Natural variation in *Pristionchus pacificus* insect pheromone attraction involves the protein kinase EGL-4

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The geographical mosaic theory of coevolution predicts that different local species interactions will shape population traits, but little is known about the molecular factors involved in mediating the specificity of these interactions. *Pristionchus* nematodes associate with different scarab beetles around the world, with *Pristionchus pacificus* isolated primarily from the oriental beetle in Japan. In particular, the constituent populations of *P. pacificus* represent a rare opportunity to study multiple specialized interactions and the mechanisms that influence population traits at the genetic level. We identified a component of the cGMP signaling pathway to be involved in the natural variation for sensing the insect pheromone ETDA, using targeted introgression lines, exogenous cGMP treatment, and a null *egl-4* allele. Our data strongly implicate *egl-4* as one of several loci involved in behavioral variation in *P. pacificus* populations. That EGL-4 homologs have been independently implicated for behavioral variations in other invertebrate models suggests that EGL-4 may act as a modulator for interspecies behavioral repertoires across large phylogenetic distances.

chemosensation | nematode | near-isogenic lines

The interactions among organisms are part of the adaptive forces shaping the evolution of species, but the molecular factors mediating the specificity of these interactions are largely elusive. The geographical mosaic theory of coevolution posits that most organisms specialize their species interactions locally. This is based on the assumption that species are groups of genetically differentiated populations and that most interacting species have nonidentical geographical ranges (1). One important aspect of these interactions involves communication between species, but it is not clear whether perception or modulation of signals can diverge at the population level or which signal transduction molecules are involved. Therefore, further insights into ecological genetics would require access to natural variation in species interactions and mature genetic tools to identify the molecular basis for population differences.

Nematodes occupy innumerable ecological niches in plants and animals. One particular type of association with invertebrates is known as necromeny, in which nematodes infest live insects and wait for their hosts to die before resuming their life cycle on the cadaver (2). It is believed that the infested insects are not harmed and provide means of dispersal and food source for the hitchhiking nematodes. *Pristionchus* nematodes associate with different scarab beetles worldwide, with *Pristionchus pacificus* isolated primarily from the oriental beetle (*Anomala/Exomala orientalis*) in Japan and in the northeastern United States (3–5). In particular, *P. pacificus* has been used as a tool to compare with the model organism *Caenorhabditis elegans* in developmental biology, genetics, and genomics (6). In addition to the oriental beetle, *P. pacificus* populations have also been isolated from soil and other scarab beetles. Thus, *P. pacificus* populations have geographically mosaic associations, representing a rare opportunity to study multiple specialized interactions and the mechanisms that influence population traits. Recent studies in the chemosensory behavior of *P. pacificus* suggest that specific beetle pheromones contribute to the unique chemoattraction profiles of closely related *Pristionchus* species (5, 7). Chemoattraction to insect and plant derived compounds also differs between *Pristionchus* species and even some *P. pacificus* strains (7). One example is the attraction to (*E*)-11-tetradecenyl acetate (ETDA), a well studied moth sex pheromone. Here, we identified a component of the cGMP signaling pathway to be involved in the natural variation for sensing ETDA, using targeted introgression lines, exogenous cGMP treatment, and a null *egl-4* allele. Interestingly, a homolog of EGL-4 in *Drosophila* was shown to play a role in natural variation of foraging behavior, indicating a conserved role of cGMP-dependent protein kinases as a modulator of interspecies behaviors.

Results

Natural Variation in Insect Pheromone Attraction. To determine the range of natural variation in *P. pacificus* chemoattraction to ETDA, we surveyed 19 *P. pacificus* strains that represent their known global distribution [Fig. 1*A* and supporting information (SI) Table S1]. In addition, we also measured attraction to (*Z*)-7-tetradecen-2-one (ZTDO), the sex pheromone of the oriental beetles recently identified as a host to *P. pacificus* populations in Japan (5). We found that attraction responses sorted by the geographical provenance of the strains showed strong attraction to ETDA in strains from the northeastern U.S., Bolivia, and Japan, most of which were derived from *Exomala* or other scarab beetles. Not surprisingly, all strains derived from *Exomala* also exhibited robust ZTDO attraction. At the same time, strains from the geographically diverse soil sources showed extremes: High attraction (Washington strain) or complete insensitivity to both pheromones (California and China strains). When compared with the California strain, the three strains from Poland, Madagascar, and Bolivia (5270) were attracted to only ETDA and not ZTDO, whereas the remaining strains display attraction to both insect pheromones. As a comparison, the strain from the closest sister species, *Pristionchus* sp. 11, showed weak attraction to both pheromones. Taken together, the natural variation in insect pheromone attraction observed in the different *P. pacificus* strains is consistent with the geographic mosaic theory of species interaction.

***egl-4* Is a Major Locus for Pheromone Sensing.** We next sought to identify the major factors mediating the natural variation in

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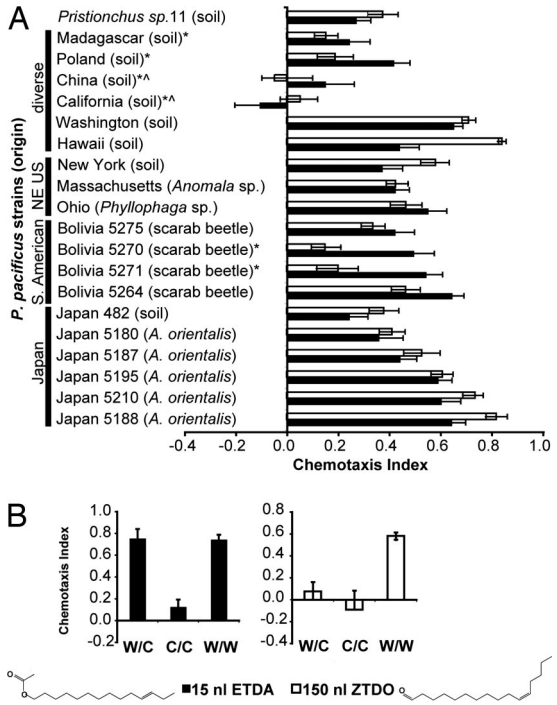

Fig. 1. Chemoattraction profiles of *P. pacificus* strains. (*A*) Natural variations in *P. pacificus* attraction toward insect pheromones *E*-11-tetradecenyl acetate (EDTA) and *Z*-7-tetradece-2-one (ZTDO). ^ and * denote no attraction toward 15 nl of EDTA and 150 nl of ZTDO and no significant difference to the control California strain (PS312), respectively (Dunnet's *posthoc* multiple comparisons test, $P < 0.05$). (*B*) Insensitivity to ETDA in the California strain is recessive with respect to Washington, whereas the insensitivity to ZTDO is a dominant trait. $n \geq 10$ replicates were performed for each condition. Error bars denote SEM. The ecological origins of each strain is indicated in parentheses. See Table S1 for details.

insect pheromone attraction for ETDA by determining the genetic loci associated with the difference between the ETDA-insensitive California and the ETDA-attractive Washington strains. We made recombinant inbred lines (RILs) starting with 250 F_2 lines, maintained them by single worm descent for 10 generations, and looked for markers associated with insensitivity to 15 nl of ETDA. Of the 206 lines that survived to F_{12} generation, we picked 22 most insensitive RILs and, after three rounds of assays, genotyped them by using SSCP markers across all chromosomes (Table S2). The region on the bottom of chromosome IV from S286 to S284 contained the highest proportion of California alleles, and genotyping at shorter intervals in this region revealed two likely regions, S591 and S284 to be associated with ETDA insensitivity. All lines contained at least a California allele at these loci. S591, a genetic marker for *Ppa-egl-4* (Fig. 2*A*; see below), was generated from routine genetic placement of putative *C. elegans* homologs of genes involved in chemosensation.

Because *Cel-egl-4* is known to regulate olfaction in *C. elegans* and natural variation in foraging behaviors in *Drosophila melanogaster* larvae and honey bees (8–10), we further investigated the extent of contribution of *Ppa-egl-4* in mediating *P. pacificus* ETDA attraction. We surmised that the Washington *egl-4* allele in the California background would enhance ETDA attraction; so we constructed near-isogenic lines (NILs) containing either the California or Washington *egl-4* locus in reciprocal genetic backgrounds by introgressing for 12 generations to obtain the lines *NIL CA1* with the *egl-4* WA allele and *NIL WA1* with the *egl-4* CA allele (see *Materials and Methods*). Using SNP markers on Supercontig 85, we ascertained that the maximum interval in the resulting California background containing the Washington donor *egl-4* locus was 110 kb (markers SRH1 to L154) (Fig. 2*A*). The ≈80-kb regions flanking the 30-kb *egl-4* locus contains nine other predicted genes with significant similarity to *C. elegans* genes (BLASTX; $e < e^{-10}$), but none was previously implicated to be involved in chemosensation (see *Materials and Methods* and Table S3). In *NIL CA1* containing the Washington *egl-4* allele, attraction to ETDA was significantly enhanced compared with the California wild-type parent (Fig. 3). Specifically, *NIL CA1* has a chemotaxis index (CI) of ≈0.4, whereas CA wild type has no response. This result suggests that the *Ppa-egl-4* locus strongly contributes to ETDA sensing. Two other independent *NILs (CA3*, *CA4*) with the *egl-4 WA* allele in California background also showed enhanced ETDA attraction compared with the California parent (Fig. S1). However, we cannot exclude the possibility that other genes within the introgressed 110-kb WA region can also contribute to variations in ETDA attraction.

To further confirm that the enhanced ETDA attraction was due to the Washington *egl-4* allele and not due to a particular interstrain NIL background, we measured the attraction of a sibling line (*NIL CA2*) that segregated for the California *egl-4* allele and found it to be less attracted to ETDA than *NIL CA1* (Fig. 3). Interestingly, *NIL CA2* still showed a small increase in ETDA attraction independent of the *egl-4* locus when compared with the California parent, suggesting that other remaining loci from the Washington donor genome may also contribute to ETDA attraction. For the *NIL WA1*, however, we were not able to completely introgress the Washington loci near the *egl-4* region spanning ≈8 cM of possible regions of incompatibility (S591–S587) (Table S2; see *Materials and Methods*). Because of this resulting donor drag (4–20 cM) from the California genome, the *egl-4* CA allele did not alter the chemotaxis response of *NIL WA1* compared with wild-type Washington (Fig. S2).

Unlike *Cel-egl-4*, which has seven known splice forms, we found only a single *Ppa-egl-4* splice form in four *P. pacificus* strains. *Ppa-egl-4* (CA) encodes a 777-aa protein with no predicted amino acid difference to *Ppa-egl-4* (WA) (Fig. 2*B*). Furthermore, both Poland and Hawaii strains with moderate ETDA attraction have predicted amino acid sequences identical to *Ppa*-EGL-4 (CA) (data not shown). Thus, there appears to be no predicted coding sequence difference in *egl-4* alleles that would account for the difference in chemoattraction among the four strains.

To ascertain the protein size of *Ppa*-EGL-4, we performed Western blot analysis, using the *Cel*-EGL-4 antibody on adult hermaphrodites. We found, consistent with the result of the RT-PCR experiments, only a single ≈95-kDa band in *P. pacificus* compared with at least two bands observed in *C. elegans* and *Pristionchus* sp. 11 samples (Figs. 2*C* and S3 *A* and *B*; *Pristionchus* sp. 11 data not shown). To determine the cellular expression pattern of *Ppa*-EGL-4, we performed immunostaining on whole *P. pacificus* adult hermaphrodites (California) and found that the EGL-4 protein is expressed in several unidentified head neurons similar to the pattern observed in *C. elegans*, consistent with its likely conserved chemosensory function (Fig. 2*D*) (11).

***Ppa-egl-4* Is Differently Expressed and Regulatory Sequences Differ Among Strains.** To determine whether regulatory sequence changes are associated with ETDA insensitivity, we compared 1.9-kb 5′ and 700-bp 3′ regions (including ≈220 bp of 3′ UTRs) of eight strains having either attractive or insensitive response to ETDA. In the 5′ region, we identified 7-bp and 9-bp deletions located 1.6 kb and 691 bp upstream of the start codon, respectively, and three separate single base pair polymorphisms shared between the two ETDA-insensitive strains California and China that are not present in the five attractive strains (Fig. S4). However, the same changes were also found in the ETDA attractive strain Poland. There were no California/China-specific polymorphisms in the 3′ downstream sequences. Thus, we could not identify shared polymorphisms in these potential regulatory

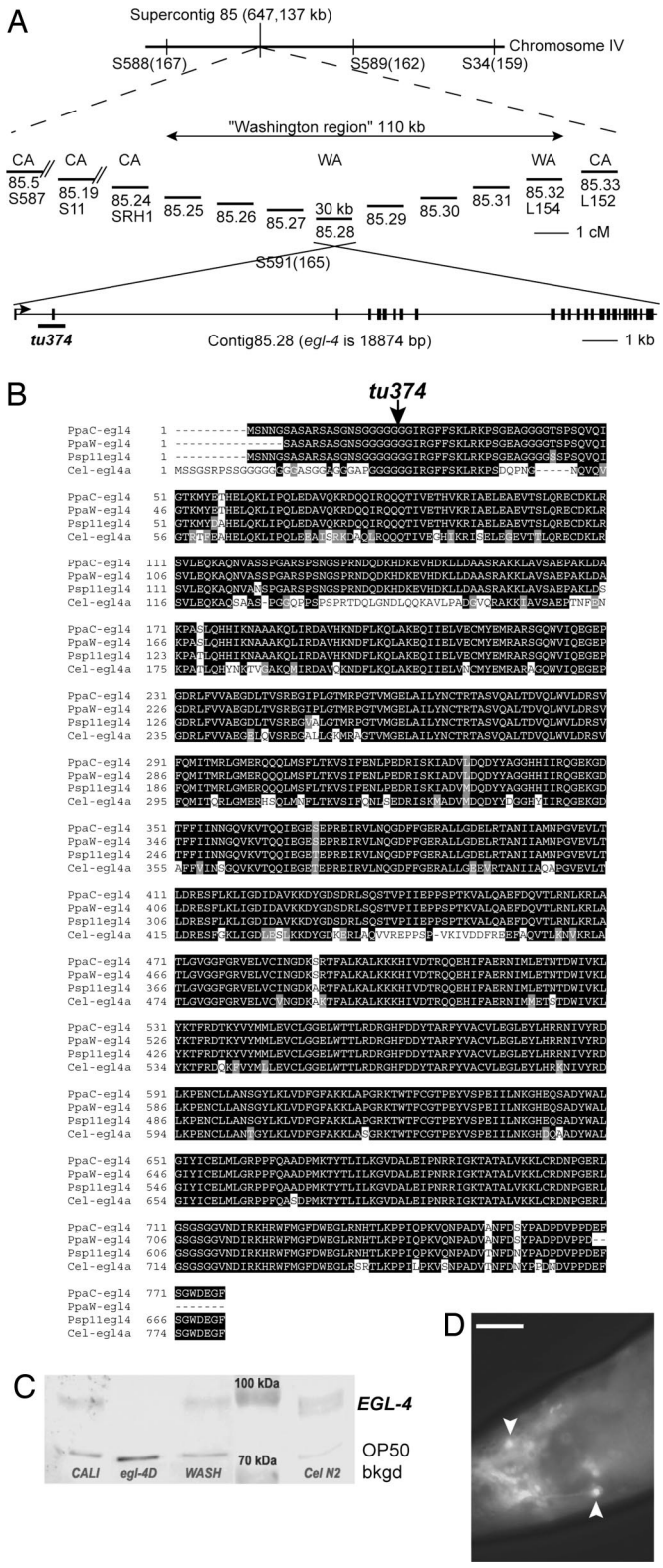

Fig. 2. *Ppa-egl-4* genome location, EGL-4 protein sequence alignment, and protein expression. (*A*) The position of *Ppa-egl-4* on integrated genetic linkage and physical maps. *Ppa-egl-4* is located at 167 cM on Chromosome IV on Supercontig 85. Nearby subcontigs 85.x flanking the contig85.28 containing *egl-4* are shown (≈30 kb, not to scale) with the genotyped strain background indicated above. The only region containing the Washington donor sequence is between contigs 85.25 and 85.32, spanning ≈110 kb. The 780-bp deletion containing the second exon (*tu374*), which results in frame shift and a premature stop codon, is shown below the diagram of the *egl-4* gene structure. Genetic distances are indicated in parentheses. (*B*) Amino acid sequence alignment of *egl-4* orthologs among *P. pacificus* strains California and Washington, *C. elegans* N2, and *Pristionchus* sp. 11. (*C*) Western blot of whole adult hermaphrodite protein extracts, using an anti-*Cel*-EGL-4 antibody. Additional loading and antibody controls are shown in Fig. S3 *A* and *B*. (*D*) *P. pacificus* California adult hermaphrodites immunostained with anti-*Cel*-EGL-4 antibody show staining in head neurons. (Scale bars, 20 μm.)

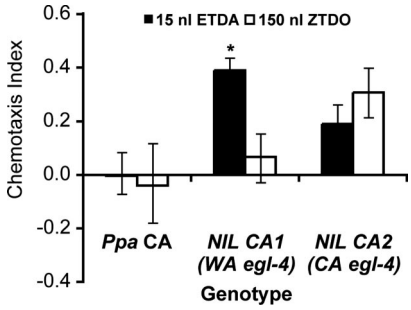

Fig. 3. Chemoattraction of near-isogenic lines (NILs). Chemoattraction of NILs of *Ppa-egl-4* in California (CA) background segregating for *egl-4* Washington (WA) (*NIL CA1*) or *egl-4* CA (*NIL CA2*) alleles show that the *egl-4* WA locus confer partial but significant enhancement of attraction toward 15 nl of ETDA but not 150 nl of ZTDO compared with both the parental California strain and a sibling line with a *egl-4* CA locus (*NIL CA2*). *, $P < 0.05$, Dunnett's *posthoc* multiple comparisons test and Tukey's HSD tests. More than 20 replicate assays were performed for each genotype on at least four separate days. Additional NILs are shown in Fig. S1. Error bars denote SEM.

regions that are exclusive to ETDA-attractive and insensitive strains. This suggests that either independent changes mediate the difference between the insensitive strains and the attractive strains and/or that additional regulatory regions or genes are required for this effect.

Given the sequence differences in the promoter region of *Ppa-egl-4*, we proceeded to determine whether *Ppa-egl-4* expression is different between the California and Washington strains. Quantitative real time reverse transcriptase-PCR (qRT-PCR) of the young adult stage showed slightly higher expression of *Ppa-egl-4* in Washington than in the California strain (Fig. 4*A*). Interestingly, J4 and adult hermaphrodites that show the small but significant difference in *egl-4* expression represents those stages in which the chemotaxis assays were performed. A pattern of low *egl-4* transcript levels associated with low or no ETDA attraction was also observed in the Madagascar and China strains (Fig. 1; see also Fig. S8).

Exogenous cGMP Up-Regulates *Ppa-egl-4* and Phenocopies the Pheromone-Attractive Strain. Exogenous cGMP has been shown to increase the activity of cGMP-dependent protein kinases (PKGs) and thereby affect development and behavior in *C. elegans* and the honey bee (10, 12). To test whether exogenous cGMP can also increase *egl-4* transcript level in the adult California worms, we treated worms briefly with a membrane permeable cyclic guanosine monophosphate, cGMP (8-bromo-cGMP). We found that exogenous cGMP treatment resulted in a 2-fold increase in the transcript level of *egl-4* in the California but not the Washington strain (Fig. 4*B*). To test whether this increase in *egl-4* level directly enhances chemoattraction to the two insect pheromones, we similarly treated young adult stage *P. pacificus* with cGMP for 1 h before chemotaxis assays. We found that cGMP treatment indeed increased the attraction toward ETDA in a cGMP concentration dependent manner in the California but not the Washington strain (Fig. 4*C*). We found a similar enhanced attraction to ZTDO in the California strain (data not shown). In contrast, cGMP treatment of the *NIL* lines

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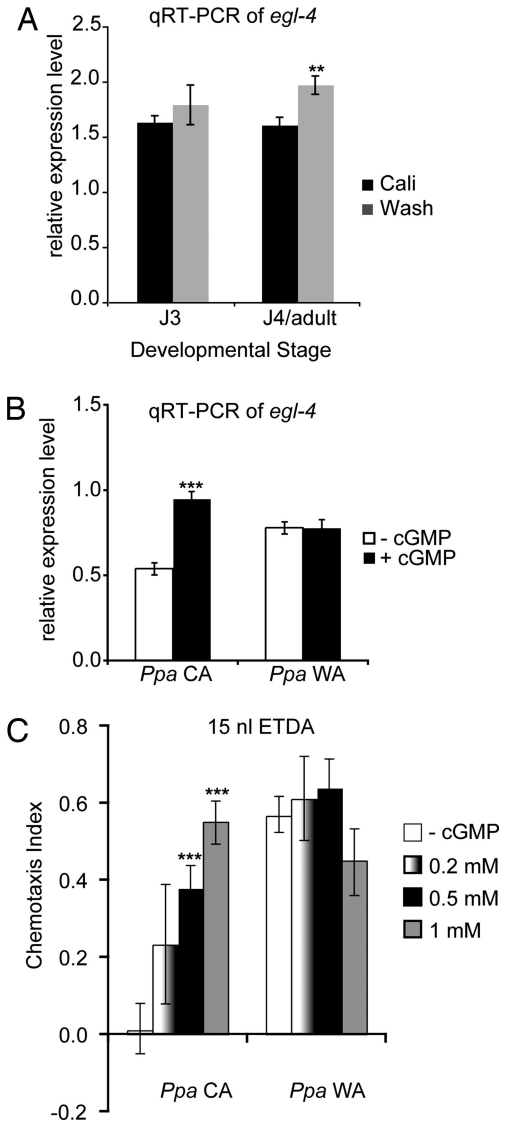

Fig. 4. *Ppa-egl-4* expression level and enhanced attraction with exogenous cGMP. One-hour incubation in 8-bromo-cGMP (+cGMP) is compared with mock treatment in buffer (−cGMP). (*A*) qRT-PCR measurement of whole worm *egl-4* expression in two postembryonic stages of *P. pacificus* California and Washington (N6 cDNA). Relative expression level is defined as: [(level of *Ppa-egl-4*/*Ppa-beta-tubulin*) × 100]. **, significant difference in *egl-4* expression levels between California and Washington, $P < 0.01$ by *t* test). (*B*) Exogenous cGMP [0.5 mM] significantly increased *egl-4* expression level in the California but not the Washington strain (Q_t cDNA). (*C*) Increasing concentrations of exogenous cGMP enhanced attraction to 15 nl of ETDA pheromone in the California but not the Washington strain. Ten to 15 replicate assays were performed for each condition over >3 separate days. ***, significant difference between mock and cGMP treated populations, $P < 0.001$ by two-sampled *t* test. Error bars denote SEM.

enhanced ETDA attraction only in the *NIL* carrying the *egl-4 CA* allele, suggesting that the response to exogenous cGMP is primarily due to regions *cis*- to the *egl-4 CA* locus. (Fig. S5). However, exogenous cAMP treatment had no effect on EDTA or ZTDO attraction (Fig. S6). To show that exogenous cGMP does not result in indiscriminate enhanced attraction to all odors, we investigated the effects of exogenous cGMP on the responses to strong *C. elegans* attractants known to be appreciably less attractive to *P. pacificus* Washington (7). We found that cGMP did not significantly alter the chemoattraction of *P. pacificus* California to 150 nl of butanone, benzaldehyde, or isoamyl alcohol or 15 nl of diacetyl or pentanedione (Fig. S7). Finally, to test whether exogenous cGMP can also enhance ETDA attraction in strains with no or low ETDA attraction and low endogenous *egl-4* transcript levels, we found that cGMP can enhance the ETDA attraction of *P. pacificus* strains Madagascar and China (Figs. S8 and S9). These results suggest that the following effects of exogenous cGMP in *P. pacificus*: (*i*) it is specific for certain odors, such as insect pheromones, and does not cause hyperattraction and (*ii*) interstrain difference in ETDA attraction is partly due to modulation of odor signals through *egl-4* expression levels rather than in pheromone receptors.

***Ppa-egl-4* Null Allele Abolished cGMP-Dependent ETDA Attraction.** To provide molecular evidence that the *Ppa-egl-4* locus in the California background is involved in the cGMP-dependent chemoattraction to insect pheromones, we analyzed an *egl-4* loss-of-function allele in *P. pacificus* California. We generated a mutant allele, *egl-4D* (*tu374*), which contains a 780-bp deletion of the second exon and parts its flanking introns (Fig. 2*A*). This deletion is predicted to result in a transcript of 111 bp, leading to an early stop, and was confirmed to not express any EGL-4 protein by Western blot analysis of whole worms (Figs. 2*C* and Fig. S3*B*). We found that, as in mock cGMP treatments, *tu374* remain unattracted to both ETDA and ZTDO showed no altered chemotaxis toward other *P. pacificus* attractants myristate and β-caryophyllene (Fig. S10). In addition, *tu374* led to a defect in odor adaptation in myristate attraction, reduction in diacetyl attraction, and in nonchemotaxis physiological phenotypes, such as fewer egg count, lower fecundity, and smaller body size (Fig. 5*A*, Figs. S11 and S12, and Table S4). Most importantly, the resulting *Ppa-egl-4* null mutant was no longer attracted to ETDA after cGMP treatment. *egl-4* is therefore required for exogenous cGMP dependent ETDA attraction (Fig. 5*B*). In contrast, *tu374* attraction to ZTDO after cGMP treatment remained unaltered, demonstrating that the enhanced ZTDO attraction by exogenous cGMP does not involve *egl-4* and ZTDO attraction depends on other loci (Fig. 5*C*).

Discussion

Our data strongly implicate the cGMP-dependent protein kinase EGL-4 as being involved in natural variation in *P. pacificus* host insect pheromone attraction. Although cGMP signaling is required for many physiological processes, we have shown that the effect of a brief exogenous cGMP treatment in *P. pacificus* is very specific to ETDA and dependent on *egl-4*. In *C. elegans*, the *egl-4* locus itself is known to have complex transcriptional and post-transcriptional regulations that can further modulate the cGMP signaling. RT-PCR and sequence analyses of the *P. pacificus* whole genome sequence confirmed the existence of several *C. elegans* orthologues in the cGMP signaling pathway in addition to *egl-4*, such as seven transmembrane G protein coupled receptors, G proteins (*odr-3*, *gpa-3*, and *goa-1* homologs), guanylase cyclases, and cyclic nucleotide-gated cation channels (*tax-2* and *tax-4* homologs). Because many functional homologs in the cGMP pathway are present in *P. pacificus* and some EGL-4 functions such as odor sensing and adaptation are conserved in *C. elegans*, EGL-4 is likely to be also regulated at various levels and have multiple functions in *P. pacificus* chemosensation.

In addition to chemosensory defects, *C. elegans egl-4* alleles have been isolated for a wide range of defects, from egg laying to body size, and for both enhancers and suppressors for constitutive dauer formation (8, 11, 13, 14). The *Ppa-egl-4* allele *tu374* has a noticeable reduction in diacetyl attraction, in adaptation to the odor myristate and in egg laying and body size, suggesting that there are certain conserved functions of EGL-4 between *P. pacificus* and *C. elegans*. Surprisingly, the loss of *egl-4* in *P. pacificus* caused a reduction in body size, in contrast to the large body phenotype of loss-of-function *egl-4* alleles in *C. elegans*. The small body phenotype was

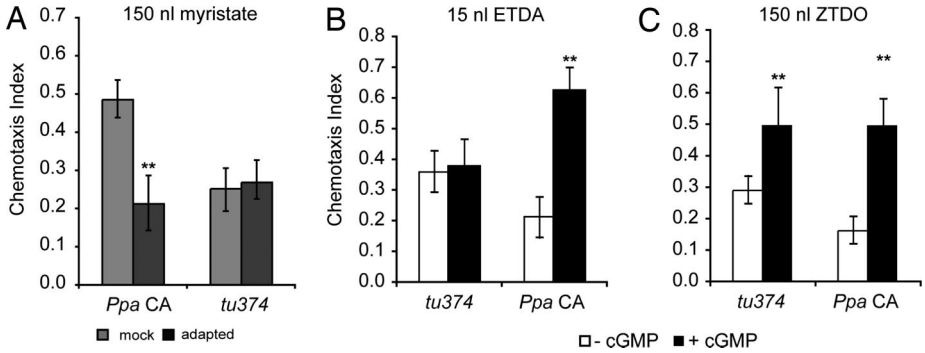

Fig. 5. Chemosensory behavior of *Ppa-egl-4* null mutant *tu374*. (*A*) *tu374* showed lack of adaptation to myristate after one hour incubation with 0.5% of myristate (vol/vol). **, significant difference between mock and myristate exposed populations, $P < 0.01$ by two-sampled *t* test. (*B* and *C*) *tu374* deletion also abolished cGMP-dependent attraction to ETDA (*B*) but not to ZTDO (*C*). **, significant difference between mock and cGMP treated populations, $P < 0.01$ by two-sampled *t* test.

also observed in the F_1 progeny from 45% of *PpaW-egl-4* morpholino injected Washington animals. In contrast, the (−)ETDA phenotype was not observed in these F_1 progeny, perhaps because of the refractory nature of neurons to morpholino. The precise contribution of *Ppa-egl-4* to variations in *P. pacificus* chemosensation, however, may be multifaceted and dependent on the genetic background. Such sheer diversity of *egl-4* alleles in *C. elegans* found for processes which can potentially affect fitness in the wild—chemoattraction, dauer formation, foraging behavior, body size—highlights the intrinsic capacity of EGL-4 to coordinately fine-tune existing behavioral and developmental programs.

We speculate that strains isolated from soil in which beetles and other insect larvae spend a considerable period of their life cycles contain both nonnecromenic and necromenic *P. pacificus* populations. More work will be needed to address the selective pressure for attraction to different insect pheromones, but we can already surmise that distinct genetic factors are responsible for attraction toward the two pheromones based on the following: (*i*) natural variation in the attraction of the two pheromones are uncoupled, because several strains attracted to ETDA lack attraction toward ZTDO (Poland and Bolivia 5270 and 5271). In particular, the Poland strain is genetically very similar to California; however, unlike the California strain, it shows significant attraction to ETDA. (ii) The Washington genotype is dominant with regard to chemoattraction toward ETDA but recessive in ZTDO attraction (Fig. 1*B*). (iii) Loss of *egl-4* function in *tu374* abolished only cGMP-dependent attraction to ETDA but not ZTDO. This separation of signaling pathways has allowed us to compare the role of *egl-4* in attraction to these two insect pheromones and to highlight the specificity of cGMP signaling pathways.

Given that *P. pacificus* is a selfing species that can allow a single individual to colonize and produce a population in a new habitat, more studies will be needed to understand the precise interaction of EGL-4 with other allelic variations in the cGMP signal transduction pathway. Modulation of primary signals by multifunctional proteins, such as protein kinases, may be a beneficial strategy for coordinating multiple phenotype changes rapidly. Thus, it is tempting to speculate that the modulation of odor signals by different *P. pacificus* populations may happen more often than the turnover of novel odor receptors in the context of mosaic species interactions. This is consistent with our finding that EGL-4 and its homologs in two nematodes, *P. pacificus* and *C. elegans*, and in *D. melanogaster* function as a conserved modulator of instinctive behaviors at the population level.

Materials and Methods

Nematode Maintenance and Genetics. All *P. pacificus* strains were maintained at 20–23°C as described in ref. 7. All *Pristionchus* strains have been inbred by single hermaphrodite descent for at least 10 generations and are preserved as frozen stock available upon request. We surveyed proportionally more strains isolated in Japan and Bolivia, because more than half of *P. pacificus* strains were derived from these locations. To ascertain whether the chemoattraction to ETDA and ZTDO was genetically dominant, we assayed the interstrain cross progeny of the pheromone-insensitive California strain (PS312) and pheromone-attracted Washington (PS1843) strain (Fig. 1*B*). Wild-type California males were crossed to the phenotypically marked Washington *dpy-like* lines, and wild-type Washington males were crossed to the marked California *unc-1*. We scored the resulting non-*unc* or non-*dpy* F_1 progeny in chemoattraction assays. As controls, we used both wild-type strains and the intrastrain cross progeny, i.e., Washington × Washington *dpy-like* and California × California *unc-1*.

Recombinant inbred lines (RILs) were produced by single worm descent from 250 F_2 progeny of crosses between Washington hermaprodites (attractive) and California males (insensitive). In *P. pacificus*, multiple recombinations occur per chromosome per generation. We constructed nearly isogenic lines (NILs) (15, 16) from initial crosses between Washington hermaphrodites and California males. F_1 progeny were repeatedly crossed to California (recurrent parent), followed by selfing to homozygosity and selecting those with the homozygous Washington *egl-4* allele (donor parent) and vise versa. The final genotypes of the NILs are shown in Table S2. (For details, see *SI Text*.)

Primer sequences are listed in Table S5.

Chemoattraction Assay. Population chemoattraction assays were performed on 8.5-cm NGM agar plates as described in ref. 7. Chemotaxis differs significantly between *P. pacificus* and *C. elegans*; *P. pacificus* chemotaxis is slower, partly because of slower locomotion, and the attractive concentration range for *P. pacificus* is also much narrower, ≈10-fold. The chemotaxis index (CI) for a given condition is a summary of at least 10 replicate assays with ≈100 J3 to adult nonstarved worms per assay measured over three days to minimize effects of batch variation. More than 99% pure (*E*)-11-tetradecenyl acetate (ETDA) and (*Z*)-7-tetradecen-2-one (ZTDO) pheromones were obtained from Sigma–Aldrich and Bedoukian Research, respectively. All attractants were diluted in pure ethanol. The CI values for ZTDO and ETDA assays were recorded after a 9- to 15-h incubation at 23°C. To treat *P. pacificus* with exogenous 8-bromo-cGMP or 8-bromo-cAMP (as HCl salt; Sigma), worms were washed once with M9 buffer, incubated with 500 μM cGMP or cAMP from a 20 mM stock diluted in dH_2O for 1 h at room temperature, washed again with 20x volume of M9 buffer, and then loaded onto assay plates.

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Supporting Information

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SI Text

Chemoattraction Assays. To test for the effects of cGMP on NILs, we used 15 nl of ETDA as attractant for lines in California background (same as in Fig. 3), and 1.5 nl of ETDA was used for *NIL WA1* in the Washington background because of the already strong chemoattraction to 15 nl of ETDA in *NIL WA1* without cGMP treatment (Fig. S1). Because only well fed nematodes were used in our assays, and that mock, cGMP, and adaptation treatments all involved soaking worms in M9 buffer for at least 1 h, differences to normal chemotaxis assay (<30 min in buffer) may incur as the result of response to prolonged starvation (17). In particular, the chemoattraction of *NIL CA* lines and wild-type California to ETDA and pentanedione were enhanced by such incidental starvation treatments, respectively.

RIL Contruction. Randomly picked single F_2 larvae from crosses between California and Washington strains were transferred to new plates for at least 10 generations before analysis (19). The high rate of RIL extinction (18%) is higher than other RILs constructed from similar crosses between California and Hawaii, or California and a Japanese strain (ref. 1 and G. Bento unpublished data), suggesting the existence of several heterozygous incompatible loci between California and Washington, which contain ≈4.5% nucleotide polymorphism between their genome sequences (D. Dieterich, unpublished results). We assayed 200 surviving F11 RILs and selected 22 lines most insensitive to 15 nl of ETDA. After confirming insensitivity for three generations ($F_{11–13}$), the 22 selected line were mapped by using standard SSCP mapping markers (single-stranded conformation polymorphism) (2). We scored markers at ≈20–50 cM intervals. The two regions with the most California haplotypes (≈15 or 16 of 22 lines having the homozygous California alleles in regions I and II, respectively) mapped to the bottom of chromosome IV, with region I delineated as S286–S587 (150–166 cM) and region II as >S290 (>176 cM).

NIL Contruction. *egl-4* was genotyped with the intragenic SSCP marker S591, and after the eighth introgression cross, also selected for the desired reciprocol background genotype at the flanking markers with markers S34 and S587 (≈7 cM interval). For *NIL WA1*, which we could not reduce the donor *egl-4* allele to a smaller interval similar to *NIL CA1*, we found no recombinants between the markers S591(*egl-4*) and S587 after examining 197 individuals in the F_{10} and F_{12} generations. Although we were able to isolate individuals that were heterozygous for S591 and homozygous Washington for S587, these heterozygotes segregated only for Washington or heterozygous genotypes, but not the desired California genotype, suggesting possible regions of incompatibility. The expected region of donor parent genome retained in the recurrent parent background after 12 introgression crosses is 15.4 ± 11 cM on chromosome IV (7.6% of 203 cM) and 0.10 cM in all nonlinked chromosomes (0.01% of 880 cM total) as calculated by the formula: $2((1 - e^{-tLM/2})/tL_M)$, where t is the number of backcrosses and L_M is the length of the marker chromosome (3) (summarized in Table S2)

Molecular cloning of *egl-4*. A BLASTX search, using *C. elegans* Wormpep160 freeze with various contigs of the finished 9× coverage *P. pacificus* California genome, identified contig85.28 as a clear 1–1 ortholog for the *C. elegans egl-4* gene (www.pristionchus.org). *Ppa-egl-4* contains a ≈2.4-kb coding region with 24 exons spanning ≈19 kb of genomic sequence. Full-length *Ppa-egl-4* from PS312 and PS1843 strains were obtained by using RH12031/RH11818 (first round); RH12032/RH11819 (second round) primers from random hexamer primed (N_6) cDNA. Overlapping PCR products containing the 3rd–24th exons were used to amplify *egl-4* cDNA from JU138 (Hawaii) and RS106 (Poland) strains (RH11820/RH12550; RH11819/RH12589 for the 5′ 1.6 kb and RH11818/RH12548; RH11821/RH12162 for the 3′ 700-bp fragments). No alternative splice forms from either N6 or polyT primed cDNAs were ever detected in the four *P. pacificus* strains using these primers. 5′ and 3′ RACE reactions were performed by using the SL1 and polyT primers as described in ref. 4. By contrast, at least three splice forms were detected from *Pristionchus* sp. 11 cDNAs, using similarly positioned RH14744/RH14746 (first round) and RH14745/RH14747 (second round) primers. However, the N_6 cDNA contains a longer transcript detected only by RH12587/RH13990 primers. The cDNA of the paralog of *Ppa-egl-4, Ppa-C09G4.2*, was isolated with RH13113/RH13114 (first round) and RH13115/RH13116 (second-round) primers. The accession numbers for the genes mentioned are EU375876–EU375890.

EGL-4 Protein Expression. *C. elegans* anti-EGL-4 antibody corresponding to amino acids 35–138 was a gift from M. Fujiwara and Y. Ohshima (Kyush University, Kyushu, Japan) (5, 6). Approximately 30 adult *P. pacificus* hermaphrodites were washed briefly in M9 and lysed at 80°C in 80 μl of Laemmli lysis buffer for 5 min. Five to 15 μl of the lysates were loaded onto 8% SDS/PAGE gels, electroblotted onto nitrocellulose membranes, and immunostained with the EGL-4 antibody. The membrane was incubated in TPBS (PBS buffer without $MgCl_2$, 0.05% Tween-20, and 2% BSA) for 1 h followed by incubation with a 1:5,000 dilution of EGL-4 antibody for >12 h at 4°C with gentle rocking. The primary anti-ALPHA-TUBULIN antibody (human) (Dianova) was used as a loading control to detect 57kD antigen. The membrane was then incubated with 1:1,000 dilution of anti-rabbit IgG alkaline phosphotase conjugate (Dianova) and washed three times in 1 h. Color visualization was done with BCIP/NBT solution (Sigma). EGL-4 immunostaining of whole California strain J4 to adult stage nematodes was done by using the Finney-Ruvkun protocol as modified for *P. pacificus* (7, 8) available under "Protocol" on www.pristionchus.org/wikionchus.

***egl-4* Deletion Mutant.** To obtain a loss-of-function deletion mutant, we mutagenized with trimethylpsoralen-UV and screened ≈1.06×10^6 genomes in the PS312 background (9), using the primers RH11962/RH11964 (first round) and RH11963/RH11965 (second round), targeting 1817 bp of the genomic region containing the first two exons (94°C for 30 s, 58°C for 20 s, 72°C for 3 min, 30 cycles, with 1:3 dilution of template for second round PCR). We isolated a mutant with a 779-bp deletion of the entire second exon and flanking introns that resulted in a putative early stop. This line *tu374* was outcrossed to PS312 four times, using PCR genotyping before commencing analyses. See Table S5 for primer sequences.

Quantitative Reverse Transcriptase-PCR (qRT-PCR). Developmentally staged RNA was obtained from various strains of *P. pacificus* J3 and J4/young adults using synchronizing eggs by bleaching or letting gravid adults lay eggs for 24 h at 20°C. J4/young adult samples do not contain laid eggs and J1 embryos. RNA was isolated with TRIZOL, treated with RQ1 DNase (Promega), and reverse transcribed with random hexamer (N_6) or polyT (Q_t)

primers and Stratagene SuperScript II. Approximately 1–2 μg of RNA from ≈3,000 worms per sample were used for synthesizing cDNA in 20-μl reactions, resulting in ≈1:20 dilutions used for each qRT-PCR. qRT-PCR samples were derived from at least two cDNA synthesis reactions.

Real-time quantitative PCR was performed by using the Roche LC480 Light Cycler, SYBR Green PCR mix, and the manufacturers software (Roche). *Ppa-egl-4* primers RH12548 and RH12549 were used to amplify a 86-bp *egl-4* coding fragment from cDNA samples (165 bp in gDNA) with a annealing temperature of 58°C for 45 cycles. AG11112 and AG11113 were used to amplify beta-tubulin as a control gene. *Egl-4* is less abundant in N_6 primed than Q_t primed cDNA but both type of cDNAs gave similar results. Relative expression level of *egl-4* is defined as: ([level of *Ppa-egl-4*/*Ppa-beta-tubulin*]x100). Inter-strain comparisons were calculated from the mean ratios of relative expression of *egl-4* between a non-California and a California strain sample within the same PCR runs (fold over California expression). 8-bromo-cGMP treated worms were incubated with cGMP for 1 h, washed in 8× volume of M9 buffer, and then RNA extracted after another hour to allow for transcription. Mock-treated worms undergo the same procedure in buffer without cGMP.

Egg Count and Body Length Measurements. PS312 (California) and *Ppa-egl-4* deletion mutant (*tu374*) young adult hermaphrodites were picked onto NGM plates seeded with one-day old OP50. These individuals were picked onto fresh plates every 18–24 h two times at 20°C and their eggs counted. Larvae that hatch and survive to J4 stage after three days were scored as the number of progeny. Body size measurements were performed with MetaMorph software (Universal Imaging) on a Zeiss AxioPlan 2 Nomarski microscope. Wild-type and mutant worms were grown concurrently and their body lengths (mouth tip to anus) were measured from age days 4–6 at ≈24-h intervals.

Morpholino Knockdown. 0.5 mM morpholino oligo (Gene Tools) targeting translational initiation in *egl-4 WA* was injected into ETDA(+) *P. pacificus* Washington young adult hermaphrodites. We observed low incidences of the small body phenotype reminiscent of the null *Ppa-egl-4* allele in the F_1 progeny within the first 48 h of injecting (45 ± 7% of *egl-4* morpholino injected parents had small F_1 progeny, with 7 ± 2% F_1 penetrance for small body, n = 104 injected animals). Later progeny of these morpholino injected worms and coinjected morpholino and RNAi (5′ 900 bp of *egl-4 WA*) worms, and the negative control Ppa-LET-23 morpholino injected worms did not show such small body phenotype.

Statistical Analyses. Statistical analyses, using two-sampled Student's t test and One-Way ANOVA, were done in Microsoft Excel. Differently treated nematodes were always derived from the same population pool and analyzed by two-sampled t test, i.e., cGMP-treated versus mock-treated. JMP software 5.1 (SAS Institute) was used to perform Wilcoxon rank sums test for discrete data. After a significant ANOVA with multiple comparisons ($P < 0.05$), we used post hoc tests to identify pairwise differences of means between specific groups (Dunnett's test for comparing to a control group or Tukey–Kramer HSD test). Alpha was set at 0.05.

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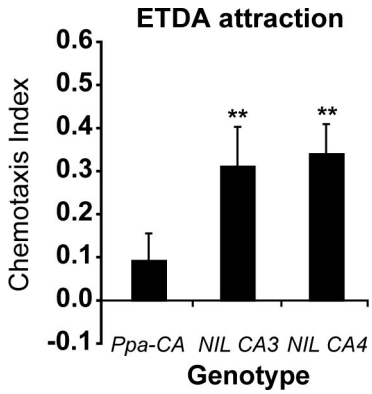

Fig. S1. Chemoattraction of additional *NIL CA* lines (CA3 and CA4) containing the *egl-4* WA locus also showed enhanced ETDA attraction compared with the parental California strain. (**, $P < 0.01$, Dunnet's *posthoc* multiple comparisons test). Error bars denote SEM.

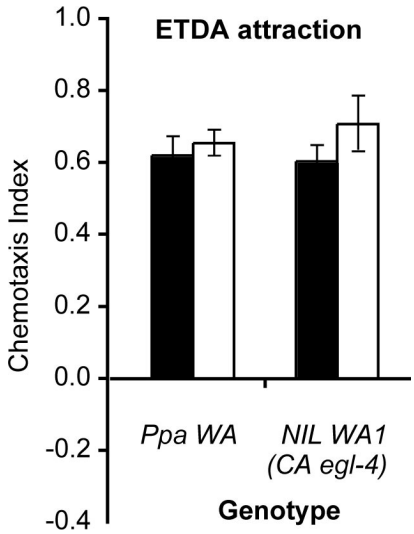

Fig. S2. Chemoattraction of NIL of *Ppa-egl-4* in Washington (WA) background having the *egl-4* CA allele showed no difference to parental Washington strain. >15 replicate assays were performed for each genotype on at least four separate days.

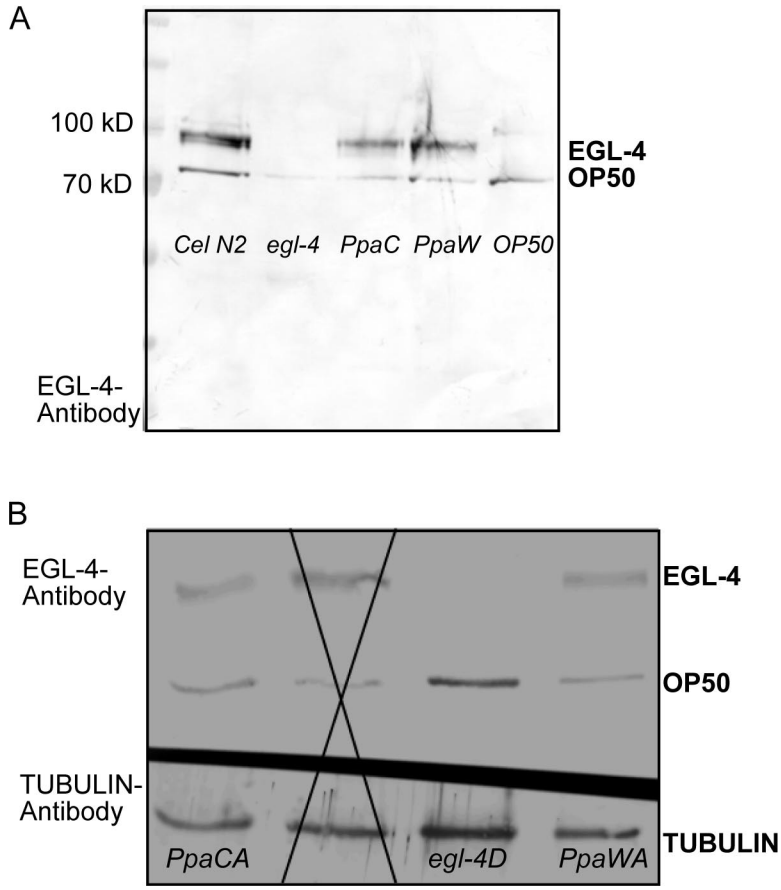

Fig. S3. Western blots of whole adult hermaphrodite protein extracts ($n = 30$ for each genotype), using an anti-*Cel*-EGL-4 antibody. (*A*) The EGL-4 antibody is specific for ≈95-kDa doublet bands found in *C. elegans N2* wild-type (lane 1) but not the loss-of-function *egl-4* mutant *n479* (lane 2; a smaller band <70 kD is visible) (14) or the OP50 *Escherichia coli* food source (lane 5, nonspecific ≈80-kDa and >100-kDa bands). The EGL-4 antibody cross hybridizes with a single ≈95-kDa protein in *P. pacificus* strains California and Washington (lanes 3–4). (*B*) (*Upper*) Using the EGL-4 antibody, no 95-kDa antigen corresponding to the *Ppa*-EGL-4 was detected in the *Ppa-egl-4* deletion mutant (*egl-4D*, lane 3) compared with the *P. pacificus* wild-type strains (lanes 1 and 4). Lane 2 is not relevant. *Bottom*: A protein loading control of the same blot, using a human *alpha-tubulin* antibody (57kD antigen) showed approximately equal loading in all lanes.

ETDA ATTRACTION	STRAIN	SEQUENCE	
-ETDA	CALI	TATTGTTAGATCAGATAAA	(-1840 bp)
-ETDA	CHNA	TATTGTTAGATCAGATAAA	
+ETDA	POLD	TATTGTTAGATCAGATAAA	
+ETDA	HAWA	TATTATTAGATCAGATAAA	
+ETDA	WASH	TATTATTAGATCAGATAAA	
+ETDA	BOLI	TATTATTAGATCAGATAAA	
+ETDA	JAPN	TATTATTAGATCAGATAAA	
+ETDA	MADG	TATTATTAGATCAGATAAA	
-ETDA	CALI	TCCGACTGAATAGCAGACGAAGAAAC	(-1660 bp)
-ETDA	CHNA	TCCGACTGAATAGCAGACGAAGAAAC	
+ETDA	POLD	TCCGACTGAATAGCAGACGAAGAAAC	
+ETDA	HAWA	TCCGACTGAATAGCAGACGATGAAAC	
+ETDA	WASH	TCCGACTGAATAGCAGACGATGAAAC	
+ETDA	BOLI	TCCGACTGAATAGCAGACGATGAAAC	
+ETDA	JAPN	TCCGACTGAATAGCAGACGATGAAAC	
+ETDA	MADG	TCCGACTGAATAGCAGACGATGAAAC	
-ETDA	CALI	TCGTAATTTC-------CGGAAAGGA	(-1617 bp)
-ETDA	CHNA	TCGTAATTTC-------CGGAAAGGA	
+ETDA	POLD	TCGTAATTTC-------CGGAAAGGA	
+ETDA	HAWA	TCGTAATTTCCGGCCTTCGGAAAGGA	
+ETDA	WASH	TCGTAATTTCCGGCCTTCGGAAAGGA	
+ETDA	BOLI	TCGTAATTTCCGGCCTTCGGAAAGGA	
+ETDA	JAPN	TCGTAATTTCCGGCCTTCGGAAAGGA	
+ETDA	MADG	TCGTAATTTCCGGCCTTCGGAAAGGA	
-ETDA	CALI	ATTTAGACGGAGAGAATGA	(-1518 bp)
-ETDA	CHNA	ATTTAGACGGAGAGAATGA	
+ETDA	POLD	ATTTAGACGGAGAGAATGA	
+ETDA	HAWA	ATTTAGACGGAGAGAGTGA	
+ETDA	WASH	ATTTAGACGGAGAGAGTGA	
+ETDA	BOLI	ATTTAGACGGAGAGAGTGA	
+ETDA	JAPN	ATTTAGACGGAGAGAGTGA	
+ETDA	MADG	ATTTAGACGGAGAGAGTGA	
-ETDA	CALI	CCGACCATTATAT---------CTGTACATAC	(-691 bp)
-ETDA	CHNA	CCGACCATTATAT---------CTGTACATAC	
+ETDA	POLD	CCGACCATTATAT---------CTGTACATAC	
+ETDA	HAWA	CCGACCATTATATTTTCCACATCTGTACATAC	
+ETDA	WASH	CCGACCATTATATTTTCCACATCTGTACATAC	
+ETDA	BOLI	CCGACCATTATATTTTCCACATCTGTACATAC	
+ETDA	JAPN	CCGACCATTATATTTTCCACATCTGTACATAC	
+ETDA	MADG	CCGACCATTATATTTTCCACATCTGTACATA	

Fig. S4. Differences in putative regulatory 1.9-kb upstream sequences from *P. pacificus* strains. Only common differences between ETDA-insensitive strains (California and China) and ETDA-attractive strains (Hawaii, Washington, Bolivia RS5275, Japan RS5195, and Madagascar) are shown. Poland strain is the most genetically similar strain to California based on nuclear and mitochondrial sequences of 84 isolated strains.

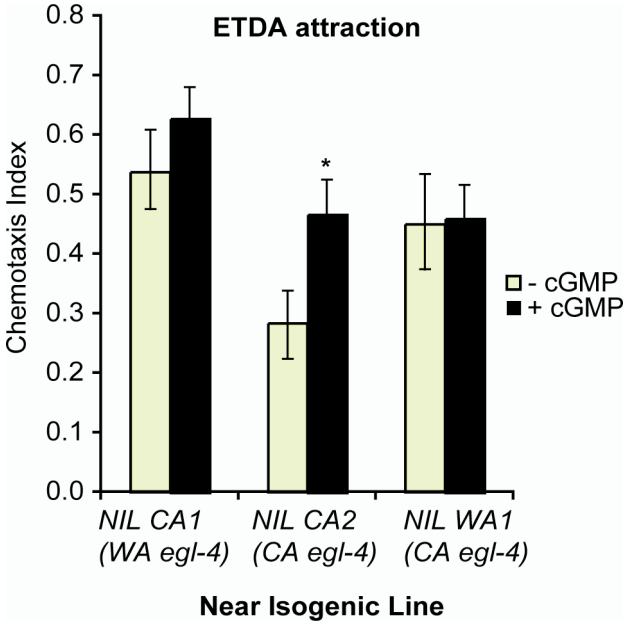

Fig. S5. The effects of 500 μM cGMP on the chemoattraction to ETDA in *NILs*. 15 nl of ETDA for *NILs CA1* (*egl-4 WA*) and and *CA2* (*egl-4 CA*) and 1.5 nl of ETDA for *NIL WA1* (*egl-4 CA*) were used. 15 replicate assays were performed for each genotype on at least three separate days. *, significant difference between mock and cGMP treated populations, $P < 0.05$ by two-sampled *t* test.

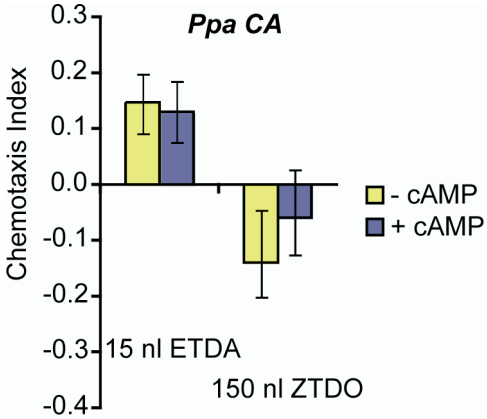

Fig. S6. 500 μM exogenous 8-bromo-cAMP did not enhance pheromone attraction in *P. pacificus* California (in contrast to 8-bromo-cGMP).

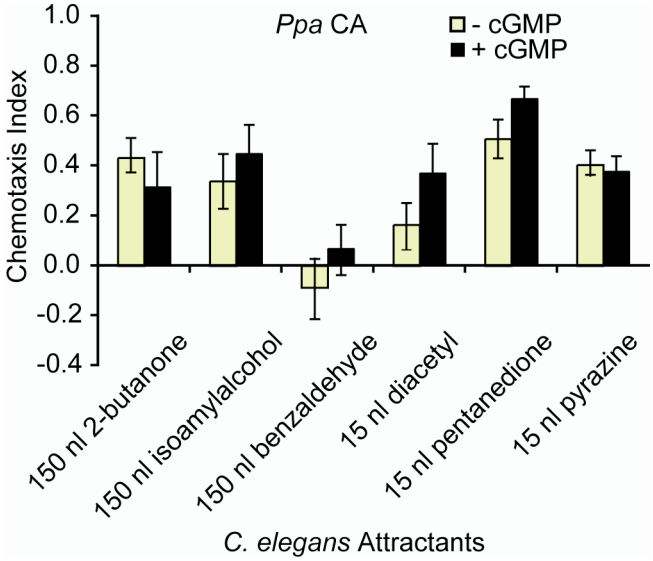

Fig. S7. Exogenous cGMP treatment of *P. pacificus* California did not enhance attraction to 150 nl of known *C. elegans* attractants 2-butanone, isoamyl alcohol, benzaldehyde, or 15 nl of diacetyl or 2,3-pentanedione.

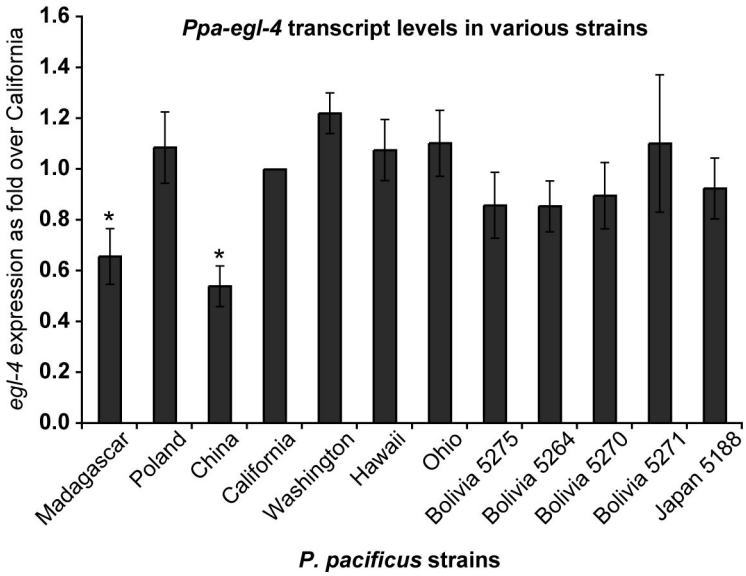

Fig. S8. qRT-PCR measurements in various *P. pacificus* strains. Average *Ppa-egl-4* transcript levels (*egl-4*/*tubulin*) are indicated relative to that of California ($n = 13$ reactions). *, expression of *egl-4* in Madagascar and China strains are significantly different from all other strains by ANOVA followed by pairwise comparisons, using Student's *t* test, $P < 0.05$.

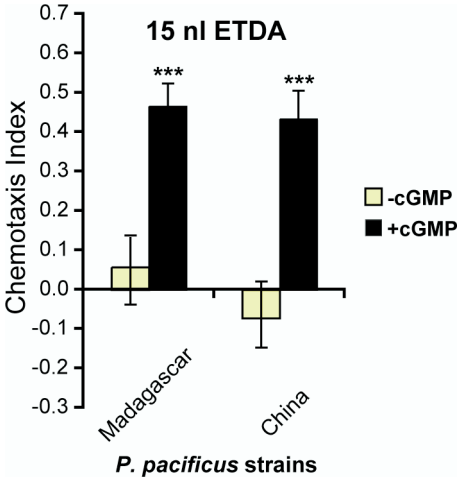

Fig. S9. Exogenous cGMP treatment can also enhance ETDA attraction of *P. pacificus* strains Madagascar and China, which showed low endogenous *egl-4* expression levels and ETDA attraction. ***, significant difference between mock and cGMP treated populations, $P < 0.001$ by two-sampled *t* test.

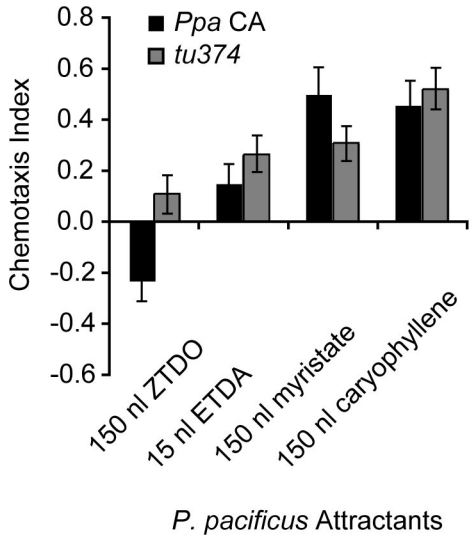

Fig. S10. *tu374* retained wild-type chemotaxis to *P. pacificus* attractants ZTDO, ETDA, myristate, and β-caryophyllene.

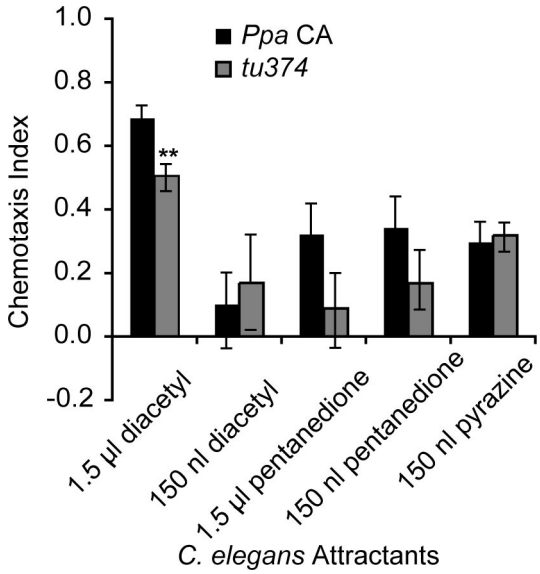

Fig. S11. *tu374* retained wild-type chemotaxis to most shared attractants with *C. elegans*, but attraction to diacetyl was slightly reduced. **, $P < 0.01$ by Student's *t* test.

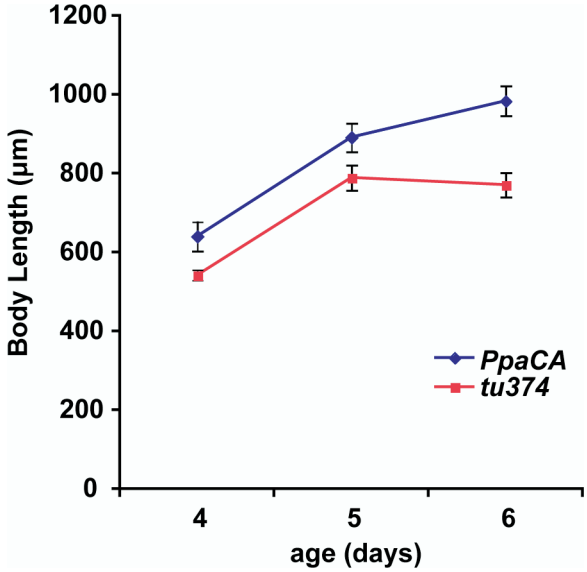

Fig. S12. Body length measurements of J4 and young adults at 24-h intervals showed *tu374* to be significantly shorter than wild-type California on all 3 days (≈78–88% of wild type) at 20°C ($n = 15$ for day 4 and $n = 20$ for days 5–6). Error bars denote 95% confidence intervals and all differences between the two strains are significant by Student's t test ($P < 0.001$).

Table S1. *Pristionchus pacificus* and *Pristionchus* sp. 11 strains

Strain	Geographical locality	Ecological origin
RS5228*	Japan (Mount Hiei)	soil
JU150†	Madagascar (Antananarivo)	soil
RS106†	Poland (Augustow)	soil
JU723†	China (Longsheng, Guangxi)	soil
PS312†	California (Pasadena)	soil
PS1843†	Washington (Port Angeles)	soil
JU138†	Hawaii (Captain Cook)	soil
SB5880†	New York	soil
RS5131†	Massachusetts (Carver)	*Exomala* sp.
RS5134†	Ohio (Wooster)	*Phyllophaga* sp.
RS5275	Bolivia (Santa Cruz)	scarab beetles
RS5270	Bolivia (Santa Cruz)	scarab beetles
RS5271	Bolivia (Santa Cruz)	scarab beetles
RS5264	Bolivia (Santa Cruz)	scarab beetles
JU482†	Japan (Hakone)	soil
RS5180†	Japan (Hakone)	*Exomala orientalis*
RS5187†	Japan (Hakone)	*Exomala orientalis*
RS5195†	Japan (Hakone)	*Exomala orientalis*
RS5210†	Japan (Hakone)	*Exomala orientalis*
RS5188†	Japan (Hakone)	*Exomala orientalis*

*All strains are *Pristionchus pacificus*, except for RS5228, which is a strain of *Pristionchus* sp. 11.

†These strains were described in refs. 1 and 2.previously . With the exception of RS106, all ''RS'' strains were isolated from field trips aimed to search for the natural habitat of *P. pacificus*. Bolivian strains came from multiple species of ″scarab beetles.″

1. Herrmann M, Mayer W, Hong RL, Kienle S, Minasaki R, Sommer RJ (2007) The nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) is associated with the Oriental beetle *Exomala orientalis* (Coleoptera: Scarabaeidae) in Japan. *Zoological Science*, 24:883–889.
2. Zauner H, *et al.* (2007) Distinct patterns of genetic variation in *Pristionchus pacificus* and *Caenorhabditis elegans*, two partially selfing nematodes with cosmopolitan distribution. *Mol Ecol* 16:1267–1280.

Table S2. SSCP markers used for genetic mapping of 15 nl ETDA insensitivity in RILs, their genetic locations, and genotypes in near isogenic lines NILs.

Linkage group	Marker	Genetic location, cM	*NIL CA1,2* (*egl-4* WA)	*NIL CA3* (*egl-4* WA)	*NIL CA4* (*egl-4* WA)	*NIL WA1* (*egl-4* CA)
I	S449	23	CA	CA	CA	WA
233 cM	S563	79	CA	CA	CA	WA
	S450	90	—	—	—	—
	S254	132	CA	CA	CA	WA
	S209	223	CA	CA	CA	WA
II	S97	23	—	—	—	—
170 cM	S598	47	CA	CA	CA	WA
	S50	57	—	—	—	—
	S325	73	—	—	—	—
	S558	106	CA	CA	CA	WA
	S289	135	CA	CA	CA	WA
III	S108	11	—	—	—	—
118 cM	S299	45	CA	CA	CA	WA
	S263	78	—	—	—	—
	S238	96	CA	CA	CA	WA
IV	S210	0	—	—	—	—
203 cM	S49	5	CA	CA	CA	WA
	S273	51	CA	CA	CA	WA
	S132	76	CA	CA	CA	WA
	S221 (C09G4.2)	77	CA	CA	CA	WA
	S298	77	CA	CA	CA	WA
	S320	84	CA	CA	CA	WA
	S344	126	CA	CA	CA	WA
	S286	150	—	—	—	—
	S285	156	CA	CA	CA	WA
	S34	159	CA	CA	CA	WA
	S589	162	CA	CA	CA	CA*
	S591 (*egl-4*)	**165**	**WA***	**WA***	**WA***	**CA***
	S11	166	CA	CA	CA	CA*
	S587	166	CA	CA	CA	CA*
	S588	167	—	—	—	—
	S590	169	—	—	—	—
	S290	176	—	—	—	—
	S288	182	CA	CA	CA	WA
	S284	203	CA	CA	CA	WA
V	S247	20	—	—	—	—
167 cM	S223	53	CA	CA	CA	WA
	S327	101	—	—	—	—
	S200	129	—	—	—	—
	S503	144	CA	CA	CA	WA
X	S57	22	CA	CA	CA	WA
198 cM	S438	22	CA	CA	CA	WA
	S422	83	—	—	—	—
	S419	130	CA	CA	CA	WA
	S259	156	—	—	—	—

NIL CA lines share the same genotype. —, not genotyped for NILs. Underlined markers were genotyped in NILs. C09G4.2 is a putative paralog of *egl-4*. Boldface text indicates targeted donor *eg1-4* locus.
*Regions containing the donor genotypes.

Table S3. Predicted *P. pacificus* genes based on *E* values of $<e^{-10}$ (Wormpep160) in the region contig 85.25 to contig 85.32

Predicted gene (predicted function)	TBLASTX E value
cyp-14 (cytochrome P450)	e^{-12}
glt-3 (amino acid glutamate transporter)	e^{-23}
ATP pathway	e^{-29}
CE27192 (?)	e^{-19}
CE36059 (UTP-galactose transporter)	e^{-12}
CE27512 (nuclear transport)	e^{-16}
CE36898 (?)	e^{-11}
CE33241 (ATPase)	e^{-22}
CE36718 (?)	e^{-17}

?, unknown function in *C. elegans*.

Table S4. Number of eggs and brood size in California wild type and *tu374*

Strain	Eggs, no.*	Progeny, no.*	Hatching, %
Wild type	155 ± 5	149 ± 4	96
tu374	139 ± 5	107 ± 4	77

Average and SEM values for total eggs and live progeny produced over three days at 20° C ($n = 34$ each).

*Significant differences between the two genotypes for the number of eggs laid and progeny are $P = 0.017$ and $P < 0.0001$, respectively, using Wilcoxon one-way test. The *P. pacificus* CA wild type holds ≤2 eggs in uterus and may mask the egg-laying phenotype that is more apparent in the *C. elegans* N2 wild type, which holds more eggs.

Table S5. List of primers used (5′ → 3′)

Primer	Sequence	Purpose
RH11962	aacaatattttcgggctatg	*Ppa-egl-4* Deletion screen
RH11964	atgagaagtgatgatcgttg	*Ppa-egl-4* Deletion screen
RH11963	aatcatgtctgtaagcagtc	*Ppa-egl-4* Deletion screen
RH11965	actatctctcaactgacaac	*Ppa-egl-4* Deletion screen
RH12548	GCAGGAACATATATTCGCAG	*Ppa-egl-4* cDNA; qRT-PCR
RH11818	GAATCCCTCATCCCATCCAGAG	*Ppa-egl-4* cDNA
RH12589	GTTCCGTGACACCAAGTATG	*Ppa-egl-4* cDNA
RH11819	ATCTGGAGGCACATCGGGATC	*Ppa-egl-4* cDNA
RH11820	cagGTGCAAATCGGCACGAAG	*Ppa-egl-4* cDNA
RH12550	AGGATAATTTCCGGCGAGAC	*Ppa-egl-4* cDNA
RH11821	TGTACGAAACGCACGAACTGCAG	*Ppa-egl-4* cDNA
RH12162	GAAGACAATTCTCCGGCTTG	*Ppa-egl-4* cDNA
RH12549	TGTCACGGAACGTTTTGTAC	qRT-PCR *Ppa-egl-4*
AG11112	CTCGGAGGAGGAACTGGATC	qRT-PCR *Ppa-beta-tubulin*
AG11113	GACCGTGTCAGAGACCTTAG	qRT-PCR *Ppa-beta-tubulin*
RH14744	ATGAGCAACAACGGCTCTGC	*P. expectatus egl-4* cDNA
RH14745	CTCTGCGAGGTCCGCGAGCG	*P. expectatus egl-4* cDNA
RH14746	TAGTTAAGTAGTCGAACCAG	*P. expectatus egl-4* cDNA
RH14747	AAGAGGAGAACGACGATTAG	*P. expectatus egl-4* cDNA
RH12587	AAGCGCGATCAGCAAATTCG	*P. expectatus egl-4* cDNA
RH13990	TGCCACCTTAGTGGGACTGG	*P. expectatus egl-4* cDNA
RH15339	GCGAAATAGTGAGTGTGCTC	2 kb 5′ *Ppa-egl-4* sequence
RH15305	GTAGGCGATCCACTCTTAC	2 kb 5′ *Ppa-egl-4* sequence
RH15340	CTTGCGGACCTCGCTGAGGC	2 kb 5′ *Ppa-egl-4* sequence
RH15304	GCAACTCTCTCGGTGCTC	2 kb 5′ *Ppa-egl-4* sequence
RH15719	GCTGATCCCGATGTGCCTCC	0.7 kb 3′ *Ppa-egl-4* sequence
RH15720	GTAAAGGATAACGAACCAAAC	0.7 kb 3′ *Ppa-egl-4* sequence