images showing the gonad structures of the nematodes in used in this study. Bar = 50µm. One gonad arm of each nematode is highlighted in pink with the rachis, oocyte and embryo regions labeled where they are visible. A) *C. elegans N2,* B) *Oscheius FVV-2.* C) *Rhabditis* sp., D) *Mesorhabditis* sp., E) *Acrobeloidies* sp. For the bidelphic species: *C. elegans*, *Oscheius* and *Rhabditis* sp., the other gonad arm is behind the intestine and is not visible. *Mesorhabditis* sp. and *Acrobeloides sp.* have monodelphic gonads. See text for additional explanations.

domain aligns poorly with arthropod and vertebrate SID-like sequences, thus for the protein alignment only regions from transmembrane domain 2 to 11 were used (Tomoyasu et al., 2008).

Phylogenetics: Bayesian phylogenetic trees were generated using MrBays (v3.1.2) (Ronquist and Huelsenbeck, 2003). The most appropriate model of *sid-1* sequence evolution was determined to be the GTR+G model as selected by Akaike Information Criterion in JModeltest version 0.1.1 (Posada, 2008). Maximum parsimony (MP) and distance trees were derived using the PHYLIP package, bootstrap analyses was performed using 1000 pseudo-replicates of the dataset (Phylogeny Inference Package) version 3.68 (Felsenstein, 1993). The accession numbers of the sequences used in the phylogeny are: XP_789210, XP_002941891, XP_416544, XP_001367317, NP_001152891, NP_060169, XP_001235205, NP_758461, NP_001035545, XP_001380860, BAH22347, PP50833, XP_003093368, EGT59237, XP_002645379, ABU75284, XP_974254, XP_974836, NP_001103253, XP_395167, XP_001615484, NP_001106736, NP_001106735, BAF95807, ABP98803, XP_001951907, XP_001901528, AW773473, DN153307, CJA17163, EGT42616, NP_504372, XP_003113953, XP_002636380.

RESULTS

Feeding RNAi: We used standard *C. elegans* feeding RNAi techniques (see Materials and Methods) to knockdown endogenous *dpy-5*, *unc-54*, *sqt-1A*, and *sqt-1B* genes to test the effectiveness of RNAi by feeding in the grassland nematodes (Table 3). For the *Oscheius sp.* and *Rhabditis* sp. feeding RNAi experiments, no Dpy and less than 1% of Unc animals (n>500) were observed amongst worms fed *dpy-5* or *unc-54* dsRNA, respectively (Table 3). Similarly, *Acrobeloides* sp. feeding RNAi nematodes grown on bacteria expressing dsRNA complimentary to each of the two *sqt-1* sequences were indistinguishable from controls fed bacteria containing an empty vector. A small proportion (3/243) of

TABLE 3. Percentage of nematodes scored that have the expected knockdown phenotype based on the corresponding mutant phenotype in *C. elegans* (total number of worms scored is shown in parenthesis).

Feeding RNAi Species	*dpy-5*	*unc-54*	*sqt-1A*	*sqt-1B*
C. elegans	87.8 (608)	90.6 (625)		
C. briggsae	0 (703)			
Oscheius sp	0 (781)	0 (689)		
Rhabditis sp	0 (452)	0.3 (706)		
Mesorhabditis		1.2 (243)		
Acrobeloides sp			0 (305)	0 (49)
Microinjection				
C. elegans	86.9 (780)	74 (872)		
C. briggsae	92.4 (582)			
Oscheius sp	0 (1186)	0 (709)		
Rhabditis sp	0 (227)	0 (167)		
Mesorhabditis		4.6 (393)		

Mesorhabditis fed bacteria expressing *unc-54* dsRNA displayed the expected paralyzed phenotype. As expected from previously published observations (Winston et al., 2007) no Dpy individuals were observed when the satellite model *C. briggsae* was fed *dpy-5* dsRNA expressing bacteria (Table 3). Reference experiments targeting *unc-54* and *dpy-5* indicate that *C. elegans* is highly sensitive to feeding RNAi, with ~90% (n>600) of progeny displaying the expected knockdown phenotypes (Table 3). These experiments demonstrate the *Oscheius* sp. FVV-2, *Rhabditis* sp., *Mesorhabditis* sp. and *Acrobeloides* sp. are insensitive to feeding RNAi targeted toward two endogenous genes using standard *C. elegans* feeding RNAi techniques.

RNAi by injection: For the initial reference experiments using *C. elegans* and *C. briggsae*, robust knockdown phenotypes were observed greater than 73% of the progeny from an injected hermaphrodite displaying the expected mutant phenotype (Table 3). For the grassland nematodes, injection of dsRNA into the *Oscheius* sp. and *Rhabditis* sp. distal gonad did not result in progeny with the expected knockdown phenotypes (Table 3). For *Mesorhabditis* a modest 4.6% of progeny from injected females displayed the paralyzed *unc-54* phenotype. To further explore the potential of an RNAi mechanism in *Mesorhabiditis* sp. we next attempted to knockdown the *Mesorhabditis* large subunit of RNA polymerase II (*ama-1*). This highly conserved gene has a well-defined embryonic lethal knockdown phenotype and has previously been used to test for the effectiveness of RNAi in non-model nematodes (Aboobaker and Blaxter, 2003). In contrast to reference experiments, reproductive output was unaffected by *ama-1* RNAi in *Mesorhabditis* sp., *Oscheius* sp. and *Rhabditis* sp (Fig. 4).

Real time quantitative Reverse Transcribed PCR measurement of target gene expression: One possible explanation for the lack of robust RNAi knockdown phenotypes in feeding and microinjection experiments is that the genes targeted by RNAi in the grassland soil nematodes have become functional diverged from their counterparts in *C. elegans* and sequence similarity would therefore be a poor predictor of RNAi phenotype. To address this we used quantitative RT-PCR (qRT-PCR) to directly measure targeted gene expression in the progeny from an individual nematode injected with dsRNA. RNAi in *Oscheius* sp, FVV-2,*Mesorhabditis* sp and *Rhabditis* sp. resulted in no reduction in targeted *unc-54* expression relative to water injected controls (Table 4; p>0.05). Reference experiments with *C. elegans* showed a significant reduction in *unc-54(RNAi)* expression (~25 fold reduction) relative to the water injected control nematodes (Table 4; p<0.05). The normalized expression levels of targeted gene in *dpy-5* (RNAi) *Oscheius* sp. and *Rhabditis* sp. animals were also not significantly different to that of untreated controls (Table 4; p>0.05). *C. briggsae dpy-5(RNAi)* animals showed a significant reduction in expression (p<0.05), with a normalized expression ratio of 0.007 relative to the water injected controls. These experiments provide further evidence that *Oscheius* sp. FVV-2, *Rhabditis* sp. and *Mesorhabditis* species does not possess robust RNAi response when standard *C. elegans* techniques are adopted.

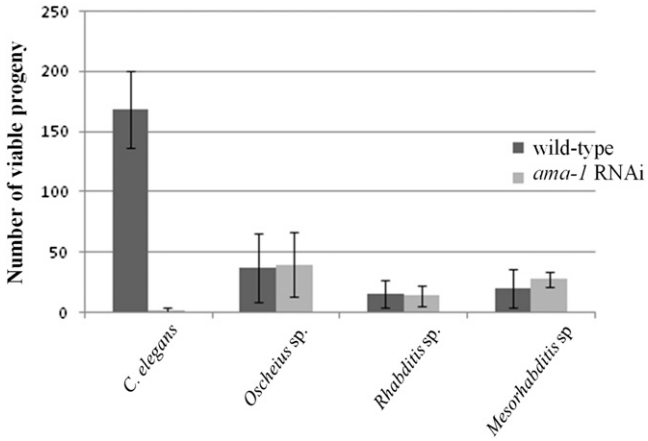

FIG. 4. Viability assay following RNAi knockdown of *ama-1* by microinjection in *C. elegans*, *Oscheius* sp, *Rhabditis* sp, and *Mesorhabditis* sp. The average number of surviving offspring from a nematode injected with either water (control) or dsRNA to the endogenous *ama-1* gene of that species. Only the N2 RNAi treatment significantly (t-test p>0.01) reduced progeny viability. Error bars indicate standard deviation.

TABLE 4. qPCR determined relative expression ratios[a] of grassland soil nematode genes targeted by RNAi (SEM for average delta-CT values of the RNAi target gene is shown in parenthesis).

species	*dpy-5*	*unc-54*
C. elegans	NT	0.04 (0.3)*
C. briggsae	0.007 (0.83)*	NT
Oscheius sp. FVV-2	0.6 (0.57)	0.53 (0.3)
Rhabditis sp.	1.48 (1.4)	1.2 (0.02)
Mesorhabditis sp.	NT	0.57 (0.2)

[a] Target gene expression in uninjected animals was set at one and data was normalized using the expression of the conserved *ama-1* gene, thus an expression ratio of 1.0 indicates no change in expression in response to RNAi.

* indicates significance difference from water injected control (p<0.05) as determined by the REST qPCR analysis package (Pfaffl et al., 2002). NT is not tested.

Phylogeny of the systemic RNAi channel gene sid-1: The trans-membrane channel protein SID-1 is thought to allow cell-to-cell movement of the dsRNA silencing signal in *C. elegans*, and thus is required for systemic RNAi in this species (Winston et al., 2002). As we were unable to amplify *sid-1* sequences (results not shown) from the grassland nematodes, the absence of a *sid-1* could explain why these species have a poor RNAi response. We attempted to improve on previous studies (for example: Tomoyasu et al., 2008; Xu and Han, 2008) by using DNA sequences to increase the number of phylogenetic sites available to recover *sid-1* evolutionary relationships. The resulting Baysian phylogenetic tree in Figure 5 shows a nematode-specific *sid-1* clade is formed with *B. malayi*, *X. index,* and *H. bacteriophora* sequences at its base, however, the placement of sequences from these latter three species has only modest support (0.63, 0.63, and 0.74, respectively). A second well-supported nematode clade contains the *P. pacificus* and *Caenorhabditis tag-130* orthologs. Multiple independent duplications of the ancestral *sid*-like sequence have occurred in the arthropod clade, giving rise to lineage specific gene expansions seen in *Tribolium* and *B. mori*. The vertebrate sequences are divided amongst two well supported (0.89) SIDT1 and SIDT2 gene lineages that formed by a duplication at the base of the vertebrate lineage. Consistent with previous analyses (Tomoyasu et al., 2008; Xu and Han, 2008) there is little resolution at the important node that describes the relationship between arthropod and vertebrate *sid*-like sequences and the *Caenorhabditis sid-1* and *tag-130* gene lineages. Distance and Parsimony trees derived from this alignment had the same arrangements of nodes when bootstrap support was significant (>80%) (not shown).

Discussion

In this study we investigate the feasibility of using RNAi as a tool to study gene function in four ecologically relevant nematodes isolated from Tallgrass prairie soils in the mid-western United States. We show that introduction of dsRNA by injection or by feeding bacteria expressing dsRNA, as is standard when working with *C. elegans*, do not result in a robust knockdown phenotype in three species of Rhabditidae and a single species from Cephalobidae. This conclusion is supported by results from reference experiments with *C. elegans* and *C. briggsae*, where a robust RNAi response

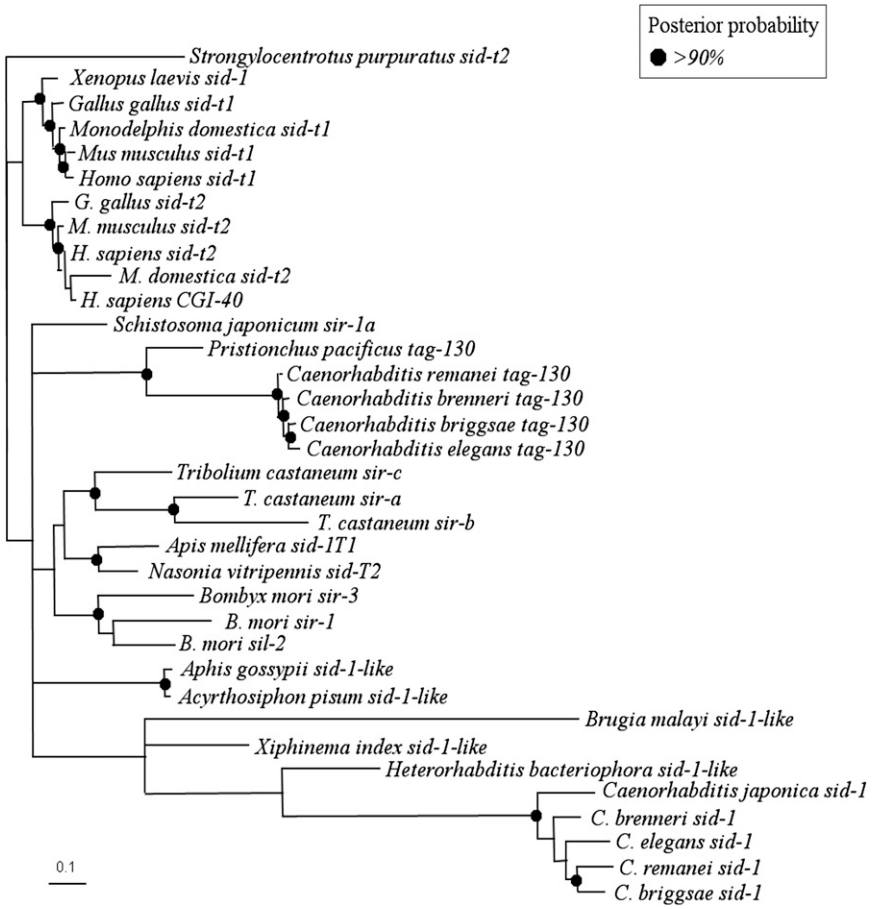

Fig. 5. Bayesian phylogenetic tree of vertebrate, arthropod and nematode *sid*-like genes. The tree was derived from an alignment of *sid*-like coding regions 3rd codon positions removed to reduce the effect of homoplasy. Posterior probability values greater than 90% are indicated by the filled circles.

could be reproducibly detected using the same experimental methods as adopted on the grassland soil nematodes. Although qRT-PCR assays detected a decrease in *Mesorhabditis* sp. and *Oscheius* sp. FVV-2 expression following dsRNA microinjection, this effect was between 10 to 70-fold less potent than that observed in *C. elegans* and *C. briggsae*. We also observed a low percentage (4.6%) of *Mesorhabditis* sp. with the expected *unc-54* movement defect, thus this nematode species may have a weak or poorly penetrant RNAi response for *unc-54*. A complicating issue with *Mesorhabditis* is that this nematode is gonochoristic (male-female species); in many cases injected females do not lay eggs possibly because they did not mate. The poor RNAi response of *Mesorhabditis* relative to *C. elegans*, combined with the low fecundity, would make this nematode an ineffective model species for functional studies using RNAi. In the following paragraphs, we discuss three potential reasons we did not observe a robust RNAi knockdown effect in these grassland soil nematode species.

1) RNAi functions in these nematodes just as in C. elegans, *but the genes we chose are either not transcribed, or do not have similar knock-down phenotypes.* Although the grassland nematode genes targeted in the RNAi assays have sequence homology to *unc-54*, *dpy-5*, *ama-1*, and *sqt-1* in in *C. elegans*, the relatively small regions of sequence overlap and the lack of genome data from the grassland soil nematodes make it impossible to conclusively predict the expected knockdown phenotypes. The qRT-PCR assays address this problem by directly measuring mRNA levels of genes targeted by RNAi, rather than relying on a visible phenotype. However, as the RNA used in the qRT-PCR experiments was obtained by pooling 25 progeny from a treated nematode, a knockdown effect occurring in a small proportion of progeny would be largely 'hidden' by gene expression in unaffected individuals present in the sample. Potentially this problem could be overcome by individually testing progeny from an injected nematode using single worm qRT-PCR. However, it is important to recognize that the utility of RNAi for functional studies necessitates a high level of penetrance of the knockdown phenotype, thus the identification of an RNAi effect in a small percentage of the progeny would not be of practical research value.

2) RNAi functions in these nematodes as in C. elegans, *but the signal is prevented from expanding across cells due to incompatible morphology or deficient environmental uptake mechanisms.* RNAi by injection is a robust method for introducing dsRNA into *Caenorhabditis* nematodes, even those that lack an environmental RNAi response. During our initial characterization of the grassland soil nematodes used in this study we found that the number of pre-oocytes in the distal gonad that would be available to take up the dsRNA varies between species. Therefore, a detailed examination of gonad morphology may prove to be a good initial screen for identifying nematodes suitable for the microinjection based RNAi. The failure of feeding RNAi to elicit a knockdown response in the grassland soil nematodes tested was less surprising (Viney and Thompson, 2008). Environmental RNAi in *C. elegans* is dependent on *sid-2*, a gene that has thus far only been identified in the genome of *C. briggsae* and *C. remanei*; both of these species are insensitive to RNAi response by feeding and soaking and are thought to possess non-functional *sid-2* orthologs (Winston et al., 2007). Also, Dalzell et al (2011) demonstrate that most genes responsible for the uptake and spread of dsRNA in *C. elegans* are absent from parasitic nematodes. This observation suggests that the *C. elegans* mechanism of environmental RNAi may have arisen relatively recently, and the environmental RNAi observed in *Heterorhabditis bacteriophora* (Ciche and Sternburg, 2007) and several parasitic nematodes has evolved independently (Aboobaker and Blaxter, 2003; Issa et al., 2005; Geldhof et al., 2006; Kotze and Bagnall, 2006; Visser et al., 2006).

3) Systemic RNAi does not function in these species as it does for C. elegans. The presence of a *sid-1* gene in an organism's genome has been proposed as a potential indicator of a systemic RNAi mechanism (Dong and Friedrich, 2005), however, based on comparisons between the *sid-1* and *tag-130* genes we agree with the conclusions of Tomoyasu et al., (2008) that this observation is likely deceptive. A probable key event in the evolution of *sid-1* was the timing of the *sid-1/tag-130* divergence (Fig. 5). If the duplication that produced these two gene lineages occurred before the arthropod/nematode divergence, the *sid*-like genes of arthropods could be related to either the *sid-1* or *tag-130* ancestor. Alternatively, if the *sid-1/tag-130* divergence occurred within the nematode lineage it is possible that *sid-1* was later recruited for its role in systemic RNAi. Although our phylogenetic analyses (Fig. 5) did not differentiate between these two possibilities, we favor the latter hypothesis based on the following observations. Firstly, a number of organisms with apparent systemic RNAi mechanisms lack *sid-1* in their genomes (Xu and Han, 2008). Secondly, arthropod and vertebrate *sid*-like sequences are structurally similar to the CeTAG-130, especially the first 200 amino acids that form the extracellular region of the protein, which is clearly unique in the case of *Caenorhabditis* SID-1. Thirdly, both CeTAG-130 and the three *Tribolium sid*-like genes do not appear to play a role in RNAi (Tomoyasu et al., 2008). Finally, nematodes are the only group that contains both TAG-130 and SID-1 members (Fig. 5), so the most parsimonious explanation for this observation would be that the divergence occurred within the nematode lineage. The alternative hypothesis, that the *tag-130/sid-1* duplication occurred in the ancestor of nematodes and arthropods, would require the extinction of either the *sid-1* or *tag-130* genes at the base of the arthropod lineage (Fig. 5).

The grouping of the *H. bacteriophora sid-1 like* sequences with the *Caenorhabditis sid-1* genes in our phylogeny (Fig. 5) hints at a conserved role of these genes given that *H. bacteriophora* also has a robust RNAi response. Currently, we are not aware of any published findings on the susceptibility of *X. index* to RNAi, but given the presence of a *sid-1* sequence related it will be interesting to determine if this is the case for this economically important pest nematode. As more genome sequences become available for *H. bacteriophora* and *X. index*, as well as other nematodes that form deep branches in the phyla, the additional sequences data will hopefully improve the SID-1 phylogeny (Fig. 5).

RNAi has been adopted by many researchers as the method of choice for studying gene function in non-model nematode taxa. However, few groups have carefully described the characteristics of the RNAi response directly in the organism under investigation. Perhaps then it is not surprising that several studies have reported RNAi results that are difficult to interpret in light of the robust RNAi phenotypes observed in the distantly related *C. elegans*. Inconsistencies in the data can often be blamed on the significant technical challenges associated with working on nematodes that have complex life history traits (for example parasitism), however, other results point to the existence of as yet uncharacterized species-specific mechanistic differences in the RNAi pathway (Felix, 2008; Dalzell et al., 2009). Several studies have reported strong gene-specific variations in the effectiveness of RNAi; in some cases expression of the majority of genes tested was unaffected by the treatment (specifically Issa et al., 2005; Geldhof et al., 2006; Visser et al., 2006). Also, the efficiency of RNAi in *N. brasiliensis* (Hussein et al., 2002) and *H. glycines* (Sukno et al., 2007) has been shown to be highly dependent on which part of the coding region is used to generate the dsRNA. Studies in *B. malayi*, *Trichostrongylus colubriformis*, *Schistosoma mansoni* have also shown that, in contrast to results in *C. elegans* (Winston et al., 2002; Feinberg and Hunter, 2003; Tijsterman et al., 2004), siRNAs are as effective as dsRNAs in eliciting an RNAi response (Ford et al., 2009; Krautz-Peterson et al., 2010). The specificity of the RNAi mechanism appears to vary between different species as well, for example, cross-species RNAi between distantly related nematodes has recently been reported in *Panagrolaimus superbus* (using *Aphelenchus avenae* dsRNA) and *Ascaris suum* (using *C. elegans* dsRNA) (Gao et al., 2006; Reardon et al., 2010). DNA sequence homology degrades relatively quickly, so the ability of small regions of homology to initiate an RNAi response introduces the possibility that off-target effects may significantly complicate the interpretation of mutant phenotypes. This would especially be the case in species where little genomic sequence information available to bioinformatically verify the specificity of the injected dsRNA.

In conclusion, we investigated the effectiveness of RNAi in four non-model grassland nematodes and found that neither feeding nematodes bacteria expressing dsRNA nor injecting dsRNA into the gonad produced the expected RNAi knockdown phenotypes in any of the grassland soil nematodes. This is consistent with other studies that have reported limited reproducibility of RNAi in various nematode species (see Knox et al., 2007; Ford et al., 2009). Due to low effectiveness and inconsistent reproducibility, we suggest that a more primary research needs to be carried out to increase our understanding of the mechanistic differences in the RNAi pathway between species, specifically with respect to gene- and sequence- specific variations in the effectiveness of RNAi. This understanding may lead to new methodologies that improve the reproducibility and effectiveness of RNAi in non-model nematodes.

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