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Molecular evolution and quantitative variation for chemosensory behaviour in the nematode genus *Caenorhabditis*

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Abstract

***Caenorhabditis elegans* is a model organism in biology, yet despite the tremendous information generated from genetic, genomic and functional analyses, *C. elegans* has rarely been used to address questions in ecological genetics. Here, we analyse genetic variation for chemosensory behaviour, an ecologically important trait that is also genetically well characterized, at both the phenotypic and molecular levels within three species of the genus *Caenorhabditis*. We show that the G-protein ODR-3 plays an important role in chemosensory avoidance behaviour and identify orthologues of *odr-3* in *C. briggsae* and *C. remanei*. Both quantitative genetic analysis of chemosensory behaviour and molecular population genetic analysis of *odr-3* show that there is little genetic variation among a worldwide collection of isolates of the primarily selfing *C. elegans*, whereas there is substantially more variation within a single population of the outcrossing *C. remanei*. Although there are a large number of substitutions at silent sites within *odr-3* among the three species, molecular evolution at the protein level is extremely conserved, suggesting that ODR-3 plays an important role in cell signalling during chemosensation and/or neuronal cilia development in *C. remanei* and in *C. briggsae* as it does in *C. elegans*. Our results suggest that *C. remanei* may be a more suitable subject for ecological and evolutionary genetic studies than *C. elegans*.**

Keywords: *Caenorhabditis*, chemotaxis, Gprotein, molecular evolution, ODR-3, quantitative genetics

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Introduction

One of the primary challenges facing ecological genetics is bridging the boundaries of ecological context, phenotypic expression, gene function and molecular variation. For many organisms, the ecological significance of a particular set of phenotypes is compelling, yet tools for genetic analysis may be wanting. Other organisms may provide tremendous genetic and genomic resources, yet our knowledge of the ecological significance of these features is often very limited. The nematode *Caenorhabditis elegans* clearly falls into this latter group of organisms. *C. elegans* was the first animal to have its whole genome sequenced (The C. elegans Sequencing Consortium 1998) and is a model organism for genetics, neurobiology and developmental biology (Riddle *et al*. 1997). However, despite its global distribution, the environmental and ecological factors that regulate its biology in these widely dispersed habitats are largely unknown. This oversight is caused both by the fact that most biologists who study *C. elegans* are primarily focused on molecular and/or cellular phenomena and because these small transparent nematodes have turned out to be fairly difficult to collect and study in their natural soil habitat. Further, as seen below, genetic analysis of natural populations of *C. elegans* suggest serious limitations in using this species for population genetic studies. This does not mean, however, that the tremendous power of this model system cannot be brought to bear on some fundamental questions in ecological genetics.

C. elegans is an androdioecious (primarily self-fertilizing) nematode that lives in soil. It interacts with its environment through chemical and mechanical (touch and temperature) stimuli. Chemosensation is used in locating food, avoiding predators, pathogens and toxins (reviewed in Troemel 1999), in male-mating (Liu & Sternberg 1995) and

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egg-laying (Daniels *et al*. 2000). Chemosensation, through the detection of a dauer pheromone (Golden & Riddle 1982, 1984), also regulates development in response to food level and population density (reviewed in Riddle & Albert 1997). *C. elegans* detects a wide array of chemicals using only ≈ 30 chemosensory neurones. By comparison *Drosophila* requires 1000 neurones to detect the same number of chemicals, whereas mice use 10^7 neurones (Troemel 1999). The neurobiological system of *C. elegans* is peculiar in so far as different chemoreceptors are co-expressed in each chemosensory neurone (Troemel *et al*. 1997). *C. elegans* is therefore remarkably capable at chemosensation and, as the primary modality through which these nematodes interact with their environment, chemosensation is an obvious focal point for studies of ecological and behavioural genetics.

The chemosensory pathway

Extensive genetic analyses have helped to identify the molecular mechanisms underlying the chemosensory signalling pathway, which is now well characterized from the receptor down to particular effectors (Fig. 1). The chemosensory receptors that directly interact with odorants fall into four gene families (Troemel *et al*. 1995; Bargmann 1998; Troemel 1999). The largest family comprises ≈ 700 genes, including that for ODR-10, the only receptor for which the odorant (diacetyl) is known (Sengupta *et al*. 1996). All of the more than 1000 receptors possess a seven-transmembrane domain and are coupled to G proteins, which help transduce the extracellular odour signal into an internal cellular response (Simon *et al*. 1991). From a whole genome analysis, Jansen *et al*. (1999) identified 20 G_α, 2 G_β and 2 G_γ proteins in *C. elegans* and found a homologue in each of the four vertebrate α subunit classes, with 16 α genes being unique. After the G protein is activated, it can act as an effector through several different signalling pathways (Fig. 1). It has recently been shown that the guanylyl cyclase ODR-1 mediates olfaction and odour discrimination as a downstream effector of a G protein-coupled receptor (L'Etoile & Bargmann 2000), particularly for odorants sensed by the AWC neurone (Bargmann *et al*. 1993). The entire guanylyl cyclase family (29 members) may in fact represent a new family of chemoreceptors (Yu *et al*. 1997). Further downstream (Fig. 1), the cGMP produced by these cyclases acts on cyclic nucleotide-gated channels, such as those encoded by *tax-2* and *tax-4* genes (Coburn & Bargmann 1996; Komatsu *et al*. 1996) to generate neural signalling. Alternatively, the G protein may act directly on the cation channel, such as OSM-9 (Fig. 1; Colbert *et al*. 1997). The entire structure of the pathway is likely to be fairly complex, as revealed by recent studies on the regulation of G protein signalling by RGS proteins (Hajdu-Cronin *et al*. 1999; Dong *et al*. 2000; Chase *et al*. 2001; van der Linden *et al*. 2001).

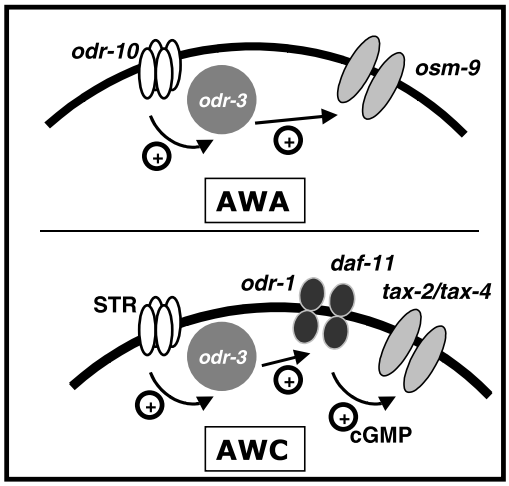

Fig. 1 Chemosensory pathways in *Caenorhabditis elegans*. In the AWA neurone, whose dendritic process extends to just below the amphid sensory structure at the nose of the nematode, odours detected by a seven transmembrane domain STR receptor (such as ODR-10) generate a conformational change that activates the G protein (ODR-3), which in turn can activate a cation channel (OSM-9). In AWC neurones, the receptor (STR) to ODR-3 G protein connection is the same, but a cGMP second messenger is generated by a guanylyl cyclase (ODR-1 and DAF-11), which in turn activates the cyclic-nucleotide gated ion channel (TAX-2/TAX-4). After Troemel (1999).

In studying variation in individual behaviour and genetic variation across the signal transduction pathway, we have chosen to focus on the central player in this system, the G protein. The G_α protein ODR-3 (Roayaie *et al*. 1998) is required for olfactory function mediated through separate pathways by the amphid wing chemosensory neurones AWA and AWC. In AWA neurones, response to diacetyl sensed by ODR-10 requires ODR-3, which regulates the cation channel OSM-9 (Troemel *et al*. 1997; Roayaie *et al*. 1998). In the AWC neurones, response to benzaldehyde also requires ODR-3. Once the odorant binds to its receptor, activated ODR-3 regulates the nucleotide-gated channel TAX2/TAX4 through the activation of the guanylyl cyclases DAF-11 (Roayaie *et al*. 1998) and ODR-1 (L'Etoile & Bargmann 2000), which then produce cGMP as an internal second messenger. The AWA and AWC neurones regulate the response to the majority of volatile chemoattractants to which *C. elegans* responds (Bargmann *et al*. 1993). Avoidance of noxious chemicals is probably equally relevant from an ecological point of view. Here *odr-3* also plays an important role, as it is expressed in the ASH neurone, which is necessary for an avoidance response to many chemicals (Bargmann *et al*. 1993; Roayaie *et al*. 1998; Troemel 1999; Hilliard *et al*. 2002). Linoleic acid is a fatty acid with nematicidal properties that also acts as a chemorepellant (Stadler *et al*. 1994). Interestingly, linoleic acid

Table 1 Strains used in this study. All are new sequences except N2 *odr-3* (The C. elegans Sequencing Consortium 1998) and AF16 *odr-3* (the Sanger Institute and the Genome Sequencing Center, Washington University, St Louis, unpublished)

Strain	Classification	Origin	*odr-3* Accession no.
N2	*C. elegans*	Bristol, UK	AY7008192
AB3	*C. elegans*	Adelaide, Australia	AY146566
BO	*C. elegans*	Bergerac, France	AY146564
CB4855	*C. elegans*	Palo Alto, CA	AY146559
CB4856	*C. elegans*	Hawaii	AY146562
CB4857	*C. elegans*	Claremont, California	AY146561
CB4932	*C. elegans*	UK	AY146560
DH424	*C. elegans*	El Prieto Canyon, CA	AY146558
RC301	*C. elegans*	Freibourg, Germany	AY146563
TR403	*C. elegans*	Madison, WI	AY146565
EM464	*C. remanei*	Brooklyn, NY	AY146577
PB235	*C. remanei*	Dayton, OH	AY146571
PB237	*C. remanei*	Dayton, OH	AY146570
PB241	*C. remanei*	Dayton, OH	AY146574
PB244	*C. remanei*	Dayton, OH	AY146573
PB245	*C. remanei*	Dayton, OH	AY146572
PB255	*C. remanei*	Dayton, OH	AY196906
PB257	*C. remanei*	Dayton, OH	AY146569
PB258	*C. remanei*	Dayton, OH	AY146568
PB261	*C. remanei*	Dayton, OH	AY146575
PB266	*C. remanei*	Dayton, OH	AY146576
PB293	*C. remanei*	Dayton, OH	AY146567
AF16	*C. briggsae*	Ahmedabad, India	AY146578
HK104	*C. briggsae*	Okayama, Japan	AY146579
DH1300	*C. briggsae*	NA	AY146580
VT847	*C. briggsae*	Hawaii	AY146581

has been isolated from live cultures of various species of nematicidal *Bascidiomycetes* (Stadler *et al*. 1994).

Here, we examine the genetic basis of variation in chemosensory response by first investigating the levels of genetic variation for chemotaxis across a worldwide distribution of natural isolates of *C. elegans* and within a single population of a related outcrossing species, *C. remanei*. Second, we show that the G protein ODR-3 plays a central role in this response. Finally, and most centrally, we examine the molecular evolution and population genetics of this gene through a comparison of within-species variation and divergence among *C. elegans*, *C. remanei* and a third congener *C. briggsae*.

Materials and methods

Nematode strains

Behavioural assays were conducted using 26 strains of *Caenorhabditis remanei* collected contemporaneously from the same population in Ohio (kindly supplied by S. Baird, Wright State University). Of these, 11 strains were randomly selected for further genomic analysis. An additional strain of *C. remanei* from New York was also used for comparison. Ten natural isolates of *C. elegans* and four of *C. briggsae* with worldwide distributions were also used in the genetic analysis. These strains, along with their classification, place of origin and sequence Accession nos, are listed in Table 1. *C. remanei* strains were each inbred for at least six generations of brother–sister mating to minimize segregating within-strain variation. The *C. elegans odr-3(n2150)* mutant strain CX2205 derived from an N2 background (Roayaie *et al*. 1998) was used in the mutant assays and compared with the response of the N2 wild-type strain. *C. remanei*, *C. elegans* and *C. briggsae* strains were maintained under the same conditions, following standard protocols, at 20 °C on 1.75% NGM-Lite media and fed with *Escherichia coli* OP50 strain (Brenner 1974). Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center.

Phenotypic assays

Behavioural assays commenced with the transfer of a single individual at the L4 larval stage to the centre (origin) of a 10 cm Petri dish containing 1.6% BBL agar (Benton-Dickinson) and the addition of 1 μL of a 1% linoleic acid (Sigma) solution (freshly diluted in ethanol) adjacent to the worm. The position of the individual was

then recorded at 1-s intervals for a total of 4 min using a compound microscope equipped with a video camera and a motorized stage controlled by a computer running Image Pro Plus (Media Cybernetics) image analysis software (Pierce-Shimomura *et al*. 1999; Ajie *et al*. manuscript in preparation). Each recorded path was subsequently analysed so that three summary chemotaxis traits could be quantified (Ajie *et al*. manuscript in preparation). Directness is defined as the ratio of the beeline distance between the origin and the individual's final position to the total path length travelled. A turn was defined as a change in direction of 90° or more, and the average number of turns per minute was recorded. Velocity was measured as the average instantaneous velocity maintained over the 4-min period. Controls in the absence of a repellent were obtained following the same protocol, but substituting 1 μL of pure ethanol for the repellent solution.

Replicate measures from at least 10 individuals from each strain were used to estimate the within and between-strain variance components. For the *C. remanei* population, standard estimates of quantitative genetic parameters for completely inbred lines were used (Falconer & Mackay 1989). Variation among the *C. elegans* natural isolates was quantified using the Q_{ST} parameter (Spitze 1993). Variance estimates and significance tests on these parameters were calculated using a bootstrap approach with 10 000 resampling events (Phillips & Arnold 1999).

Identification of C. briggsae *and* C. remanei odr-3 *homologues*

The *C. elegans* ODR-3 protein was blasted against the *C. briggsae* whole genome shotgun assembly version cb25.agp8 (the Sanger Institute and the Genome Sequencing Center, Washington University, St Louis, unpublished) using the TBLASTN program (Altschul *et al*. 1990) from the Sanger Institute's *C. briggsae* BLAST server (http://www.sanger.ac.uk/Projects/C_briggsae/blast_server.shtml). A contig (FPC2220) of 1 915 539 bp giving a protein fragment matching the 112 first amino acids of *Ce odr-3* protein was then isolated from the database. A DNA sequence of 5000 bp (from contig's position 632561–637561), in which nucleotide 632561 corresponds to the first position of codon of the protein fragment, was then pulled from the contig. This DNA sequence was then aligned by eye with *Ce odr-3* using BIOEDIT (Hall 1999). After alignment, a sequence of 2219 nucleotides of the 5000 previously selected was retained. The *C. remanei odr-3* sequence was obtained through amplification with conserved primers as outlined below.

DNA extraction and amplification

Worms were harvested from plates when the population grew large and were washed twice in 1 mL of distilled water. Genomic DNA was extracted using the CTAB protocol (Winnepenninckx *et al*. 1993). Pelleted DNA was resuspended in 30 μL of distilled water.

Amplification of *C. remanei* and *C. elegans odr-3* sequences, respectively, required three and four sets of primers (Table 2) designed from *Ce odr-3* sequence (except primers KY26F and KY26R which were designed from *Cr odr-3* sequence). Amplification of *C. briggsae odr-3* sequences required four sets of primers designed from *C. briggsae* AF16 *odr-3* sequence previously identified from the *C. briggsae* database (Table 2; see below). In all cases, primers were designed so that sequences amplified by the different sets of primers overlapped, thereby verifying that the right gene was targeted by each set of primers. Amplifications were processed in a final volume of 50 μL, with 2.5 μL DMSO, 5 μL mix of dNTPs at 6.6 mM, 5 μL of Buffer 10× (Q-Biogen), 0.6 μL of each primer at 50 μM, 0.3 μL of Q-BioTaq (Q-Biogen), and between 3 and 5 μL of template DNA. Polymerase chain reaction (PCR) conditions were: hot start 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min. Five microlitres of PCR products were checked for appropriate size using gel electrophoresis with the molecular weight marker XIV (Roche Molecular Biochemicals) on a 1.5% agarose gel. PCR products were then purified on a 1.5% agarose gel using QIAquick® Gel Extraction Kit (QIAgen).

Sequencing

Sequencing was processed on a ABI 310 automated sequencer and the sequencing reactions were carried out using BigDye™ Terminator Cycle Sequencing kit 1× from ABI with the following conditions: 30 cycles of 94 °C for 40 s, 55 °C for 25 s and 60 °C for 4 min. Sequencing reactions were purified on Sephadex™ G-50 Fine columns and cleaned with ethanol. The primers used for amplification were used for sequencing with additional internal primers, allowing the whole sequence to be overlapped and confirmed on both strands.

Sequence alignment and variation

Cr and *Cb odr-3* DNA sequences were aligned by eye with *Ce odr-3* using BIOEDIT (Hall 1999) and confirmed with reference to the ORF using the standard genetic code (Osawa *et al*. 1992; Jukes & Osawa 1993). Difference matrices for the DNA sequences were calculated using BIOEDIT. Nucleotide diversity (π; Nei 1987) was measured and tests of selection (Tajima 1989; Fu & Li 1993) were conducted using the DNASP Version 3.53 (Rozas & Rozas 1999). Neighbour-joining trees (Saitou & Nei 1987) were calculated using MEGA Version 2.1 using a Kimura 2-parameter model (Kumar *et al*. 2000).

Table 2 List of primers and their respective position within the *Ce odr-3* sequence (when based upon this sequence). *Ce odr-3* was amplified using primers F1a/Rev1a, F2a/Rev2a, F2b/R1a and OVLF/OVLR. *Cr odr-3* was amplified using primers F1a/Rev1, KY26F/KY26R, and F2a/R1a. *Cb odr-3* was amplified using primers CRO12F/CRO11R, CB27F/CB43R, MY1F/MY1R and NV85F/NV85R. Internal primers were used for sequencing, thus allow the whole sequence to be checked on both strands. Primers PROCE2F/PROCE1R and PROCB1F/PROCB1R were used to amplify *odr-3* upstream region within *Caenorhabditis elegans* and *C. briggsae*, respectively.

Name	Sequence 5′–3′	Sens/Position among *Ce odr-3*
F1a	GAAAATTCGGAAGGTAACGC	forward/22
Rev1	AGAATCTGGAAGTTGATATTCGCTA	reverse/511
F2a	ATAATTGTCAGCGTATATCTG	forward/983
Rev2a	AAGAATCATTGCTGTTGAAAGG	reverse/2311
F2b	GTAATTCAACTTGGTTCCTTTC	forward/2274
R1a	AAATTCTTCTGAATAATTGTATC	reverse/2630
Rev1a	TCGGAAATAGCAGTAATGAA	reverse/1415
F1c	CCTGGCGTGAAGAAAGCATT	forward/458
Rev1ac	TTCATTACTGCTATTTCCGA	reverse/1396
KY26F	AATTCAATCTTTATGGGCTG	forward*
KY26R	AATTTCACTTCTACGACACC	reverse*
OVLF	AAAGAGATAAGGAAATGCTG	forward
OVLR	CAATAAACCAATAACCTACC	reverse
CRO12F	AGAACTCAGAAGGTAATGCC	forward†
CRO11R	ATCTATTCTCAGTCTTTCTC	reverse†
CB27F	TTATGGGCAGATCCAGGAGT	forward†
CB43R	AGAAATCAAAGAGTACAAAG	reverse†
MY1F	CAGCAAGTATGGTTATTCAG	forward†
MY1R	AGAGTGGCTAGAACTATC	reverse†
NV85F	TCAGGATATTCTCTACTCTC	forward†
NV85R	CAATGACACTTGAAATGACA	reverse†
PROCE2F	ACATTGTTATATTTTGCTCTCC	forward‡
PROCE1R	CAGCTCGCTTATCCGCATTC	reverse‡
PROCB1F	CTTGATTTTTTGTGCCTGCC	forward‡
PROCB1R	TAGACTTTCCACATTCTCCG	reverse‡

*Used only for *C. remanei odr-3* amplification and sequencing.
†Used only for *C. briggsae odr-3* amplification and sequencing.
‡Used to amplify and sequence *odr-3* upstream region.

odr-3 *Promotor identification*

Approximately 500 bp upstream from the first position of *odr-3* coding region (CDS) were obtained from clone C34D1 (The *C. elegans* Sequencing Consortium 1998) and contig FPC2220 (the Sanger Institute and the Genome Sequencing Center, Washington University, St Louis, unpublished), yielding primers PROCE2F/PROCE1R and PROCB1F/PROCB1R (Table 2) used to amplify the *odr-3* upstream region in *C. elegans* and *C. briggsae* strains, respectively. *C. elegans* N2 and *C. briggsae* AF16 *odr-3* upstream regions herein identified were aligned with CLUSTAL W (Thompson *et al*. 1994) using default parameters. Prediction of TATA boxes within these two sequences was performed separately using the Hamming-Clustering method (Milanesi *et al*. 1996) implemented as a part of the Webgene site (http://www.itba.mi.cnr.it/webgene). Two potential TATA boxes were found within *Ce* N2 *odr-3* upstream region and three were found within *Cb* AF16 *odr-3* upstream region. Potential TATA boxes were then mapped onto the alignment. Among the several candidates, 5′-TTTATACTTC-3′ from the *Ce* N2 *odr-3* upstream region and 5′-TCCATATCTC-3′ from the *Cb* AF16 *odr-3* upstream region were found to be aligned, and can therefore be considered to constitute the basal promoter of *odr-3* in these two species.

Results

Quantitative genetic variation for chemotaxis within and among populations

There is significant variation in chemosensory avoidance behaviour both among the worldwide distribution of *Caenorhabditis elegans* and within the *C. remanei* population (Fig. 2). Somewhat surprisingly, levels of variation among *C. elegans* isolates are comparable with those in a single population of *C. remanei* (Fig. 2), although they are both fairly low on an absolute scale. For example, the level of among-population genetic differentiation for *C. elegans* for velocity is $Q_{ST} = 0.14$ (SE = 0.07; $P = 0.0010$), whereas the broad-sense heritability for velocity within the *C. remanei* population is $H_2 = 0.10$ (SE = 0.04; $P = 0.0011$). Similarly, the *C. elegans* Q_{ST} for turn rate is 0.06 (SE = 0.03; $P = 0.0090$) and the *C. remanei* H_2 is 0.04 (SE = 0.025; $P = 0.0254$) for the same character. In contrast, there is no evidence for genetic differentiation among the *C. elegans* populations

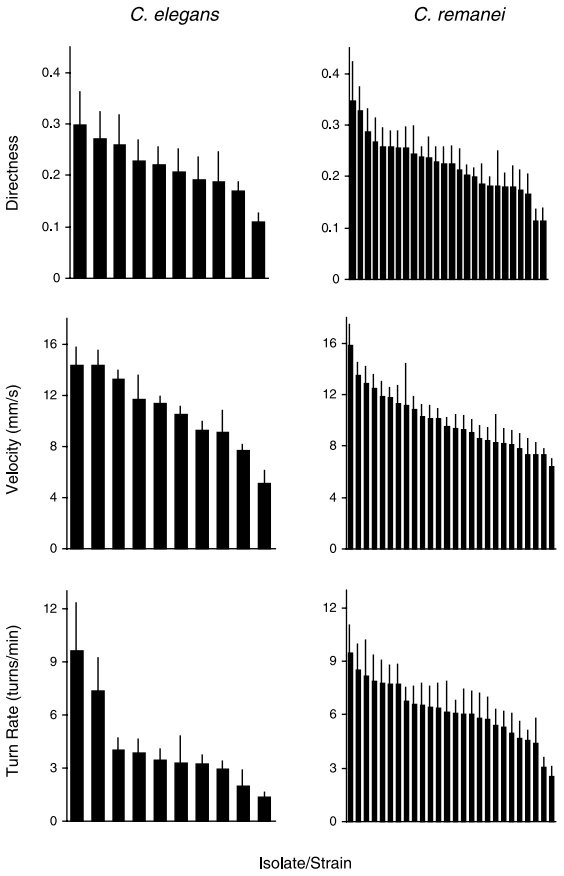

Fig. 2 Genetic variation for avoidance behaviour to linoleic acid among natural isolates of *Caenorhabditis elegans* and within a single population of *C. remanei*. Note that the total range of variation is similar across the two groups despite the difference in geographical sampling. Samples are arbitrarily rank ordered within measures to allow comparison across species.

for directness (Q_{ST} = 0.01; SE = 0.02; P = 0.3672), although there is a little variation within the *C. remanei* population (H_2 = 0.04; SE = 0.023; P = 0.0249). It is important to note that the estimates for genetic variation within the *C. remanei* population are based on variation among inbred lines. Preliminary line-crosses (Ajie & Phillips, unpublished) suggest that there is a great deal of inbreeding depression within this population, so these values are likely to be underestimates.

Avoidance behaviour in odr-3 *mutants*

Individuals with a mutation in the ODR-3 G protein are less directed in their avoidance behaviour (Fig. 3). Indeed, when compared with the wild-type N2 strain, *odr-3* mutants were less directed in behaviour whether or not the repellant linoleic acid was present (t_{373} = 4.53, P < 0.0001). There is no significant difference between the response of the *odr-3* mutant in the presence or absence of the repellant

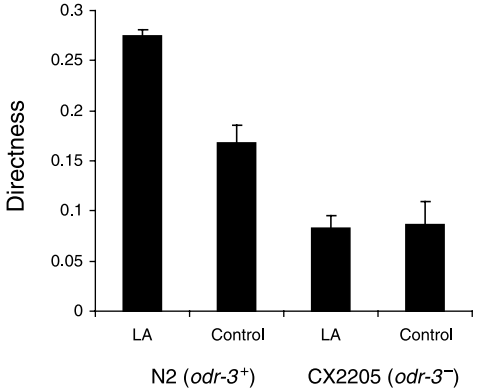

Fig. 3 Influence of the ODR-3 G protein on avoidance of the chemical linoleic acid. Wild-type (N2) and mutant (CX2205) derived from the wild-type background were tested in the presence (LA) and absence (control) of linoleic acid. The wild-type shows more directed behaviour in moving away from the repellant, whereas the mutant responses in the treatment and control situations are no different from one another.

(t_{18} = 0.17, P = 0.8666), whereas there is a marked increase in directness in the presence of the repellant for the wild-type (t_{389} = 3.98, P < 0.0001). Thus, there is both a global effect of the mutation on chemotaxis as well as a specific lack of an avoidance response to linoleic acid. There were no significant differences in linoleic acid-specific response for either velocity or turn rate in these mutants.

Genomic organization of Cr *and* Cb odr-3

We have identified *odr-3* homologues in *C. briggsae* and *C. remanei* (see Materials and methods) on the basis of amino acid identity. *odr*-3 length in *C. briggsae* (AF16) is 2219 bp, its orthologue in *C. remanei* (PB257) is 2134 bp (Table 3). *odr-3* orthologues share a conserved architecture in *C. elegans*, *C. remanei* and *C. briggsae*. Indeed, despite important composition and length differences in the five intronic sequences that the three orthologues harbour, the position of the introns within *odr-3* is conserved among the three species (Fig. 4). Lengths between orthologous exons are the same in the three species (Table 3).

odr-3 *comparison within* C. elegans

Sequence comparison of 10 strains with a worldwide distribution revealed almost no variability within *C. elegans*. Only two differences (of 2608 bp) were found between N2 and the Hawaiian strain CB4856 at positions 632 (intron II) and 1947 (intron IV), and no divergence was found at the protein level. No other differences were detected between N2 and any of the other isolates. Total nucleotide diversity (π_t) for *C. elegans* is 0.08×10^{-3}, whereas diversity at silent sites (π_{si}) is 0.11×10^{-3}.

Table 3 Structure of *odr-3* orthologues in *Caenorhabditis elegans*, *C. briggsae* and *C. remanei*. Exon sequences have the same length for the three species. Positions of exons for each orthologue are given relative to their own sequence

		C. elegans (N2)		*C. briggsae* (AF16)		*C. remanei* (PB257)	
Exon	Length	Start	End	Start	End	Start	End
I	161	1	161	1	161	1*	161
II	306	214	519	213	518	216	521
III	129	975	1103	1241	1369	1151	1279
IV	130	1320	1449	1609	1738	1514	1643
V	273	2234	2506	1824	2096	1742	2014
VI	72	2581	2652	2148	2219	2063	2134*

**Cr odr-3* sequences are incomplete at the endpoints, and so the positions at these points are inferred from the other conserved sequences.

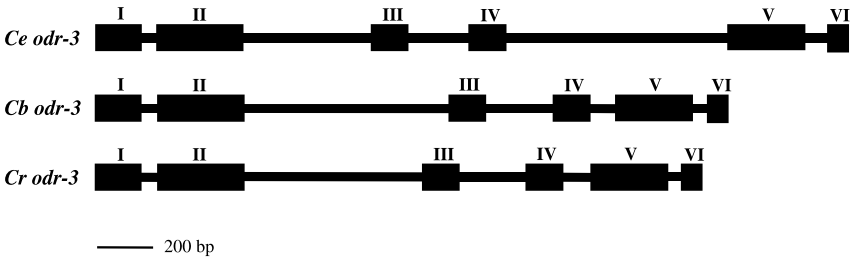

Fig. 4 Gene structure of the *Caenorhabditis remanei* and *C. briggsae* ODR-3 G proteins compared with that of *C. elegans odr-3*. Exons are shown as black boxes, lines represent introns. Numbers indicate orthologous exons. Despite variation in intron length, the architecture between the three orthologues is highly conserved: the relative position and splice sites of the introns are retained across the three species. Orthologous exons are also of the same length.

odr-3 *comparison within* C. briggsae

As with *C. elegans*, little variability was found within the four worldwide isolates of *C. briggsae* for the 2136 bp fragment analysed. HK104 differs from the other strains by five nucleotides, all located in introns. VT847 differs from the other strains at one position, also located in an intron, and no difference was found between the strains AF16 and DH1300. Total nucleotide diversity (π_t) for *C. briggsae* is 1.2×10^{-3}, whereas diversity at silent sites (π_{si}) is 1.9×10^{-3}. Although still small, these values are ≈ 15 times those for *C. elegans*.

odr-3 *comparison within* C. remanei

Comparison of *odr-3* sequences (2060 bp) within 11 *C. remanei* strains from the same population from Ohio and the additional strain from New York revealed divergences that range from 0.1% (PB237–PB266) to 3.1% (PB241–PB266). Although most differences consist of single nucleotide substitutions, it is interesting to note the presence of a deletion of eight nucleotides in intron II for the PB241 sequence. Variability was found both within introns and exons but is mainly located within introns; particularly within intron II (Fig. 5). Twelve positions were found to be variable within the cDNA, whereas 76 variable positions were identified within the introns ($\pi_t = 12.9 \times 10^{-3}$). Although almost all the differences located within the coding region of the gene are silent ($\pi_{si} = 20.7 \times 10^{-3}$), one position was found variable at the amino acid level within *C. remanei*. In PB255 methionine replaces lysine found in the other strains at position 46. Interestingly, this change in PB255 affects the motif GXXXXGKS associated with guanine nucleotide binding (Kaziro *et al*. 1991; Simon *et al*. 1991). There were 10 different haplotypes for the 11 sequences from the Ohio population, with a minimum number of 5 recombination events separating them (Fig. 5). Note that the diversity values for this single population of *C. remanei* are more than one and two orders of magnitude larger than those for the worldwide distributions of *C. briggsae* and *C. elegans*, respectively.

Comparison between Ce, Cr *and* Cb odr-3 *sequences*

Intron length and sequence are highly variable among *C. elegans*, *C. remanei* and *C. briggsae odr-3* orthologues and are impossible to align unambiguously. We therefore used the cDNA to assess the level of divergence for *odr-3* among these three species. Sequence comparisons show less divergence between *Cb odr-3* and *Cr odr-3* (11.6% for AF16–PB293 comparison) than between *Ce odr-3* and *Cr odr-3* (13.5% for N2–PB293 comparison). The same pattern is found in the overall structure of the gene (Fig. 4). The interspecific variability found in the coding region between

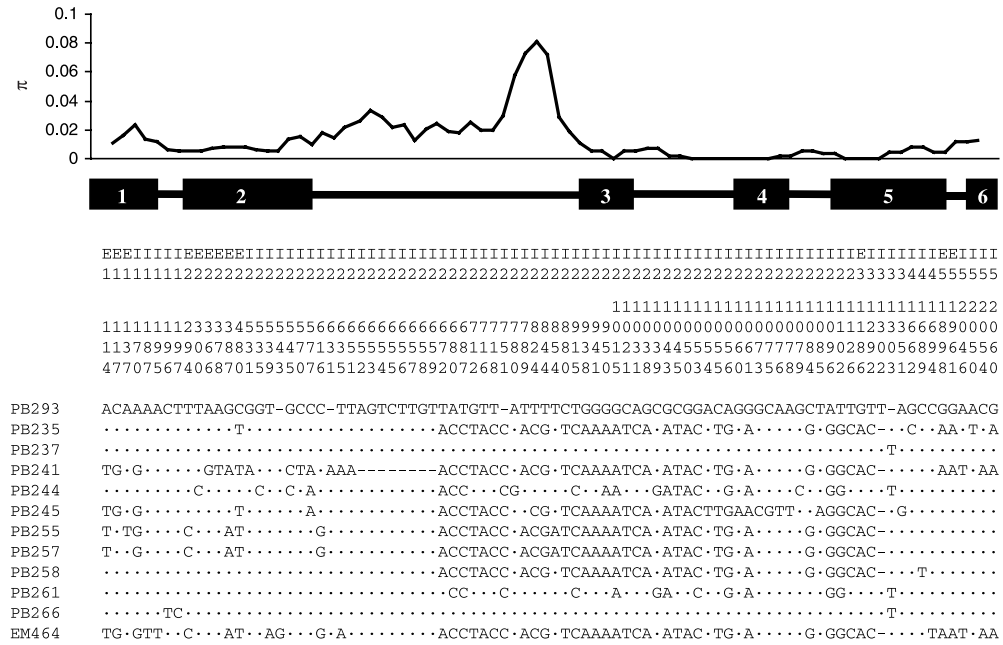

Fig. 5 Nucleotide diversity for the ODR-3 G protein within a single population of *Caenorhabditis remanei* from Ohio (PB strains) and a single sample from New York (EM464). The graph shows the nucleotide diversity (π) along the length of the gene, as calculated using a sliding window of 100 bp. Diversity is also presented with respect to its location within the gene: base positions are given between the diagram of the gene and the sequence, e.g. E1, 114 = position 114 in exon 1. All nucleotide differences are silent except for position 137 in the first exon of PB255.

Cr odr-3 and its orthologues in *C. elegans* and *C. briggsae* is huge compared with the intraspecific variability found within *Cr odr-3* cDNA. For example, even for the most divergent pair comparison (PB241–PB244) only 10 positions were found to be variable as compared with 120 and 134 variable positions between PB241 and AF16 and between PB241 and N2, respectively. The pattern of divergence within and between species is shown in Fig. 6. Although the variability between the cDNA sequences is important from the standpoint of understanding the divergence among these species, the translated *odr-3* sequences show a very high degree of conservation. *C. elegans* and *C. remanei* ODR-3 differ by only one amino acid, whereas *C. briggsae* ODR-3 differs from the *C. elegans* and *C. remanei* proteins by two amino acids (with the exception of PB255 which also differs from all the other strains at position 46, see above for more details). In *Cb* ODR-3 protein arginine replaces lysine found at position 127 in *Ce* ODR-3, and asparagine and alanine replace serine found in *Ce* ODR-3 protein, respectively, in *C. remanei* and *C. briggsae* proteins at position 303. There have obviously been significant selective constraints on protein sequence evolution in this gene. The average ratio of nonsynonymous to synonymous substitutions in *odr-3* (K_a/K_s) among these three species is 0.012. The relative lack of nonsynonymous changes precludes a formal analysis of selection within and between species.

Comparison of odr-3 *upstream region*

We have identified the *odr-3* basal promotor in *C. elegans* and *C. briggsae* (see Materials and methods). 5′-TTTATACTTC-3′ and 5′-TCCATATCTC-3′ can be considered to be the *odr-3* TATA box in *C. elegans* and *C. briggsae*, respectively. Upstream regions differ extensively between *C. elegans* and *C. briggsae* (divergence = 69%). Alignment of 539 bp of *odr-3* upstream region starting at position –1 of the CDS revealed no variability within *C. elegans* ($\pi_t = 0$). Alignment of 565 bp of the *Cb odr-3* upstream region, also starting at position –1 of the CDS, revealed low levels of variability ($\pi_t = 1.8 \times 10^{-3}$). Two positions were found to differ in HK104, and one position is different in VT847, but none of these changes affect the TATA box identified previously or the other potential TATA boxes found using Hamming-Clustering. Lack of variability in the cDNA of both of these species precludes the use of more sophisticated tests of divergence in this region, such as the HKA test (Hudson *et al*. 1987), however, levels of variability within the *C. briggsae* promoter region are comparable with the level of diversity for silent sites across the rest of the gene, indicating that there is no clear signal of selection acting within this region. The levels of divergence between *C. briggsae* and *C. elegans* in the promoter region and for synonymous sites in the coding region are also similar, consistent with a hypothesis of weak selection on the promoter region.

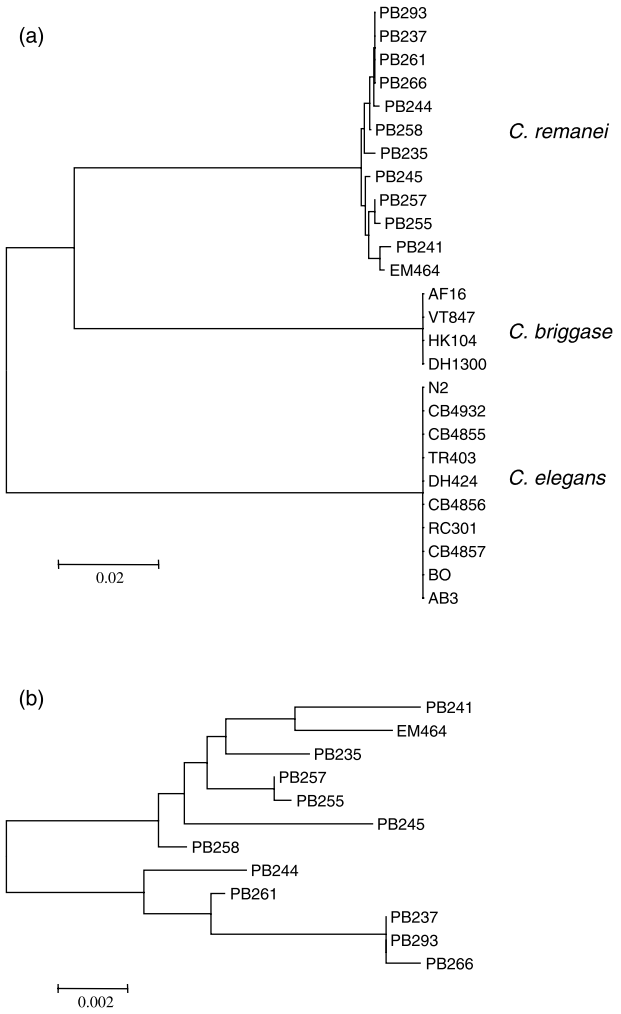

Fig. 6 Unrooted neighbour-joining trees showing the divergence among the *odr-3* sequences analysed in this study. (a) Divergence among species for the aligned cDNA sequences. Each of the major nodes shows 100% bootstrap support. (b) Divergence among samples from the *Caenorhabditis remanei* populations using the sequence for the entire gene.

Discussion

Within-species genetic variation

Both the quantitative genetic and the molecular population genetic results paint the same picture: there is very little variation among *Caenorhabditis elegans* isolates, whereas there can be substantially more variation within populations of *C. remanei*. *C. elegans* and *C. briggsae* are both primarily selfing hermaphrodites, although both species also produce males at low frequencies that can cross with the hermaphrodites. Although their role in natural populations is still unknown, under laboratory conditions males are at a reproductive disadvantage and are rapidly lost (Chasnov & Chow 2002; Stewart & Phillips 2002). In contrast, *C. remanei* is a gonochoristic, obligately outcrossing species. Comparisons of cross-fertilizing and self-fertilizing species have found that the latter tend to harbour less genetic variability than the former (Charlesworth & Wright 2001). Recently, Graustein *et al*. (2002) found a significant relationship between mating system and nucleotide diversity within *Caenorhabditis* for several genes involved in germline proliferation and sex determination, as well as a mitochondrial gene. Thus, the absence or relative absence of intraspecific variability for chemotaxis and at the *odr-3* locus within *C. elegans* is consistent with several previous results (Thomas & Wilson 1991; Koch *et al*. 2000; Graustein *et al*. 2002). Graustein *et al*. (2002) conclude, however, that the reduction of diversity they observed was greater than the twofold reduction expected from the difference between selfing and outcrossing. We also observe a much larger difference in diversity: a more than 160-fold difference between *C. elegans* and *C. remanei*.

More importantly, the influence of mating system on genetic variation applies to the expected change *within* populations. The *C. elegans* isolates sampled here have a worldwide distribution (Table 1). With partial selfing, the effective population size of new variants is reduced by increased homozygosity and reduced recombination with other loci under selection (Charlesworth & Wright 2001). One possible explanation for the pattern of variation observed in *C. elegans* is that some genes are under strong selection (Shabalina & Kondrashov 1999), facilitating selective sweeps, while at the same time migration rates around the world are extremely high (although it is hard to imagine a reasonable migration scenario between, say, France and Australia). The high degree of conservation of *odr-3* within and between species does argue for strong selection on this locus at least. Nevertheless, a more likely explanation for the lack of variation in *C. elegans* across the genome is that the current *C. elegans* populations are derived from one or a few populations with initial reduced genetic diversity that have more recently spread throughout the world. Unless mutation rates within *C. elegans* are dramatically lower than those in *C. remanei*, the level of diversity observed within *C. remanei* would suggest that there should be much more variation among in the *C. elegans* isolates if they are anywhere close to a migration–drift equilibrium.

The pattern of quantitative genetic variation among isolates of *C. elegans* and within the *C. remanei* population is also consistent with a view of strong selection and/or recent migration. The lack of obvious functional variation at a locus that is exclusively expressed in sensory neurones suggests that chemotaxis is probably under strong selection in natural populations. If selection is similar across multiple populations, then we might expect this strong selection to be reflected at the phenotypic level by a reduction in the level of among-population variation. Whether

any measure of variation among populations is large or small must be assessed relative to the amount of within-population variation, however. Unfortunately, we do not have heritability estimates for chemosensory behaviours within *C. elegans* populations. The level of variation with the *C. remanei* population is roughly the same as that among *C. elegans* isolates (Fig. 2), however, suggesting that among population variation for chemotaxis in *C. elegans* is indeed low. Again, this could be caused either by strong selection or by recent migration. Lack of variation at silent sites within *odr-3* lends more support to the recent migration hypothesis, however.

Although a greater number of sequences of *C. remanei* isolates from various locations need to be examined, it is interesting to note that EM464 from New York is no more divergent from the Ohio strains than they are from each other (Fig. 6). Graustein *et al*. (2002) sampled more populations and observed some differentiation among *C. remanei* populations, although a simple geographical pattern is not evident from gene to gene. Given the higher levels of genetic variation found in *C. remanei*, the extent of population structure within the species should be an interesting and tractable problem.

Molecular evolution of the ODR-3 G protein

C. elegans, *C. briggsae* and *C. remanei* are estimated to have diverged ≈40 Ma (Kennedy *et al*. 1993), yet they remain very similar in morphology, behaviour and development. Comparison of *odr-3* sequences among these three species indicates a high degree of conservation for the architecture of the gene. Interspecies comparisons for other genes, *mec-3* (Xue *et al*. 1992), *ges-1* (Kennedy *et al*. 1993), *unc-119* (Maduro & Pilgrim 1996), *fog-3* (Chen *et al*. 2001), *tra-2* (Kuwabara 1996; Haag & Kimble 2000), *tra-1* (de Bono & Hodgkin 1996), *bli-4* (Thacker *et al*. 1999) and *glp-1* (Rudel & Kimble 2001), have shown that this pattern tends to be general within *Caenorhabditis*. Although overall structure has tended to be preserved, disparity in intron size and number across species has frequently been found for these genes. Interestingly, the architecture of *odr-3* by comparison is extremely conserved, as the three orthologues have the same number of exons and introns, as well as conserved splice sites. Thus both the orthologous exons and the cDNA have the same length. As has been frequently reported in *C. elegans*–*C. briggsae* comparisons, only intron length differs across the three sequences (Fig. 4).

Despite extreme morphological conservation within *Caenorhabditis*, comparison of 18*S* ribosomal DNA (rDNA) between *C. elegans* and *C. briggsae* has shown that these two species are as genetically divergent as tetrapod orders (Fitch *et al*. 1995). A large-scale genomic comparison between *C. elegans* and *C. briggsae* has estimated base identity at almost 80% in regions of high selective constraint and at 30% in other regions of the genome (Shabalina & Kondrashov 1999). Here, *Cb* ODR-3 differs from its orthologues by two amino acids, and *Cr* ODR-3 differs from *Ce* ODR-3 by only one amino acid (with the exception of PB255 which differs from all other sequences by an additional amino acid change). It is not clear whether the difference observed at position 303 of the protein in each of the three species affects its function or if this is simply the result of a high substitution rate in the corresponding DNA sequence at this position (it is not in any obviously important G protein activity-related motif). But interestingly the change in PB255 affects the motif GXXXXGKS associated with guanine nucleotide binding (Kaziro *et al*. 1991; Simon *et al*. 1991) in which lysine is replaced by methionine. However, no significant phenotypic differences were found between PB255 and the other *C. remanei* strains. Such a high degree of conservation indicates that *odr-3* is under strong selection for maintained function, and thus strongly suggests that *odr-3* plays a central role in cell signalling in chemosensation and/or in neuronal cilia development in *C. briggsae* and *C. remanei* as it does in *C. elegans* (Roayaie *et al*. 1998). Alternatively, regulatory changes might alter the function of ODR-3 more than its sequence or conformation. Upstream sequences of *odr-3* are extensively divergent between *C. elegans* and *C. briggsae*, but it is notoriously difficult to identify conserved elements in the control region. Some of this divergence may be caused by evolution of regulatory elements, but as the level of divergence and the degree of polymorphism within these regions are consistent with those observed at silent sites within the gene itself, we cannot distinguish these changes from neutral changes within the promoter.

Phylogenetic relationships

Evolutionary relationships within the genus *Caenorhabditis* are currently not well resolved. On the one hand, analysis of the 18*S* rDNA has demonstrated the consistency of a *Caenorhabditis* clade but failed to resolve the relationships within the genus (Fitch *et al*. 1995). On the other hand, a clade grouping *C. elegans* and *C. remanei* has been proposed by separate and combined analysis of RNA Polymerase II, 18*S* rDNA and domain D3 of the 28*S* rDNA (Baldwin *et al*. 1997). Sequence comparison shows that *Cr odr-3* and *Cb odr-3* are more similar to each other than either of them is to *Ce odr-3*. A similar grouping is further supported by the overall structure of the gene (Fig. 4). Hence, as found with several other genes, *fog-3* (Chen *et al*. 2001), *tra-2* (Haag & Kimble 2000), *glp-1* (Rudel & Kimble 2001), our results suggest a clade grouping *C. briggsae*–*C. remanei* with *C. elegans* as a sister taxon. However, it is noteworthy that most of the interspecific differences observed in the exons are shared pair-wise (i.e. sites with

multiple states are not common) and that the proportion of shared differences seems to be nearly equally distributed between the three species. This suggests rapid speciation of *C. elegans*, *C. briggsae* and *C. remanei* and, as a consequence, the phylogenetic signal found in *odr-3* is weak despite the large number of parsimony informative sites. Rapid speciation was also suggested by the analysis of the 18*S* rDNA (Fitch *et al*. 1995). Resolution of this issue will require more data and the inclusion of appropriate outgroups.

Phenotypic and molecular variation

Although the G protein encoded by the *odr-3* gene is clearly important for the chemosensory behaviour described here (Fig. 3), we obviously cannot attempt to create a direct link between the nucleotide and phenotypic variation observed. Perhaps the best that can be said is that it is unlikely that the genetic variation in chemotaxis observed among the *C. elegans* isolates is caused by the *odr-3* locus, as we observed almost no variation within the gene or within the proximal promoter region. Furthermore, the low level of single nucleotide polymorphism (SNP) within *C. elegans* (Koch *et al*. 2000) provides something of a mixed blessing for identifying quantitative trait loci. If a sequence difference between strains is in fact found it is more likely to be functionally significant, but the low levels of variation make mapping difficult in the first place. Moreover, the very low levels of among-population quantitative variation observed in *C. elegans* suggest that identifying meaningful phenotypic variation and ecological specialization may be difficult in many circumstances. We have shown that SNP density within *C. remanei* is fairly high (although somewhat unevenly distributed; Fig. 5). A more promising direction may be to focus more closely on *C. remanei*, with increased attention being paid to potential among-population specialization and variation (see, e.g. Baird 1999, 2002). Future attempts to couple phenotypic variation with specific molecular variation need to focus on the large number of possible candidates in the chemosensory pathway, especially the large class of chemosensory receptors (Fig. 1).

Conclusion

We know more about the genetics and organismal function of *Caenorhabditis elegans* than any other multicellular organism, with the possible exception of *Drosophila melanogaster*. Despite all of this potential power, there have been very few studies utilizing *C. elegans* to address questions in ecological genetics. This study is a first step toward bridging this gap using chemosensory behaviour, a model trait that is genetically well characterized and ecologically relevant to the organism. It may turn out that using the tools developed for *C. elegans* to study the ecological and evolutionary genetics of closely related species may be the most productive way forward.

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This work is part of an effort by the Phillips laboratory to develop *C. elegans* into a model system for ecological and evolutionary genetics. Richard Jovelin is a research associate in the laboratory whose interests focus on molecular phylogenetics. Beverly Ajie was a research associate who is currently a graduate student in the Population Biology Program at UC Davis. She is interested in the evolutionary genetics of phenotypic plasticity. Patrick Phillips is an associate professor whose work focuses on theoretical and empirical population and quantitative genetics, especially as they relate to the evolution of complex traits.
