

# Ascaroside Signaling Is Widely Conserved among Nematodes

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## Summary

**Background:** Nematodes are among the most successful animals on earth and include important human pathogens, yet little is known about nematode pheromone systems. A group of small molecules called ascarosides has been found to mediate mate finding, aggregation, and developmental diapause in *Caenorhabditis elegans*, but it is unknown whether ascaroside signaling exists outside of the genus *Caenorhabditis*.

**Results:** To determine whether ascarosides are used as signaling molecules by other nematode species, we performed a mass spectrometry-based screen for ascarosides in secretions from a variety of both free-living and parasitic (plant, insect, and animal) nematodes. We found that most of the species analyzed, including nematodes from several different clades, produce species-specific ascaroside mixtures. In some cases, ascaroside biosynthesis patterns appear to correlate with phylogeny, whereas in other cases, biosynthesis seems to correlate with lifestyle and ecological niche. We further show that ascarosides mediate distinct nematode behaviors, such as retention, avoidance, and long-range attraction, and that different nematode species respond to distinct, but overlapping, sets of ascarosides.

**Conclusions:** Our findings indicate that nematodes utilize a conserved family of signaling molecules despite having evolved to occupy diverse ecologies. Their structural features and level of conservation are evocative of bacterial quorum sensing, where acyl homoserine lactones (AHLs) are both produced and sensed by many species of gram-negative bacteria. The identification of species-specific ascaroside profiles may enable pheromone-based approaches to interfere with reproduction and survival of parasitic nematodes, which are responsible for significant agricultural losses and many human diseases worldwide.

## Introduction

Nematodes are among the most abundant animals in the world [1]. They inhabit sulfurous sediment, deep-sea trenches,

mammals, insects, plants, arctic ice, and many other habitats, making them one of the most successful groups of animals on earth [2–7]. Many nematode behaviors, such as mate finding in roots, bacteria, sand, agar, and intestines, have been studied [8]. Although there have been many attempts to identify nematode pheromones [8], identification has only been successful in very few species [9–17].

In *C. elegans*, a family of small-molecule pheromones called ascarosides regulates gender-specific attraction, repulsion, aggregation, olfactory plasticity, and entry into dauer (a stress-resistant life stage), collectively demonstrating that ascarosides mediate a wide range of *C. elegans* behaviors [10–13, 15–20]. The *C. elegans* ascarosides might be derived from ascarylose-based lipids similar to those first identified in the eggshell layer of the horse parasitic nematode, *Parascaris equorum* [21]. Related ascaroside lipids have also been identified from the human parasite, *Ascaris lumbricoides*, and the pig parasite, *Ascaris suum* [21]. The ascaroside lipid layer is thought to impart remarkable resistance to chemical degradation [22], which likely contributed to the success of ascarid nematodes as some of the most pervasive parasites in the world [21, 23]. It is not known whether ascarid nematodes secrete short-chain ascarosides similar to those identified from *C. elegans* to mediate social behaviors. Ascarosides have not yet been found in any other animal phylum [22], and thus we hypothesized that short-chain ascarosides comprise a nematode-specific chemical code mediating a variety of behaviors in many different species.

To investigate this possibility, we collected worm exudates from a wide range of both free-living and parasitic nematode species and used high performance liquid chromatography/mass spectrometry (HPLC/MS) to screen specifically for ascarosides. We found that most of the tested nematode species produce ascarosides. We further observed that even evolutionarily diverged nematodes sometimes produce the same ascarosides, suggesting that ascaroside biosynthesis is highly conserved among nematodes. Using several different chemotaxis assays, we show that different nematode species respond to distinct, but partially overlapping, sets of ascarosides.

## Results

### Ascarosides Are Produced by a Wide Range of Nematode Species

We initiated a mass spectrometry-based screen for ascarosides (Figure 1) in a wide selection of nematode species, including both free-living and parasitic (plant, insect, and mammal) nematodes. To obtain sufficiently large samples, we collected exudates from developmentally mixed nematode cultures, which contained ascarosides released by both larval and adult stages. Because previous studies have shown that *C. elegans* ascaroside production is developmental stage-specific [24], we additionally sampled parasitic infective juveniles and adults separately, when feasible. Collected nematode exudates were analyzed via HPLC/MS using a protocol optimized for the detection of ascarosides in complex metabolome samples (see Supplemental Information available

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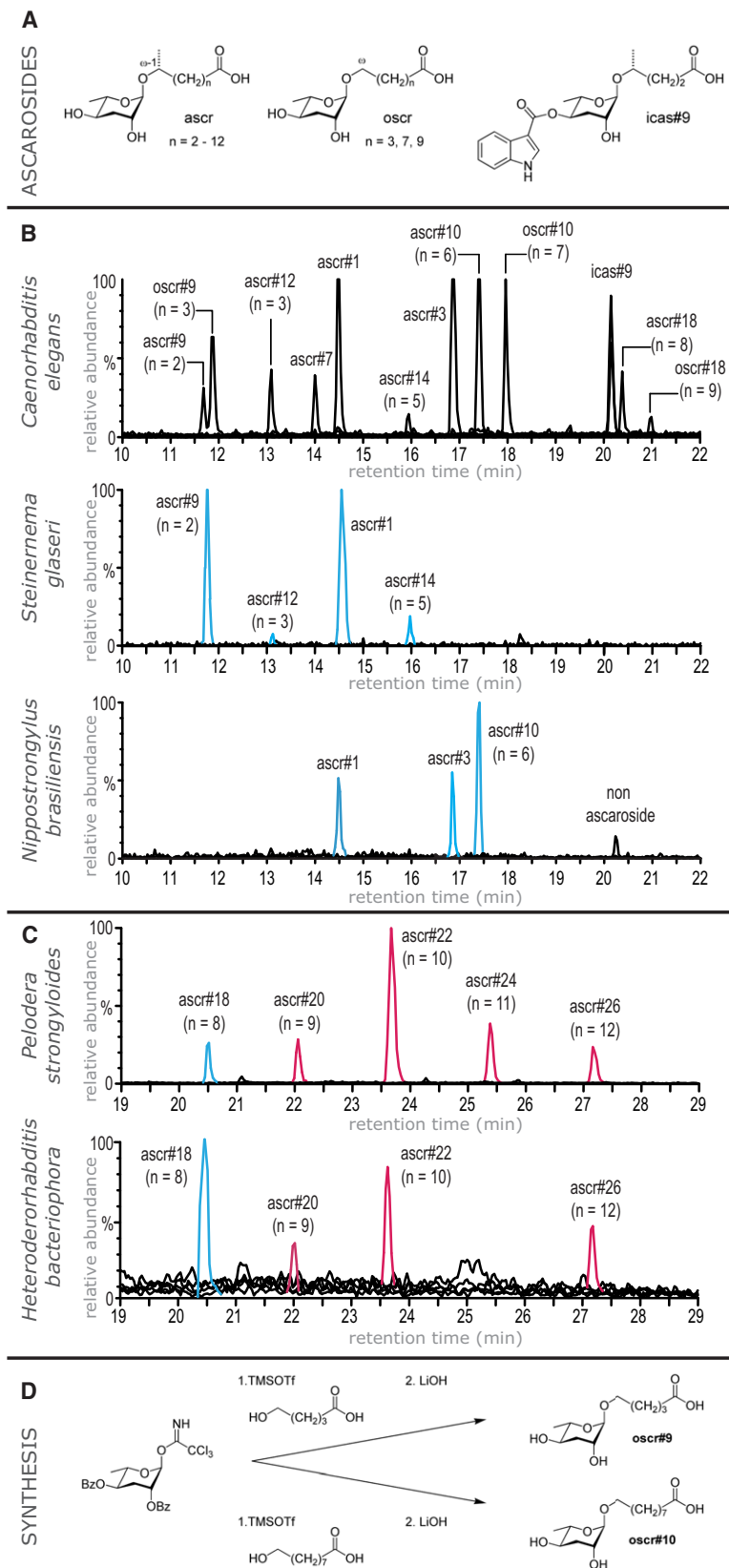


Figure 1. Structures, Detection, and Synthesis of Ascarosides

(A) Structures of simple ascarosides (ascr) and indole ascarosides (icas) previously identified from *C. elegans*. In some cases, the ( $\omega$ -1)-oxygenated ascars are accompanied by their ( $\omega$ )-oxygenation isomers (oscr). For ascaroside nomenclature, see [www.smid-db.org](http://www.smid-db.org).

(B) HPLC-MS analysis of short and medium chain ascarosides from free-living *C. elegans* (mixed-stage), insect parasitic *S. glaseri* (adult), and rat parasitic *N. brasiliensis* (adult). Analysis of *C. elegans* exudate showed the known ascarosides (ascr#1, #3, #7, #9, #10, #12, #14, and #18), the indole ascarosides, icas#3, #9, as well as several ( $\omega$ )-oxygenated isomers (oscr#9, #10, #18). The highly polar ascr#5 eluted outside of the shown retention time range. Cross-species comparison of HPLC/MS data revealed that ascarosides are also found in many other nematode species, including *S. glaseri* and *N. brasiliensis* (peaks representing compounds also abundant in *C. elegans* shown in blue).

(C) HPLC/MS identification of medium- and long-chain ascarosides in *P. strongyloides* (mixed-stage) and *H. bacteriophora* (adult). Ascr#18 (blue) is also produced in significant amounts by *C. elegans*, whereas longer chain homologs (red peaks) are abundantly produced by *P. strongyloides* and *H. bacteriophora*, but not WT *C. elegans*.

(D) Synthesis of ( $\omega$ )-oxygenated ascarosides, oscr#9 and oscr#10. See also Figure S1.

*C. elegans* [17]. This HPLC-MS-based screen revealed the presence of ascarosides in most of the analyzed nematode samples (Figure 2). In all cases, ascarosides were found to occur as mixtures of two or more compounds, with species- and life-stage-specific variation of the ascarosides' chemical structures and their quantitative ratios.

### Ascaroside Structures and Phylogeny

The majority of the ascaroside-producing nematode species we analyzed exclusively produce ascarosides that bear the ascarylose sugar at the penultimate carbon of the side chain (“ $\omega$ -1-functionalization”), except for several members of the Rhabditidae family, *Caenorhabditis sp. 7* and *Rhabditis sp.* (strain AF5), which additionally produce ascarosides in which the ascarylose is attached to the terminal carbon of the side chain (“ $\omega$ -functionalization,” e.g., oscr#9 and oscr#10 in Figure 1D) [17]. Similarly, the indole ascaroside, icas#9, (Figure 1A), which acts as aggregation signal in *C. elegans* [20], was found only in several members of the Rhabditidae family: *Caenorhabditis spp.* and *Oscheius carolinensis*. These findings suggest that  $\omega$ -functionalization and attachment of the indole carboxy group may represent innovations distal to the derivation of clade 9, whose members include the Rhabditidae family [25].

We also found clade-specific variation of the side chain lengths of ascarosides. For example, nematodes from clade 9 (*Pelodera sp.*, *Oscheius sp.*, and *Heterorhabditis sp.*) abundantly produce ascarosides with longer side chains containing 12–15 carbons, whereas species from clade 10 (*Steinernema spp.* and *Panagrellus*

online). Briefly, samples were compared to mass spectroscopic data from a library of 150 ascarosides recently identified from both wild-type (WT) and mutant strains of

*sp.*, and *Heterorhabditis sp.*) abundantly produce ascarosides with longer side chains containing 12–15 carbons, whereas species from clade 10 (*Steinernema spp.* and *Panagrellus*

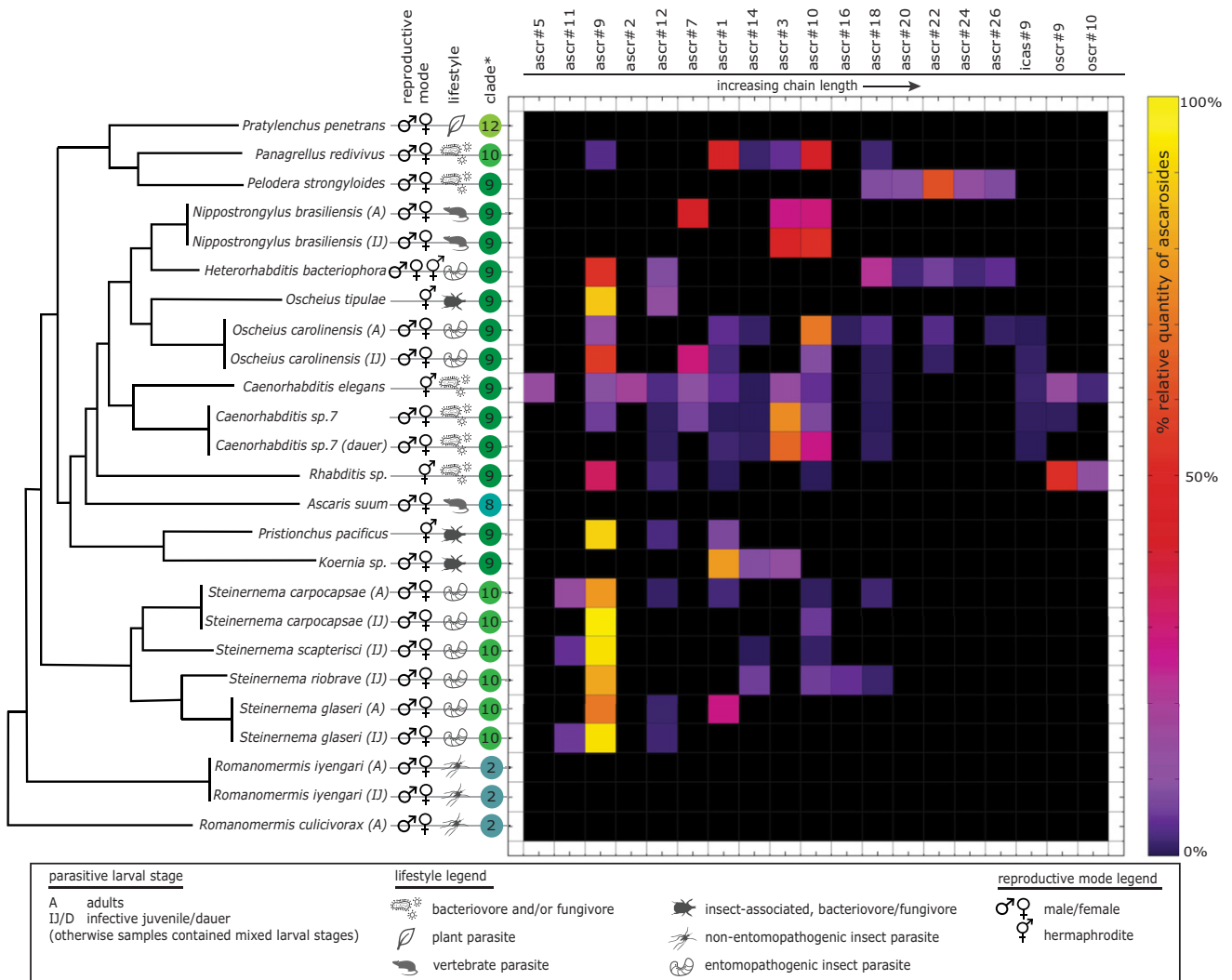


Figure 2. Ascarosides Are Produced by a Wide Range of Nematode Species

The shown plot summarizes the results from our HPLC/MS-based screen of worm exudate samples (see also Figure S2). Colors in this heatmap represent relative abundance of ascarosides detected by HPLC/MS, as indicated in the bar diagram on the right. As an example, of the ascarosides detected in *S. glaseri* infective juveniles, the total was composed of 5.7% ascr#11, 92.2% ascr#9, 2.1% ascr#12, and 0.1% ascr#10. The phylogenetic tree was constructed by comparison of small subunit ribosomal DNA (SSU rDNA) sequences obtained from GenBank. \*Clade designation adapted from Holterman et al. [25].

sp.) lack such ascarosides. Notably, species from the genus *Oscheius* and the families Steinernematidae and Heterorhabditidae comprise all known members of the entomopathogenic guild [26, 27], yet their phylogenetic separation into distinct clades is recapitulated by clade-specific trends in ascaroside chain length, rather than lifestyle. However, we also found commonalities among the ascaroside profiles of species that share similar ecological niches. For example, all species whose ascaroside blends consisted of more than 50% ascr#9 are insect-associated, including *Oscheius tipulae* (whose name derives from having first been isolated from the beetle, *Tipula paludosa* [28]), the insect-parasitic *Heterorhabditis bacteriophora*, *Oscheius carolinensis*, all *Steinernema* spp. and the necromenic insect-dwelling *Pristionchus pacificus*, suggesting that ascr#9 may play a conserved role in insect-associated nematode species.

Based on this evidence, ascaroside biosynthesis patterns appear to correlate in part with phylogeny as well as with

lifestyle or ecological niche. Furthermore, we found that ascaroside production is life-stage-dependent. Dauers and infective juveniles generally produced ascaroside blends similar to those of adults, although relative abundances of specific compounds may differ significantly. For example, *S. glaseri* adults produce high relative amounts of ascr#1, whereas this compound was undetectable in our *S. glaseri* infective juvenile samples. Generally, we found that dauers and infective juveniles release less diverse sets of ascarosides, which may reflect physiology-driven changes in ascaroside biosynthesis or secretion. For example, the dauer and IJ cuticle covering the buccal cavity and the anus may prevent ascarosides from being released, given that *C. elegans* ascarosides are likely made in the intestine [29].

We were unable to detect any of the known ascarosides in 4 of the 19 analyzed nematode species, namely *Pratylenchus penetrans*, *Ascaris suum*, and the *Romanomermis* species. It is possible that these species produce only very small

quantities of ascarosides, or that they produce ascarosides with unexpected structural features that could not be detected with our method, given that we screened for ascarosides similar to those identified from WT and mutant strains of *C. elegans*. Previous studies have reported the presence of lipid-like long-chain ascarosides in *Ascaris suum*; however, they were limited to oocytes and eggs [22, 30]. Given their highly lipophilic character, these compounds are poorly soluble in aqueous media, explaining their absence in our analysis and, furthermore, making them unlikely to serve as mobile signaling molecules. Mermithid species, such as *Romanormis spp.*, have the unique ability to store a lifetime supply of lipid-composed sustenance [31, 32]. Such differences in fatty acid metabolism may be related to the observed absence of short-chain ascarosides in *Romanormis spp.* Alternatively, ascaroside biosynthesis might not have evolved until after the *Romanormis* lineage diverged from that of the other nematode species studied here.

#### Natural Variation of Ascaroside Production in *C. elegans*

The analyses described in the preceding section revealed strong differences in the ascaroside profiles of different nematode species. Next we asked to what extent ascaroside biosynthesis is subject to natural variation within one species. For this purpose, we analyzed four genetically divergent WT strains of *C. elegans*, N2 (Bristol, UK), DL238 (Manuka, Hawaii), CB4856 (Oahu, Hawaii), and JU1527 (Orsay, France) [33]. We found that despite occupying geographically distant niches, all four strains produced similar sets of ascarosides in mostly similar ratios (Figure S2). However, we also detected several strain-specific differences. Most significantly, ascr#1 is much more abundantly produced by DL238 (Manuka, Hawaii) and JU1527 (Orsay, France) than by the other Hawaii strain CB4856 and by N2 Bristol. Furthermore, the ascaroside profile of N2 revealed much larger quantities of the  $\omega$ -oxygenated ascaroside oscr#9 than the other three strains. These results show that genetically divergent *C. elegans* natural isolates produce partially divergent ascaroside profiles; however, these intraspecific differences are very small compared to most of the differences we found between samples from different nematode species.

#### Ascarosides Mediate Distinct Nematode Behaviors

Based on the diverse functions of ascarosides in *C. elegans*, we asked whether ascarosides serve as signaling molecules in other ascaroside-producing nematode species. We tested nematode behavioral responses to synthetic samples of the detected ascarosides in several different behavior assays. We started with a retention assay (Figure 3), using automated software to detect preference or avoidance of regions conditioned with individual ascarosides (Figure S3). Not all nematode species in our study were compatible with this assay, because it requires adequate movement across a bacterial lawn. Parasitic nematodes were largely excluded as a result of their requirement of substrates similar to their host environment. We further limited this investigation to males, because in most species males were much more mobile than females or hermaphrodites, with the exception of *P. redivivus* females, which moved well and were therefore included. *Oscheius tipulae* was excluded as a result of the fact that males are only occasionally present in this hermaphroditic species. Instead, we added the closely related male-female species, *Oscheius dolichuroides*. In a previous study, samples of ~1 pmol ascr#2 and ascr#3 proved to be the most active

concentration for attraction by simple ascarosides in *C. elegans* [10]. Another study showed that 25 young adult *C. elegans* hermaphrodites secrete roughly 0.5 pmol of ascr#2 over the course of 6 hr [24]. For these reasons, we tested a similar amount, 0.6 pmol, and further tested concentrations that were higher and lower than this physiological dosage (0.6 fmol and 0.6 nmol) of ascarosides. It should be noted that, because the applied quantities of ascarosides diffuse into the agar and thereby undergo dilution, the concentrations required for attraction are likely lower than the concentrations used to apply the ascarosides in these assays.

We found that several of the tested nematode species were attracted to areas conditioned by ascarosides, particularly ascr#1, ascr#3, ascr#7, ascr#8, and ascr#10 (Figure 3). Some compounds showed activity in several species, for example ascr#3 and ascr#8, whereas other compounds were only active in a single species, such as oscr#9. In addition, activity thresholds varied between species. For example, *C. elegans* males preferred pmol-conditioned regions of ascr#10, whereas *S. glaseri* and *P. redivivus* males were only attracted to nmol-conditioned regions of ascr#10. We further observed gender-specific responses to ascarosides. The ascaroside, ascr#3, which is produced by both *P. redivivus* and *C. elegans*, attracts *P. redivivus* and *C. elegans* males, but not *P. redivivus* females, whereas previous studies have shown that ascr#3 repels *C. elegans* hermaphrodites [10]. These species- and gender-specific responses demonstrate that ascarosides may serve as signaling molecules in many nematode species.

We then investigated whether nematode-nematode interactions can be explained with the excretion of specific ascarosides. Previous studies have reported that *C. elegans* hermaphrodites fail to attract males on a 5 cm plate containing a point source conditioned by a single hermaphrodite (overnight) or on an agar-mounted slide holding hermaphrodite-incubated supernatant (five hermaphrodites in 100  $\mu$ l M9 buffer for 6 hr) [34]. We expanded both the diameter of the assay arena and the number of hermaphrodites, using a 10 cm plate and testing 1–300 hermaphrodites (Figure 4A). Results from the modified assay show that groups of *C. elegans* hermaphrodites attract males from a distance (Figure 4B). We then tested whether hermaphrodite-derived, male-attracting ascarosides ascr#2, ascr#3, and ascr#8 can account for the observed long-range chemotaxis [10, 13]. Using amounts as low as 5 fmol, we found that ascr#2 and ascr#3 elicit *C. elegans* male attraction on a 10 cm plate, whereas ascr#8 was not attractive in this long-range assay, although ascr#8 is a strong short-range attractant (Figure 4B) [13]. Our results suggest that ascr#2 and ascr#3 serve to attract *C. elegans* males from a distance, whereas ascr#8 serves to hold males within vicinity upon arrival at a point source.

Because many of the investigated nematode species produce overlapping ascaroside profiles, we asked whether production of attractive ascarosides by one species could facilitate attraction of members of a different species. To investigate whether ascarosides could lead to interspecific responses, we placed 50 females or hermaphrodites from non-*elegans* species in a holding chamber, allowing them to condition one side of a 10 cm plate for several hr. We found that *C. elegans* males chemotax toward areas conditioned by *Panagrellus redivivus* females but are not attracted by *S. carpocapsae*, *P. pacificus*, or *P. strongyloides* (Figure 4C). These behavioral observations correlated with differences in the observed ascaroside profiles of these species. Our

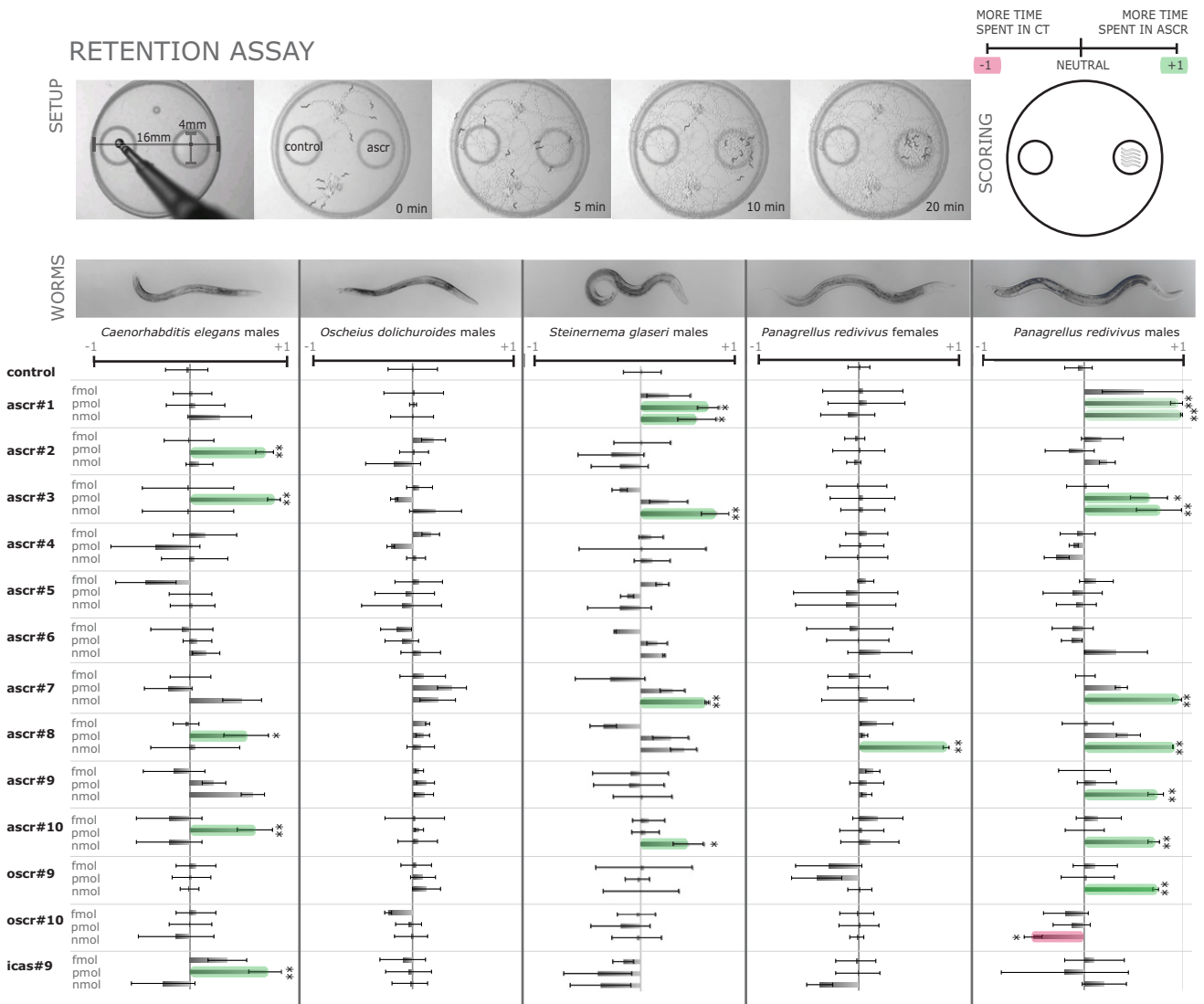


Figure 3. Species-Specific Behavioral Responses to Ascarosides

Several nematode species were scored for their response to areas conditioned with different amounts of synthesized ascarosides (0.6 fmol, pmol, and nmol) per scoring area. Their occupancy in the conditioned region was compared to occupancy in the control region for 20 min. Experiments in which nematodes spent significantly more time in regions conditioned with ascaroside are highlighted in green. Experiments in which nematodes spent significantly more time in control regions are highlighted in red, indicating avoidance of the ascaroside. Error bars are SD. Statistical significance for each value was calculated in comparison to the response to water, shown first in each set. (One-factor ANOVA followed by Dunnett's posttest, \* $p < 0.05$ , \*\* $p < 0.01$ ). See also Figure S3.

HPLC-MS analysis showed that *P. redivivus* produce ascr#3 and large quantities of ascr#10, both of which strongly attract *C. elegans* males. These compounds are absent or produced in much smaller amounts in *S. carpocapsae*, *P. pacificus*, and *P. strongyloides* (Figure 2). Instead, *S. carpocapsae*, *P. pacificus*, and *P. strongyloides* produce large quantities of ascr#9, which *C. elegans* males are not attracted to. These results suggest that ascr#3 and ascr#10 production by *P. redivivus* is one primary cause for the observed interspecies attraction. We do not know whether these species cohabitate and/or interact in nature; however, both species are commonly isolated in decomposing plant materials [35, 36].

## Discussion

Our findings suggest that ascarosides comprise a conserved family of nematode signaling molecules. Most of the analyzed

nematodes produce species-specific blends of ascarosides, and nematode responses to ascarosides depended both on their chemical structures and concentrations. The specificity of the responses to synthetic compounds as well as the observed correlation of ascaroside blend compositions with nematode intra- and interspecific attraction strongly support that ascarosides function as signaling molecules. Our simple retention and attraction assays likely uncovered only a small aspect of ascaroside function in the nematode species we studied, and future, more comprehensive analyses will require detailed consideration of the other species' ecology. The observation that genetically divergent *C. elegans* WT strains produce partially divergent ascaroside profiles indicates that combining metabolomics and genetic analyses of natural variation could make an important contribution to uncovering the genetics and evolution of ascaroside biosynthesis, perception, and downstream signaling.

## ATTRACTION ASSAY

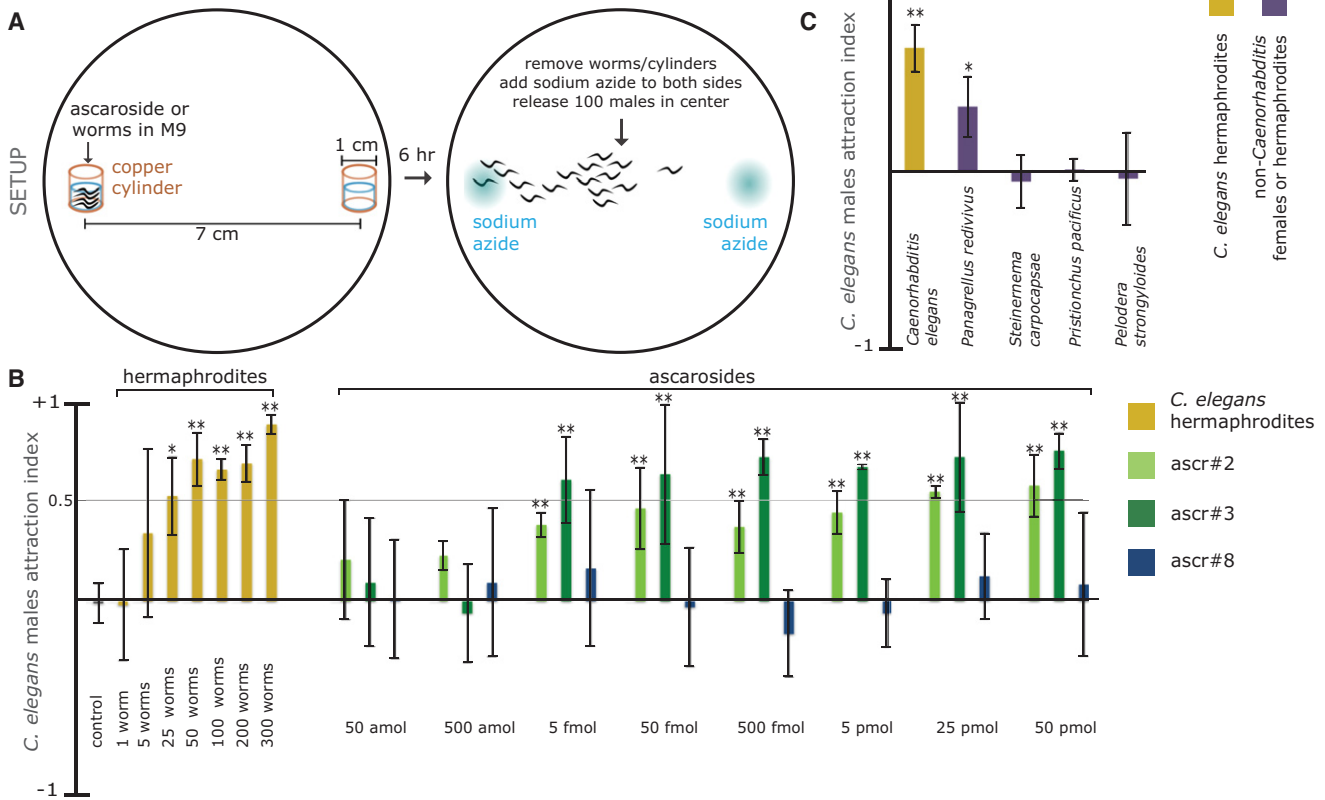


Figure 4. Attraction of *C. elegans* Males to other Nematode Species and Selected Synthetic Ascarosides

(A) Long-range attraction of *C. elegans* males to conspecific hermaphrodites was established by scoring chemotaxis to a hermaphrodite or ascaroside-conditioned point source on a 10 cm agar plate. Hermaphrodites were suspended in M9 buffer and placed within a copper cylinder on the agar plate, for 6 hr before they were removed and the paralyzing agent sodium azide was added. The same was done with an M9 buffer control in a copper cylinder, on the opposite side. *C. elegans* males were placed in the center of the plate and allowed to wander until they became paralyzed in either spot. We then calculated the attraction index (adapted from Bargmann et al. [47]), comparing numbers of worms that became paralyzed in the control versus cue region. (B) *C. elegans* males can detect and chemotax toward point sources conditioned by *C. elegans* hermaphrodites, as well as point sources of ascr#2, ascr#3, but not ascr#8. Error bars are SD. Statistical significance for each value was calculated in comparison to the control, shown in Figure 4B. (C) *C. elegans* males are attracted to a point source conditioned by 50 conspecific hermaphrodites. They demonstrate partial attraction to *P. redivivus* females, but not to *S. carpocapsae*, *P. pacificus*, or *P. strongyloides* females and hermaphrodites. (One-factor ANOVA followed by Dunnett's posttest, \* $p < 0.05$ , \*\* $p < 0.01$ ).

The conserved nature of ascaroside signaling is evocative of bacterial quorum sensing, where acyl homoserine lactones (AHLs) are both produced and sensed by many species of gram-negative bacteria [37] (Figure 5). AHLs are based on a small invariable core structure, the homoserine lactone, which bears a fatty-acid-derived side chain featuring species-specific chemical modifications [38]. Ascarosides are organized in a very similar fashion, because they are all based on the dideoxy-sugar ascarylose and feature variations in the attached fatty-acid-derived side chain [21] (Figure 5). Analogous to bacterial AHL signaling, different nematode species produce and respond to specific combinations of ascarosides. Based on recent findings describing modular assembly of ascaroside structures from a diverse array of biosynthetic building blocks [17], it is likely that many additional ascaroside structures exist in nematodes, perhaps also in species we analyzed in this study.

The shared structural organization and broad-reaching nature of both ascaroside and AHL signaling provides new insight into the syntax of biochemical communication networks. Many bacterial behaviors are mediated by quorum

sensing, such as bioluminescence, biofilm formation, virulence factor expression, antibiotic production, and motility [39]. Similarly, many *C. elegans* behaviors are mediated by ascarosides, such as mate finding, repulsion, aggregation, olfactory plasticity, and entry into a diapausal life stage [10–13, 15–20]. Furthermore, the ascaroside profile of *C. elegans* changes in response to growth and environmental perturbation [24]. Current studies of bacterial signaling involve synthesizing molecules that are structurally similar to bacterial AHLs, toward the goal of developing a new class of antimicrobial drugs that interfere with bacterial communication [40]. Developing similar approaches for interfering with nematode signaling will require more detailed structural and functional characterization of ascaroside blends. It seems likely that a better understanding of the “chemical syntax” used by nematodes will enable the design of synthetic ascaroside blends that affect nematode reproduction and survival in parasite-host models. Because nematodes are currently responsible for about \$100 billion of losses to global agriculture annually [41] and infect 3 billion humans worldwide [42], there exists a significant need for parasite control. The concept of

	NEMATODE PHEROMONES ASCAROSIDES	BACTERIA QUORUM SENSING MOLECULES N-ACYL HOMOSERINE LACTONES
STRUCTURE		
FUNCTION	holding, avoidance, aggregation, dauer formation, olfactory plasticity, long-range attraction <sup>10-13,15-20</sup>	bioluminescence, virulence, antibiotic production, biofilm formation, motility <sup>39</sup>
DISTRIBUTION	diverse range of nematode species not yet discovered in other animal phyla <sup>22</sup>  cross-species interaction ( <i>C. elegans</i> males are attracted to <i>P. redivivus</i> females, which produce the <i>C. elegans</i> attractant ascr#10)	diverse range of gram-negative bacteria species <sup>37</sup> not yet discovered outside Proteobacteria phylum <sup>45</sup>  cross-species interaction ( <i>S. enterica</i> does not make, but responds to AHLs) <sup>46</sup>

using species-specific pheromones for the management of insect pests has driven over 50 years of insect pheromone research with >100 pheromones currently utilized in insect pest management [43]. However, pheromones have been used only toward control of a single nematode species, the soybean cyst nematode *Heterodera glycines*, the only nematode species other than *C. elegans* from which pheromones had previously been identified [44]. The discovery of a conserved class of nematode pheromones will supply the basis for developing new approaches toward nematode control, in addition to providing new insights into the evolution of chemical signaling in metazoans.

#### Experimental Procedures

##### Mass-Spectrometric Analysis

HPLC-MS was performed using an Agilent 1100 Series HPLC system equipped with an Agilent Eclipse XDB-C18 column (9.4 × 250 mm, 5 μm particle diameter) connected to a Quattro II spectrometer (Micromass/Waters) using a 10:1 split. A 0.1% acetic acid acetonitrile solvent gradient was used at a flow rate of 3.6 ml/min, starting with an acetonitrile content of 5% for 5 min, which was increased to 100% over a period of 40 min. Metabolite extracts were analyzed by HPLC-ESI-MS in negative and positive ion modes

Figure 5. Similar Assembly of Signaling Molecules in Nematodes and Bacteria

*N*-acyl homoserine lactones (AHLs) are a family of small molecules that mediate bacterial quorum sensing. AHLs are based on a homoserine lactone and feature species-specific variations in the *N*-acyl chain [38]. Ascarosides are assembled in a very similar fashion, using the dideoxy-sugar ascarylose as a scaffold to which variable lipid chains are attached [21]. Both groups of signaling molecules have similar phylum-specific distribution [22, 37, 45] and play significant roles in mediating survival strategies of the producing organisms [10–13, 15–20, 39, 46].

using a capillary voltage of 3.5 kV and a cone voltage of –40 V and +20 V, respectively. Ascarosides were identified by comparison of quasimolecular ion signals (Table S2) and HPLC retention times (Figure S1C) with those of a library of 150 components identified from WT and peroxisomal beta-oxidation mutants of *C. elegans* [17]. To confirm the assignment of ascaroside structures, we used a MS/MS screen for precursor ions of *m/z* = 73.0 (Figure S1A), because previous work indicated that all ascarosides afford an intensive C<sub>3</sub>H<sub>5</sub>O<sub>2</sub> product ion upon MS/MS (argon as collision gas at 2.1 mtorr and 30 eV). Quantification of ascarosides was performed by integration of LC/MS signals from the corresponding ion traces. Relative ascaroside concentrations were finally calculated using response factors determined for synthetic standards of ascr#1, #2, #3, #5, #7, #9, #10, oscr#9, and #10, and icas#9, whereas response factors for the ascarosides that have not been synthesized yet were extrapolated based on data observed for the available standards.

##### Construction of Phylogenetic Tree

Small subunit ribosomal DNA (SSU rDNA) sequences for this analysis were obtained from GenBank. The sequences were first aligned using MUSCLE and then subsequently trimmed to facilitate comparison of sequences with varying lengths. *Steinernema monticolum* 18S

data was used as a proxy for *S. scapterisci*. The trimmed alignment resulted in 692 characters represented for all taxa. The NJ analysis was done using the Dnadist and Neighbor programs from the PHYLIP 3.68 package, using Consense to produce a majority-rule consensus tree.

##### Retention Assay

OP50 *E. coli* was grown on a standard 5 cm agar plate (made with standard Nematode Growth Medium). The 16 mm bacterial lawn was grown overnight at 20°C before being used in trials. Two 4 mm spots (0.6 μL) were placed on opposite sides of the bacterial lawn (using a transparent template to guide spot placement), and several minutes elapsed for the liquid to settle in before placing nematodes down on the assay. Recording began immediately upon worm placement. A drop (0.6 μl) of the control was placed on one side of the lawn, and 0.6 μl of the experimental cue was placed on the other side of the lawn, changing the location of the cue throughout trials, between left/right and top/bottom to avoid bias. Nematodes were isolated by gender at the L4 stage the day before being used in trials as developed adults. Worms were evenly divided and placed at two points equidistant from the foci of the scoring region (see description of total worms per trial below). Trials were recorded for 20 min and frames were collected for analysis at one frame per second. Results were averaged from at least three different trials. For every nematode species in this study, we tested different total number of worms (using water in both scoring regions) to determine the minimum number of worms necessary for consistent unbiased results over a 20 min trial. The total number of worms used in the multiple species assays

depended on that species' optimal parameters. Ten worms were used for *P. redivivus* males and females, 20 worms were used for *C. elegans* males and *O. dolichuroides* males, and 14 worms were used for *S. glaseri* males. We used automated software (Figure S3) to compare worm occupancy in each scoring region over time, then adapted the Chemotaxis Index described by Bargmann et al. [47] to score preference or avoidance to each ascaroside. One-factor analysis of variance (ANOVA) followed by Dunnett's posttest was used; 13 ascarosides were grouped according to dosage (therefore 3 dosage families, 13 members per family) per species, \**p* < 0.05, \*\**p* < 0.01.

#### Attraction Assay

Standard chemotaxis plates (10 cm) were prepared [47] and stored at 4°C until the day before use. We sectioned standard 1 cm-diameter copper pipes into 1 cm-tall segments to use for the holding chambers. The females and hermaphrodites were isolated overnight, washed 3× in M9 buffer, and then allowed to wander on a plate preconditioned with 50 µl of 1,000× streptomycin for 2 hr to kill any bacteria that might produce a false-positive attractant. The nematodes were then washed 3× and the supernatant from the last wash was placed in a copper chamber, as the control, 1 cm from the border of the 10 cm plate. The nematodes were suspended in M9 within the copper chamber, 1 cm from the border opposite the control chamber. After 6 hr, they were subsequently removed and replaced with 3 µl of 1 M sodium azide. One hundred *C. elegans* adult males were isolated overnight, washed several times in ddH<sub>2</sub>O, and placed at the center of the agar plate. After several hr, males paralyzed within 2 cm of each region were scored. We then used the Chemotaxis Index described by Bargmann et al. [47] to score male chemotaxis to either point source. One-factor ANOVA followed by Dunnett's posttest was used; interspecies analysis was grouped, hermaphrodite analysis was grouped, ascaroside experiments were grouped by chemical structure (therefore three ascaroside families, eight doses per family), and \**p* < 0.05, \*\**p* < 0.01.

#### Supplemental Information

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.03.024.

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