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Parallel evolution of domesticated *Caenorhabditis* species targets pheromone receptor genes

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Abstract

Evolution can follow predictable genetic trajectories¹, indicating that discrete environmental shifts can select for reproducible genetic changes^{2–4}. Conspecific individuals are an important feature of an animal's environment, and a potential source of selective pressures. We show here that adaptation of two *Caenorhabditis* species to growth at high density, a feature common to domestic environments, occurs by reproducible genetic changes to pheromone receptor genes. Chemical communication through pheromones that accumulate during high-density growth causes young nematode larvae to enter the long-lived but non-reproductive dauer stage. Two strains of *Caenorhabditis elegans* grown at high density have independently acquired multigenic resistance to pheromone-induced dauer formation. In each strain, resistance to the pheromone ascaroside C3 results from a deletion that disrupts the adjacent chemoreceptor genes *serpentine receptor class g (srg)-36* and *-37*. Through misexpression experiments, we show that these genes encode redundant G protein-coupled receptors for ascaroside C3. Multigenic resistance to dauer formation has also arisen in high-density cultures of a different nematode species, *Caenorhabditis briggsae*, resulting in part from deletion of an *srg* gene paralogous to *srg-36* and *srg-37*. These results demonstrate rapid remodeling of the chemoreceptor repertoire as an adaptation to specific environments, and indicate that parallel changes to a common genetic substrate can affect life history traits across species.

Caenorhabditis elegans and many other nematode species evaluate environmental conditions to choose between two alternative developmental trajectories, one leading to

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Author Contributions: P.T.M. and C.I.B. designed and interpreted experiments and wrote the paper. P.T.M. performed all genetic, molecular, and behavioural experiments, Y.X. conducted calcium imaging experiments, M.A. identified the dauer formation defect in the LSJ2 lineage, R.A.B. characterized and synthesized ascarosides, and J.L.G. contributed reagents.

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rapid reproduction and one leading to arrest in the long-lived, stress-resistant dauer larva stage. High population density, limiting food, and high temperature promote dauer larva formation⁵ (Figure 1a), a stage that corresponds to the infectious juvenile stage of parasitic nematodes. Dauer larvae do not feed or reproduce, but can survive under conditions that kill other stages, and respond to environmental improvements by exiting the dauer stage and resuming reproductive development. Although the pheromone cues that signal nematode density are normally integrated with food availability, pheromone accumulation in high-density liquid cultures causes animals to form dauer larvae despite the presence of ample food⁶. Nonreproducing dauer animals would appear to be at a disadvantage relative to those that continue to grow in these conditions. To examine adaptation to high density culture conditions, we measured dauer formation in two laboratory strains of *C. elegans*, LSJ2 and CC1, that were grown in liquid axenic media for approximately and fifty and four years, respectively, before permanent cultures were frozen down^{7, 8} (Figure 1b and Methods). Unlike wild-caught strains⁹ and the standard laboratory strain N2¹⁰, which readily form dauers in response to partially purified N2 dauer pheromone, CC1 and LSJ2 strains formed virtually no dauer larvae (Figure 1c).

N2, LSJ2, and CC1 arose from a common, inbred *C. elegans* ancestor after isolation from the wild (Figure 1b), so the pheromone resistance of LSJ2 and CC1 strains must result from new mutations that occurred in the laboratory. The genetic basis of dauer pheromone resistance was characterized by generating 94 recombinant inbred lines (RILs) between LSJ2 and N2 (Figure S1) that were genotyped at 176 informative SNPs (Table S1) identified by whole-genome sequencing of LSJ2 and N2 strains (Tables S2 and S3). Initial genetic mapping of dauer formation using N2-derived dauer pheromone preparations and the N2-LSJ2 RILs indicated that the trait was multigenic (data not shown). The active components of dauer pheromone are ascarosides, a group of small molecules with a common sugar scaffold and variable side chains¹¹⁻¹³. Four individual ascarosides that effectively induced N2 dauer formation (C3, C5, C6, and C9) did not induce dauer formation in LSJ2 or CC1 (Figure 1c). To simplify trait mapping, we examined dauer formation in response to individual ascarosides, focusing on the C3 ascaroside, whose receptors and cellular sites of actions are unknown. Among sixteen RILs exposed to 1 μ M C3, eight formed dauers at a rate comparable to N2 and eight formed dauers at a rate comparable to LSJ2 (Figure 1d). This bimodal distribution suggests the existence of a single locus that confers C3 resistance.

Quantitative trait locus (QTL) mapping using these sixteen RILs identified a single region on the X chromosome that correlated with the C3 response (Figure 1e). Mapping of the X-linked C3 resistance locus was verified by creating a near isogenic line (NIL) with the candidate region from LSJ2 introgressed into an N2 background by ten generations of backcrossing (Figure 1f). The LSJ2-N2 NIL was resistant to dauer formation induced by C3 ascaroside across a broad concentration range (Figure 1g). Unlike the parental LSJ2 strain, the LSJ2-N2 NIL formed dauer larvae in the presence of three other ascarosides (Figure 1g). These results identify an X-linked C3-resistance locus as one of several loci that confer pheromone resistance on LSJ2.

To identify the genetic changes in LSJ2 associated with C3 resistance, we sequenced the LSJ2 and N2 strains and identified all fixed polymorphisms between the two strains (Table

S2 and Table S3). The region of X associated with C3 resistance included four SNPs in intronic or intergenic regions and a 4,906 bp deletion in the LSJ2 strain that disrupts two predicted G protein-coupled receptor genes (*srg-36* and *srg-37*) (Figure 2a). A genomic clone from the N2 strain that contains both *srg-36* and *srg-37* fully rescued C3 resistance when introduced into the LSJ2-N2 NIL strain, indicating that this deletion causes the C3 resistance associated with the X-linked QTL (Figure 2b). Strikingly, *srg-36* and *srg-37* were also disrupted by a 6,795 bp deletion in the CC1 strain (Figure 2a). The deletions in CC1 and LSJ2 have different breakpoints, indicating that they occurred independently. To ask whether deletion of *srg-36* and *srg-37* also caused resistance to C3 in CC1, the region surrounding the *srg-36* and *srg-37* deletion was introgressed from CC1 into N2 to make a CC1-N2 NIL strain (Figure 1f). The CC1-N2 NIL was resistant to dauer formation induced by C3 ascaroside (Figure 2b) and its C3-resistance phenotype was rescued by a transgene covering the *srg-36* and *srg-37* genomic regions (Figure 2b). The CC1-N2 NIL readily formed dauers in response to other ascarosides (Figure S2), indicating that additional genetic mutations contribute to pheromone resistance in CC1. The existence of independent C3-resistance mutations affecting *srg-36* and *srg-37* in LSJ2 and CC1 provides strong genetic evidence linking these two chemoreceptors to dauer formation.

To determine which of the two predicted genes is associated with C3 sensitivity, we introduced *srg-36* and *srg-37* cDNAs with their respective upstream regions into the C3-resistant LSJ2-N2 NIL strain (Figure 2a). Transgenic strains expressing either cDNA formed dauer larvae in response to C3, although the *srg-37* transgene was less active than the *srg-36* transgene (Figure 2b). An *srg-37* genomic fragment also rescued dauer formation (Figure 2b). These results suggest that the SRG-36 and SRG-37 genes are at least partially redundant; either can support dauer formation in response to C3 ascaroside.

The expression patterns of *srg-36* or *srg-37* were inferred from bicistronic transcripts expressing green fluorescent protein downstream of the *srg-36* or *srg-37* promoter and cDNA. These *srg-36* and *srg-37* reporter transgenes rescued C3-induced dauer formation (Figure 2b), and were most strongly and consistently expressed in the ASI chemosensory neurons, with weak or inconsistent expression in a few other neurons (Figure 2c). *srg-36* and *srg-37* reporters were robustly expressed during the L1 stage when the dauer decision is made (Figure 2c). The ASI neurons are primary regulators of dauer formation¹⁴, and are therefore plausible sites of *srg-36* and *srg-37* action. An *srg-36* cDNA driven by the ASI-selective *srg-47* promoter rescued C3-induced dauer formation in the LSJ2-N2 NIL, but expression of *srg-36* in AFD or ASE sensory neurons did not (Figure 2b, Figure S3). These results are consistent with the hypothesis that *srg-36* acts in ASI to sense ascaroside C3 (Figure S4).

The subcellular localization of SRG-36 was examined by fusing GFP to the *srg-36* cDNA and expressing the hybrid gene from an ASI-specific promoter. This fusion protein was primarily localized in the sensory cilia of ASI (Figure 3a), suggesting a sensory function for SRG-36. The selective association of *srg-36* and *srg-37* with C3 responsiveness, and not with responsiveness to other ascarosides, suggested that they might encode C3 receptors. We tested this hypothesis by a gain of function experiment in which the *srg-36* cDNA or the *srg-37* cDNA was expressed in ASH neurons, a pair of polymodal nociceptive neurons that

direct rapid avoidance behavior¹⁵. Unlike control animals, animals expressing the ASH: *:srg-36* or ASH: *:srg-37* transgene reversed rapidly in response to 1 μ M C3 in an acute avoidance assay (Figure 3b). Neither ASH: *:srg-36*, ASH: *:srg-37*, nor control animals responded strongly to 1 μ M C6 (Figure 3b). These results demonstrate that expression of SRG-36 or SRG-37 in ASH is sufficient for C3-specific behavioral responses.

The ASH neurons respond to repulsive stimuli with increases in intracellular calcium that can be monitored using genetically-encoded calcium indicators¹⁶. Animals expressing *srg-36* or *srg-37* in ASH showed rapid, reliable Ca⁺⁺ increases in response to 1 μ M C3 ascaroside, but not to 1 μ M C6 ascaroside (Figure 3c); control animals did not respond to either C3 or C6. These results suggest that SRG-36 and SRG-37 are chemoreceptors (or subunits of chemoreceptors) that sense the C3 ascaroside. Although *srg-36* and *srg-37* are normally expressed in ASI, ASI neurons did not respond to C3 with calcium transients (data not shown). Little is known about pheromone signaling pathways in ASI, so the reason for this negative result is unclear.

LSJ2 was originally propagated in the Dougherty laboratory in the 1950s and 1960s to study nutrient requirements for nematode growth. A strain of *C. briggsae*, DR1690, that was grown in the Dougherty laboratory under the same conditions as LSJ2 also acquired resistance to dauer pheromone¹⁷ (Figure 4a). *C. briggsae* and *C. elegans* are estimated to have diverged 20-30 million years ago¹⁸. The *C. briggsae* genome encodes several genes closely related to *srg-36* and *srg-37*, but does not have one-to-one orthologs to these genes (Figure 4b). Comparing genomic DNA sequences from DR1690 with the reference *C. briggsae* strain AF16, we discovered a 33 kb deletion in DR1690 that disrupts one of the *srg* paralogs, *CBG24690*, and six other genes (Figure 4c). To determine whether this deletion affects pheromone responses, we created a near isogenic line with the *CBG24690* deletion introgressed into the AF16 reference background (DR1690-AF16 NIL). As previously reported¹⁷, AF16 readily formed dauers in response to *C. elegans* dauer pheromone, whereas DR1690 animals were resistant to dauer pheromone (Figure 4a). The *C. briggsae* DR1690-AF16 NIL formed dauers at an intermediate level compared to these two strains (Figure 4a), indicating that this region contains one of several mutations that contribute to pheromone resistance in DR1690. The DR1690-AF16 NIL was also resistant to purified ascaroside C3 compared to the parental AF16 strain (data not shown). A transgene covering the *CBG24690* genomic clone rescued dauer formation in the pheromone-resistant DR1690-AF16 NIL strain (Figure 4a). These results demonstrate that the *CBG24690* *srg* gene contributes to pheromone-induced dauer formation in *C. briggsae*.

To ask whether *CBG24690* also encodes an ascaroside receptor, we expressed a *CBG24690* cDNA in the *C. elegans* ASH neurons. Animals expressing the ASH: *:CBG24690* transgene reversed rapidly when presented with 1 μ M C3, but not 1 μ M C6 (Figure 3b). Animals expressing the *CBG24690* *srg* gene in ASH also showed rapid Ca⁺⁺ increases in response to 1 μ M C3 (Figure 3c). Unlike animals expressing *srg-36* or *srg-37* in ASH, animals expressing *CBG24690* in ASH showed weaker but reliable Ca⁺⁺ increases in response to the related ascaroside C6 (Figure 3c).

Our results suggest that *srg-36* and *srg-37*, two members of a large nematode-specific family of G protein-coupled receptors¹⁹, encode redundant receptors for the ascaroside C3. The *srg* gene family is distinct from the *srbc* gene family previously implicated in sensing ascarosides C6 and C9²⁰, suggesting that at least two of the seven chemoreceptor superfamilies of *C. elegans* can detect ascaroside pheromones. Chemoreceptors are among the fastest-evolving genes in metazoan genomes. They come from entirely different protein families in vertebrates, insects, and nematodes, and change rapidly between species²¹; only half of the chemoreceptors in *C. elegans* and *C. briggsae* are one-to-one ortholog pairs¹⁹. Despite the rapid evolution of these genes, the function of *srg*-like genes in pheromone detection has been conserved since *C. briggsae* and *C. elegans* diverged, as *C. elegans srg-36* and *srg-37*, and *C. briggsae CBG24690*, each sense C3 ascaroside to induce dauer formation. Some differences between species exist, however, as *CBG24690* also senses C6 ascaroside at concentrations that are not sensed by *srg-36* and *srg-37*. Differences in pheromone production and sensation by different *Caenorhabditis* species may allow both species-specific discrimination and general detection of *Caenorhabditis* species in the vicinity, as is observed with quorum-sensing systems in bacteria²².

These results directly demonstrate a reproducible change in the chemoreceptor repertoire in response to a discrete environmental shift. During high-density growth in the laboratory, resistance to ascaroside pheromones arose independently in *C. elegans* LSJ2, CC1, and *C. briggsae* DR1690 through changes in related *srg* genes. Although numerous single-gene mutations convey resistance to dauer formation in *C. elegans*²³, deletion of *srg* genes appears to be a favored route contributing to pheromone resistance. It is possible that specific features of the chromosomal region surrounding *srg-36* and *srg-37* predispose this region to deletion mutations, but these features would also need to be found near the *CBG24690* gene in *C. briggsae*. Alternatively, the spectrum of potential dauer-defective mutants may be constrained because the known single-gene mutations that are resistant to dauer formation have pleiotropic effects on sensory biology, stress resistance, and starvation responses that would reduce their fitness in mixed cultures²³. Global analysis of functional genetic variants suggests that evolutionarily relevant mutations are not randomly distributed, but rather cluster in specific genetic loci, or hotspot genes¹. These observations suggest that genetic trajectories during evolution are constrained and that adaptation can, at least to some extent, be predictable. One class of known “adaptive” genes are input-output genes, developmental regulators whose complex *cis*-regulatory motifs provide a molecular substrate that allows sculpting of developmental patterns^{2, 24}. *srg-36* and *srg-37* appear to fall into a second class of “adaptive” genes, including opsin genes and taste receptors^{25, 26}: sensory receptors whose diversity allows circumscribed adaptation to environmental changes without pleiotropic effects.

Methods Summary

The LSJ2 strain and the N2 laboratory strain are descended from one ancestral hermaphrodite isolated by W. Nicholas. LSJ2 was grown continuously in liquid axenic media starting in ~1957 at the Kaiser Foundation Research Institute, UC Berkeley and San Jose State University until a sample was frozen in 2009.

Dauer formation assays were performed with crude or synthesized ascarosides as described¹¹. Values report the average fraction of dauer animals 72 hours after eggs were laid on assay plates. With the exception of the mapping experiments, each strain and condition was tested in a minimum of five independent assays.

RILs were generated from reciprocal crosses between LSJ2 and an N2-derived strain and inbred for ten generations. Two laboratory-derived polymorphisms that modify the *npr-1* and *glb-5* genes in N2 affect many *C. elegans* behaviors^{7, 27}; to eliminate their effects, the cross was initiated with a strain containing 99% N2 DNA but the ancestral alleles of *npr-1* and *glb-5* from the CB4856 strain (Figure S1). These RILs were genotyped at 192 SNPs between LSJ2 and N2. The fraction of animals forming dauers in response to 1 μ M C3 ascaroside was used as a phenotype for nonparametric QTL mapping.

srg-36, *srg-37*, and *CBG24690* were ectopically expressed in ASH using the *sra-6* promoter. Vehicle control, 1 μ M of C3, or 1 μ M of C6 was dissolved in M13 buffer and presented to animals using the drop test²⁸. Each animal was scored three times for the ability to reverse in response to the stimulus. At least fifty animals were scored blindly for each strain and condition.

ASH imaging was performed in a custom-designed microfluidic device²⁹. The genetically-encoded calcium indicator GCaMP3.0³⁰ was expressed in ASH using the *sra-6* promoter.

Supplementary Material

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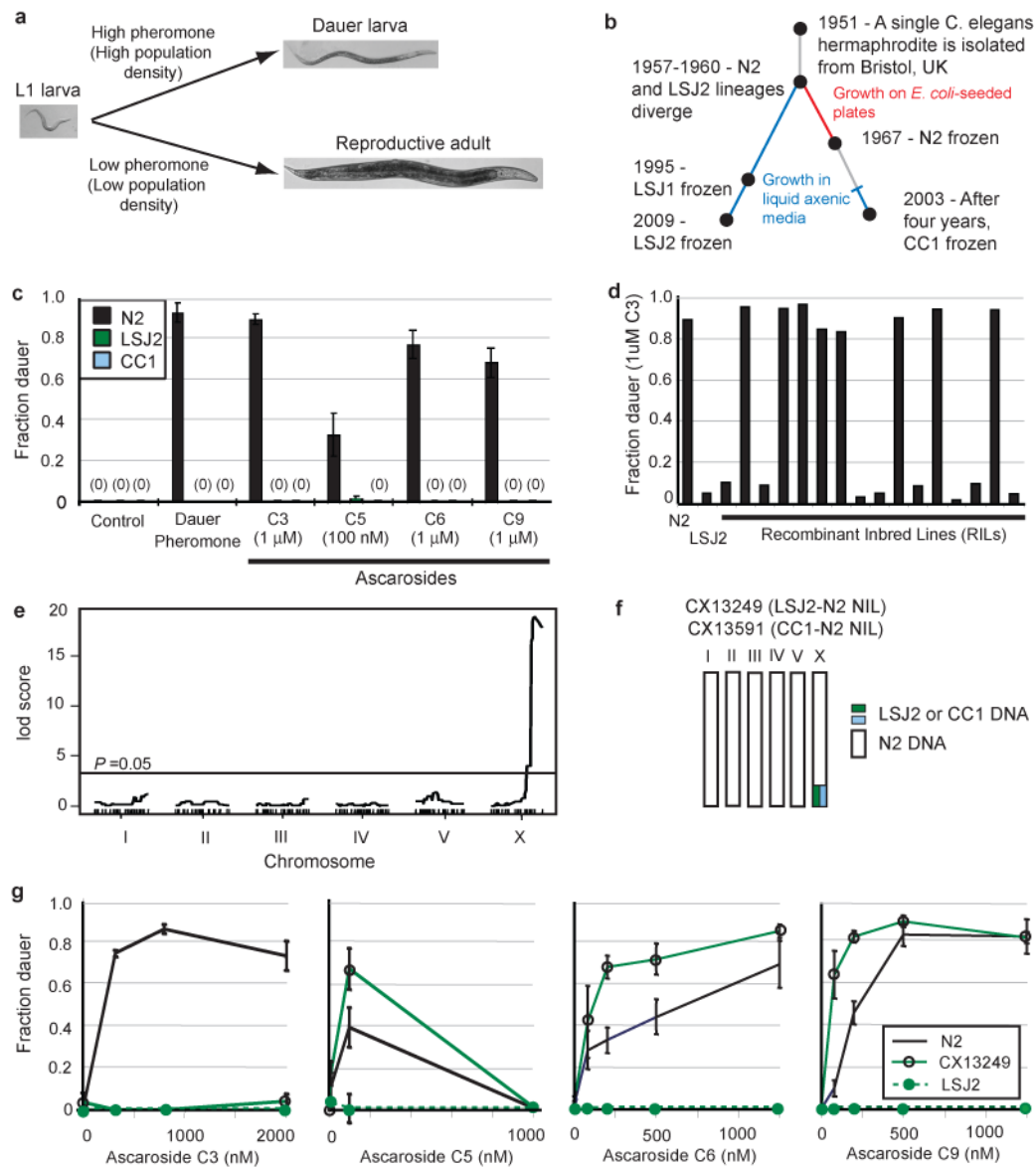
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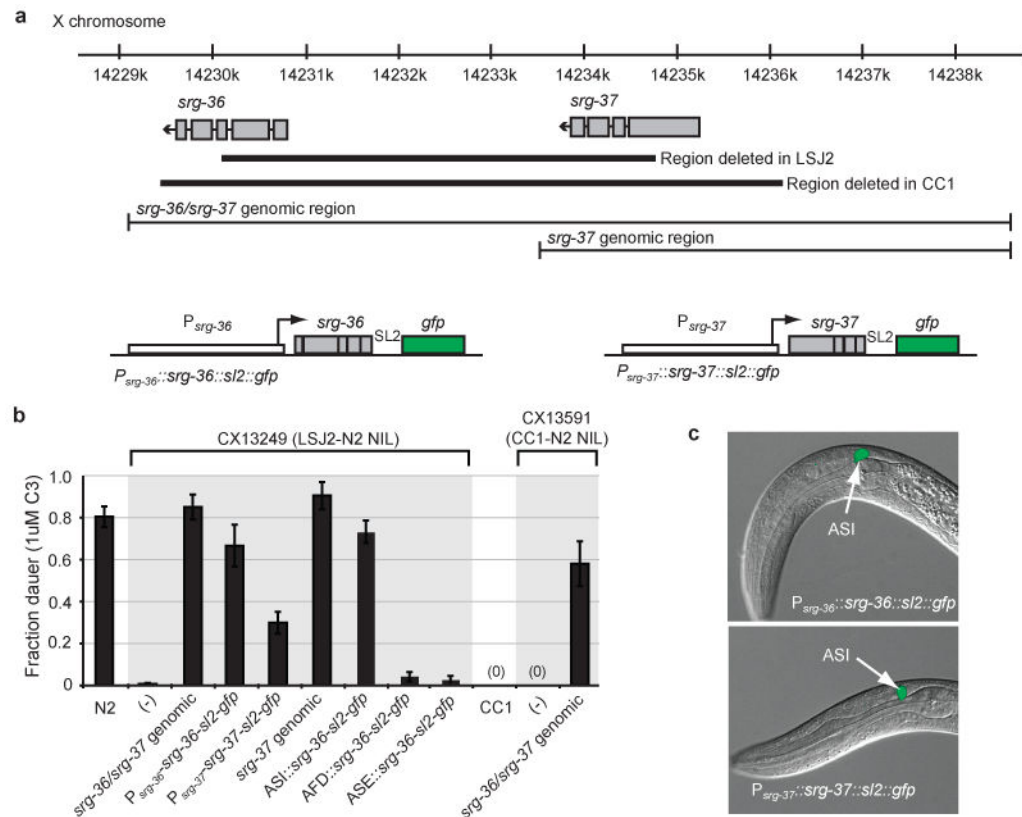
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**Figure 1.**

C. elegans cultivated in liquid are resistant to dauer pheromones. **a**, The developmental decision between reproductive growth and dauer larva formation is regulated by temperature, food, and population density. Population density is assessed by the release and sensation of ascarosides including C3, C5, C6, and C9. **b**, History of the *C. elegans* strains N2, LSJ1, LSJ2, and CC1 (see Methods). **c**, Dauer formation of N2, LSJ2, and CC1 in response to crude dauer pheromone or synthetic ascarosides. **d**, Dauer formation in response to synthetic C3 ascaroside. **e**, QTL mapping of C3 resistance. **f**, Schematic of near isogenic lines (NILs) with a small region from LSJ2 or CC1 introgressed into N2. **g**, Dauer formation in N2, LSJ2, and CX13249 strains. Error bars in all figures represent s.e.m.

**Figure 2.**

Resistance to C3 ascaroside is caused by deletion of two *srg* genes. **a**, Genomic region surrounding *srg-36* and *srg-37* on X, deletion breakpoints in LSJ2 and CC1 strains, fragments used for transgenic rescue, and design of bicistronic fusion genes. **b**, Transgenic rescue of dauer formation in response to C3 ascaroside. NIL strains used as recipients for rescue are shown in Figure 1f. ASI promoter was *srg-47* (Figure S3), AFD promoter was *gcy-8*, ASE promoter was *flp-6*. **c**, Expression of GFP from bicistronic fusion genes for *srg-36* and *srg-37* in L1 larvae, showing predominant expression in ASI sensory neurons.

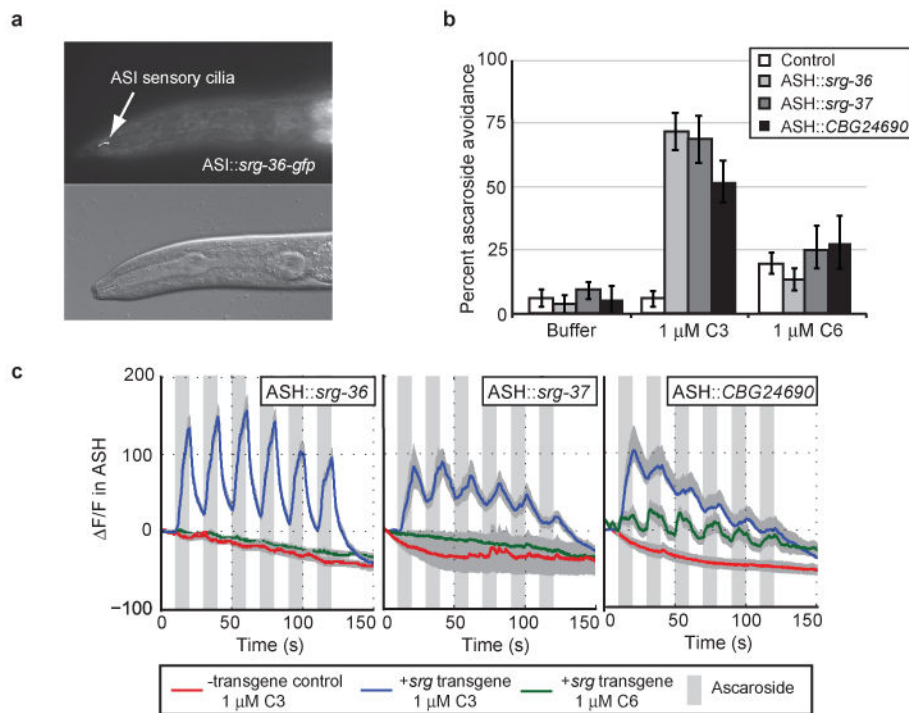


Figure 3.

The *srg* genes encode ascarside receptors. **a**, Localization of SRG-36::GFP to ASI cilia (L4 animal). **b**, Ascarside avoidance behaviours of animals with ectopic expression of *srg-36*, *srg-37*, or *CBG24690* (shown in Figure 4) in the ASH nociceptive neurons. **c**, Ascarside-induced Ca^{++} transients in ASH neurons that ectopically express *C. elegans srg-36* or *srg-37* or *C. briggsae CBG24690* in ASH. Grey bars indicate the presence of C3 or C6 ascarside, shading indicates s.e.m., n = 10 animals/condition. Ca^{++} was monitored using the genetically-encoded calcium sensor GCaMP3.0³⁰. $\Delta F/F$, percentage fluorescence change (baseline fluorescence = 100%).

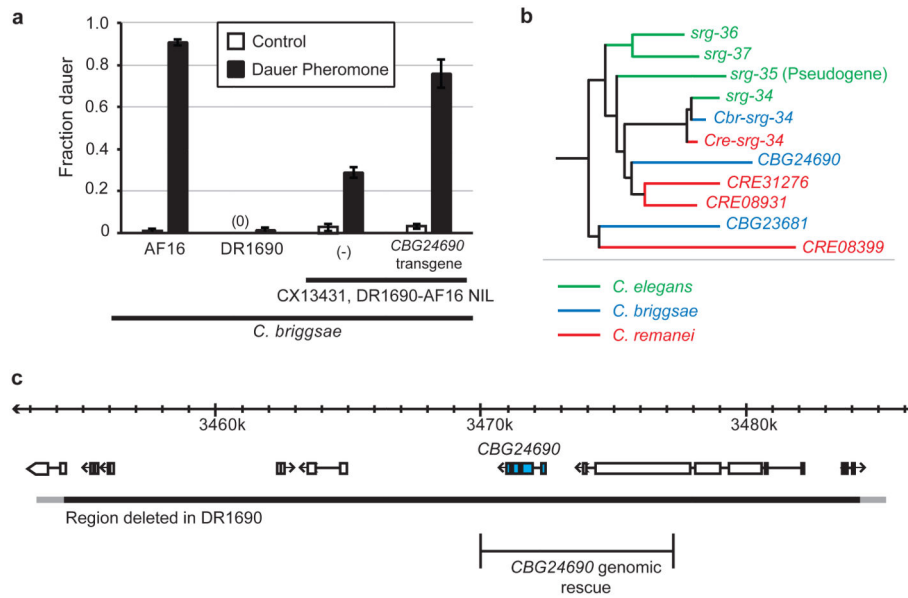


Figure 4.

Evolutionary conservation of *srg* function. **a**, Rescue of *C. briggsae* dauer formation in response to partially purified dauer pheromone by genomic fragments containing the *CBG24690* gene. CX13431 is a near isogenic line containing the *CBG24690* deletion from DR1690 introgressed into the AF16 background. **b**, Schematic of genes closely related to *srg-36* and *srg-37* from *C. elegans*, *C. briggsae*, and *C. remanei* (adapted from¹⁹). **c**, *CBG24690* genomic region from the AF16 *C. briggsae* reference strain, and location of a large deletion in the DR1690 *C. briggsae* strain that was cultivated for an extended period in liquid axenic media.