A Developmental Switch Coupled to the Evolution of Plasticity Acts through a Sulfatase

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SUMMARY

Developmental plasticity has been suggested to facilitate phenotypic diversity, but the molecular mechanisms underlying this relationship are little understood. We analyzed a feeding dimorphism in Pristionchus nematodes whereby one of two alternative adult mouth forms is executed after an irreversible developmental decision. By integrating developmental genetics with functional tests in phenotypically divergent populations and species, we identified a regulator of plasticity, eud-1, that acts in a developmental switch. eud-1 mutations eliminate one mouth form, whereas overexpression of eud-1 fixes it. EUD-1 is a sulfatase that acts dosage dependently, is necessary and sufficient to control the sexual dimorphism of feeding forms, and has a conserved function in Pristionchus evolution. It is epistatic to known signaling cascades and results from lineage-specific gene duplications. EUD-1 thus executes a developmental switch for morphological plasticity in the adult stage, showing that regulatory pathways can evolve by terminal addition of new genes.

INTRODUCTION

The evolution of morphological novelty is a major contributor to phenotypic diversity, particularly in adult stages. It has been suggested that developmental plasticity acts as a facilitator of phenotypic evolution (West-Eberhard, 2003, 2005), but the genetic and molecular mechanisms that regulate plasticity and specify novel traits have been largely elusive. Fundamental insight could be gained by an approach that integrates developmental biology with population genetics and ecology (Moczek et al., 2011). In particular, empirical studies of genetically identified developmental regulators in divergent populations and species are needed to test the significance of such regulators for phenotypic diversification.

Nematodes have a number of technical features, including genetic, genomic, and transgenic tools, which make such anistic studies practical. One model species is Pristionchus pacificus, which can be cultured on bacteria in the laboratory but lives in a necromenic association with scarab beetles in the wild (Herrmann et al., 2007). On the living beetle, P. pacificus remains in the arrested dauer stage and only resumes development with the proliferation of organisms on the dead host (Bento et al., 2010). This necromenic association has allowed the isolation of hundreds of P. pacificus strains and 30 Pristionchus species, all of which are available for population genetics and genomic studies (Herrmann et al., 2010; Morgan et al., 2012). Of particular importance is P. exspectatus, the presumptive sister species of P. pacificus, because the two species can produce sterile but viable F1 hybrids, enabling genetic studies above the species level (Kanzaki et al., 2012a). Thus, P. pacificus provides functional and genetic tools that can be coupled with micro- and macroevolutionary studies in a resolved phylogenetic context (Sommer and McGaughran, 2013; Figure 1).

Facilitating the necromenic lifestyle of Pristionchus are feeding structures that constitute an evolutionary novelty. Specifically, the mouth of P. pacificus and other Diplogastridae is equipped with moveable teeth that allow omnivorous feeding on bacteria, fungi, and other nematodes on beetle carcasses (Figure 1). These structures represent an ecologically important innovation because they are absent from Caenorhabditis elegans and other rhabditid nematodes, which are strictly microbivorous (Sudhaus and Fürst von Lieven, 2003). Moreover, the teeth exhibit developmental plasticity, whereby one of two alternative adult feeding forms is executed after an irreversible decision during larval development. The dimorphism comprises a “narrow-mouthed” or stenostomatous (St) form, which has a single, flint-shaped dorsal tooth, and a “wide-mouthed” or eurystomatous (Eu) form, which has a claw-like dorsal tooth, an opposing right sub-ventral tooth, and more complexity in its left subventral denticles (Figure 1). The Eu form is highly derived with respect to rhabditid nematodes that lack the mouth dimorphism and is the form most associated with predation (Kiontke and Fitch, 2010; Figure 1).

Under laboratory conditions, P. pacificus has a ratio of Eu-to-St animals that is influenced by environmental cues such as starvation and crowding (Bento et al., 2010; Serobyran et al., 2013). An endocrine signaling module involving Δ7-dafachronic acid (DA) and the nuclear hormone receptor DAF-12 was shown to regulate the mouth dimorphism in addition to pheromone signals (Bento et al., 2010; Bose et al., 2012; Figure 1). Although
hormonal signaling and environmental cues can shift the frequencies of the two forms in a population, they are unable to act as developmental switches. Specifically, Ppa-daf-12 mutants as well as animals treated with Δ7-DA or pheromonal small molecules have a shifted mouth-form ratio but still produce both forms. It therefore remained unknown whether any definitive switch operates in the developmental decision or whether a single form can be genetically fixed. Similarly, nothing was known about the evolution or regulation of the mouth dimorphism in other P. pacificus strains or among Pristionchus species. The capacity for genetic accommodation of a plastic trait is fundamental to the hypothesis that developmental plasticity, particularly the ability to produce an alternative morphotype, gives flexibility for responding to selection and thereby leads to the diversification of form (West-Eberhard, 2003; Moczek, 2007).

Here, we show that a developmental switch for the P. pacificus mouth dimorphism acts through a sulfatase encoded by the eud-1 gene. eud-1 mutants are completely St, whereas overexpression of eud-1 results in completely Eu populations. Differences of the mouth-form ratio in wild isolates of P. pacificus correlate with eud-1 expression, and eud-1 overexpression transforms highly St strains to have an all-Eu phenotype. Similarly, F1 hybrids between P. exspectatus and eud-1-transgenic P. pacificus are completely Eu, indicating that EUD-1 is sufficient to control the mouth-form decision in different wild populations and species. We show that the eud-1 coding region is under purifying selection.
RESULTS

Forward Genetics Resulted in Monomorphic Mutants

To test whether regulators of developmental plasticity can act as a switch in the Eu versus St mouth-form decision, we screened for mutants that would result in the complete loss of the complex, Eu form in P. pacificus. We mutagenized the California strain (RS2333), which shows a high frequency (~90%) of Eu hermaphrodites (Serobyan et al., 2013), to recover such mutants. From a mutagenesis screen of 3,850 haploid gametes, we obtained 17 mutants in which hermaphrodites were strongly eurystromatous form defective (eud). Although 13 of the 17 mutants were recessive, 4 were dominant. Specifically, homozygotes of these four mutants had a completely penetrant phenotype (0% Eu), whereas heterozygotes had an incompletely penetrant phenotype (8%–37% Eu; Figure 2). Using simple-sequence length and conformation polymorphism markers, we mapped seven of the recessive mutants to six different regions on autosomes (data not shown). In contrast, the four mutants with a dominant phenotype mapped to the same genomic interval on the X chromosome (Figure 3; see Figure S1 available online). These dominant mutants showed no other phenotype or larval lethality, suggesting the existence of a single dominant locus that acts as a specific regulator of an adult phenotypic plasticity. We named this locus eud-1.

eud-1 Encodes a Sulfatase

The isolation of four dominant alleles of eud-1 from a mutagenesis screen that is far from saturation suggests the importance of this gene. We obtained whole-genomic sequences for all four alleles by next-generation sequencing and searched for SNPs. A single 1 kb interval throughout the entire genome contained SNPs in all four mutants and was within the original mapping interval on the X chromosome (Figure 3A). Putative mutation sites of eud-1 were within the boundaries of a single predicted gene, Contig8-snap.30 (http://www.pristionchus.org), and they affected predicted exons or splice acceptors (Figures 3B and S1; Table S1). Expression of Contig8-snap.30 was observed by RNA sequencing, and all four eud-1 mutations were confirmed by Sanger sequencing.

eud-1 encodes a sulfatase putatively homologous with C. elegans sulfatase 2 (sul-2), the ortholog of human arylsulfatase A (ARSA), whose crystal structure has been solved (Lukatela et al., 1998) and active site confirmed (Waldow et al., 1999). In humans, mutations in this sulfatase lead to the lysosomal storage disorder metachromatic leukodystrophy (Schmidt et al., 1995), although no mutant phenotypes are known for Cel-sul-2. Supporting the function of Ppa-EUD-1 as a sulfatase was the location of all three coding mutations within a highly conserved active site sequence (Figure 3B). In particular, eud-1(tu451) causes an amino acid change, R99Q, identical to a mutation that virtually eliminates catalytic activity in a homologous sulfatase, ARSB, in Maroteaux-Lamy syndrome type VI (Litjens et al., 1996; Figure 3B). Furthermore, a presumptive null mutation, eud-1(tu445), characterized the only mutant with no eud-1 expression detected by RNA sequencing (Figures S2 and S3).

eud-1 Executes a Developmental Switch for the Mouth Dimorphism

We next tested whether the dominant eud-1 phenotype results from gain-of-function or reduction-of-function mutations, the latter of which would indicate that eud-1 acts as a developmental switch gene. To distinguish between these two scenarios, we performed two sets of transgenic microinjection experiments. First, we transformed the wild-type California strain with a eud-1 mutant allele, and second, we injected a wild-type eud-1 copy into eud-1 mutants. If eud-1 alleles were gain of function, transgenic animals carrying a mutant copy of eud-1 should have an all-St phenotype. In contrast, if eud-1 were haploinsufficient, eud-1 alleles would be rescued with a wild-type copy of the gene.

Two independently transformed lines carrying a eud-1(tu450) mutant construct with an egl-20::rfp (red fluorescent protein) reporter maintained a wild-type frequency of Eu animals (Figure 2). In contrast, multiple lines of eud-1(tu445) and eud-1(tu450) mutant animals independently transformed with wild-type copies of eud-1 were rescued to produce Eu animals (Figure 2). Interestingly, these lines were not only rescued but exceeded the wild-type frequency by forming >99% Eu hermaphrodites.
A third mutant allele, eud-1(tu442), was similarly over-rescued by crossing with a line of eud-1(tu450); Ex[eud-1CA] (Figure 2). These experiments show that eud-1 acts as a developmental switch for the P. pacificus mouth-form decision: zero wild-type copies of the gene in homozygous mutants resulted in all-St hermaphrodites; one wild-type copy in heterozygous mutants resulted in a low Eu frequency; two wild-type copies in the California strain resulted in a high Eu frequency; and presumed higher copy numbers in transgenic lines resulted in all-Eu hermaphrodites.

**EUD-1 Regulates Sexual Dimorphism of the Mouth-Form Phenotype**

The mouth-form phenotype of P. pacificus is sexually dimorphic, with males (XO animals) predominantly expressing the St form (Serobyan et al., 2013; Figure 2). Given that eud-1 is on the X chromosome and that eud-1 mutations are haploinsufficient, we investigated whether levels of eud-1 expression control the distinct mouth-form phenotype of males. Measurements of eud-1 expression by RNA sequencing indicated strong downregulation in males relative to mixed-stage hermaphrodites.
sister species of *P. pacificus* the role of *eud-1* not due to loss of function of *eud-1*.

Next, we wanted to know whether the regulatory network including *eud-1* could be linked to patterns of microevolutionary divergence, we searched for correlations between *eud-1* expression and natural variation in the mouth-form phenotype. First, we conducted a survey of 72 wild isolates of *P. pacificus* that have a known population structure as inferred from microsatellite markers and next-generation sequencing (Morgan et al., 2012; C.R., G. Bartelmes, A. Weller, H. Witte, W. Röseler, W.E. Mayer, C. Dieterich, and R.J.S., unpublished data). The majority of wild isolates showed a bias toward a Eu phenotype, similar to *P. pacificus* RS2333 (Figure S2). In contrast, four of these strains had a highly St phenotype (Figure S4A). To test whether expression of *eud-1* is sufficient for the formation of the Eu mouth form in wild isolates with high St frequencies, we induced overexpression of *eud-1* in two of these strains, RS5200B (India) and RS5410 (La Réunion), using a transformation construct of *eud-1*<sup>CA</sup>. RS5520B and RS5410 animals carrying copies of *eud-1*<sup>CA</sup> were converted to a higher or completely Eu phenotype (p < 10<sup>-8</sup> in all comparisons). Specifically, RS5200B; Ex(*eud-1*<sup>CA</sup>) and RS5410; Ex(*eud-1*<sup>CA</sup>) had 39%–100% and 51%–99% Eu animals, respectively (Figure S2). In contrast, transgenic lines carrying the *eud-1*<sup>tu450</sup> null allele maintained a high St frequency (≤6% Eu; Figure 4B), and animals that were RFP negative and thus had lost the *eud-1*<sup>CA</sup> array were also highly St (≤7% Eu) in all but one line; Figure 4B). Similarly, transforming *P. pacificus* strains RS5200B and RS5410 with constructs of their own alleles, *eud-1*<sup>RS2333</sup> and *eud-1*<sup>RS5410</sup>, respectively, resulted in higher (58%) to almost completely (98%) Eu phenotypes (p < 10<sup>-5</sup>; Figure 4B). Finally, using RNA sequencing, we measured the expression of *eud-1* of the two strains and found that expression of the gene was higher than in mutants, although less than half of that observed in RS2333 (Figure S2). Together, these experiments show that a switch including *eud-1* regulates the dimorphism in naturally variant populations. Moreover, the phenotype of highly St strains is not due to loss of function of *eud-1* but most likely due to the control of its expression.

**The EUD-1 Switch Operates in Wild Populations of *P. pacificus***

To determine if a developmental switch gene identified by forward genetics could be linked to patterns of microevolutionary divergence, we searched for correlations between *eud-1* expression and natural variation in the mouth-form phenotype. First, we conducted a survey of 72 wild isolates of *P. pacificus* that have a known population structure as inferred from microsatellite markers and next-generation sequencing (Morgan et al., 2012; C.R., G. Bartelmes, A. Weller, H. Witte, W. Röseler, W.E. Mayer, C. Dieterich, and R.J.S., unpublished data). The majority of wild isolates showed a bias toward a Eu phenotype, similar to *P. pacificus* RS2333 (Figure S2). In contrast, four of these strains had a highly St phenotype (Figure S4A). To test whether expression of *eud-1* is sufficient for the formation of the Eu mouth form in wild isolates with high St frequencies, we induced overexpression of *eud-1* in two of these strains, RS5200B (India) and RS5410 (La Réunion), using a transformation construct of *eud-1*<sup>CA</sup>. RS5520B and RS5410 animals carrying copies of *eud-1*<sup>CA</sup> were converted to a higher or completely Eu phenotype (p < 10<sup>-8</sup> in all comparisons). Specifically, RS5200B; Ex(*eud-1*<sup>CA</sup>) and RS5410; Ex(*eud-1*<sup>CA</sup>) had 39%–100% and 51%–99% Eu animals, respectively (Figure S2). In contrast, transgenic lines carrying the *eud-1*<sup>tu450</sup> null allele maintained a high St frequency (≤6% Eu; Figure 4B), and animals that were RFP negative and thus had lost the *eud-1*<sup>CA</sup> array were also highly St (≤7% Eu) in all but one line; Figure 4B). Similarly, transforming *P. pacificus* strains RS5200B and RS5410 with constructs of their own alleles, *eud-1*<sup>RS2333</sup> and *eud-1*<sup>RS5410</sup>, respectively, resulted in higher (58%) to almost completely (98%) Eu phenotypes (p < 10<sup>-5</sup>; Figure 4B). Finally, using RNA sequencing, we measured the expression of *eud-1* of the two strains and found that expression of the gene was higher than in mutants, although less than half of that observed in RS2333 (Figure S2). Together, these experiments show that a switch including *eud-1* regulates the dimorphism in naturally variant populations. Moreover, the phenotype of highly St strains is not due to loss of function of *eud-1* but most likely due to the control of its expression.

**Hybrid Crosses Show a Conserved Role for *eud-1* across Species**

Next, we wanted to know whether the regulatory network including *eud-1* could also be linked to macroevolutionary patterns of the mouth dimorphism in *Pristionchus*. To determine the role of *eud-1* in other species, we recruited the gonochoristic sister species of *P. pacificus*, *P. exspectatus*. Specifically, we designed hybrid-cross experiments to test whether the *eud-1* switch operates above the species level. First, we identified a strain (RS5522B) of *P. exspectatus* to be completely St (<1% Eu) (Figures 4C and 4E). This strain resulted from ten generations of random inbreeding from a parental strain originally having a high Eu frequency in females (71% Eu; data not shown). Additionally, we found that *eud-1* expression in this highly St strain was lower (Figure S2). To test whether a greater number of *eud-1* copies could induce the Eu form, we crossed this strain to males of wild-type (RS2333) and *eud-1*<sup>tu450</sup>; Ex(*eud-1*<sup>CA</sup>) lines of *P. pacificus*. Nontransgenic hybrid females had intermediate mouth-form frequencies, presumably due to genetic complementation (Figure 4C). In contrast, female hybrids carrying the *eud-1*<sup>CA</sup>; egl-20::rfp array were completely (100%) Eu (Figure 4C), and transgenic hybrid males were almost completely (90%) Eu (Figure 4E). Similarly, transgenic hybrid F1 females with *P. pacificus* mothers, marked by *Ppa-unc-1*, and *P. exspectatus* fathers were 100% Eu (Figure 4C).

We next tested whether multiple copies of *Pex-eud-1* could convert the phenotype of *F* hybrids between *P. pacificus* and *P. exspectatus*. To do this, we transformed the *P. pacificus* mutant *eud-1*<sup>tu450</sup> with the allele from *P. exspectatus*, which resulted in complete overexpression (100% Eu; Figure 4D), and then crossed the transgenic line to *P. exspectatus*. Transgenic hybrid F1 females were completely (100%) Eu (Figure 4D). The results of hybrid crosses therefore indicate that the *eud-1* switch is evolutionarily conserved across species. However, given that overexpression studies can affect multiple developmental pathways in an uncontrollable manner, we complemented our transgenic experiments with studies of genetic variation and gene function in nontransgenic experiments.

**The eud-1 Locus Shows Strong Signs of Purifying Selection**

The genetic and transgenic experiments described above indicate a role for *EUD-1* in shifting the balance between the two mouth forms in different *P. pacificus* populations and in *P. exspectatus*. However, given that these experiments were based on transgenic animals with a largely uncontrolled number of gene copies in transgenic arrays, the results do not distinguish whether the activity of the *EUD-1* protein differs between strains and species or whether the observed phenotypic differences were due to elements regulating *eud-1*. To discriminate between these possibilities, we analyzed the genetic variation at the *eud-1* locus among 104 resequenced *P. pacificus* strains and *P. exspectatus* (Figure 3C). We found 44 synonymous and 23 nonsynonymous substitutions among the 104 *P. pacificus* strains, although no substitutions were predicted to be deleterious. Normalizing by the number of synonymous and nonsynonymous sites within *eud-1*, these values translate into a dN/dS ratio of 0.15, indicating that 85% of nonsynonymous substitutions in *eud-1* were purged from populations quickly. Consistent with this finding was a low dN/dS ratio (0.11) between *P. pacificus* and *P. exspectatus* (Figure 3C). Thus, the coding region of *eud-1* shows signatures of purifying selection.

Similarly, we searched for signs of selection in the 7 kb upstream region of *eud-1* that is sufficient to drive *eud-1* expression. Tests for selection of noncoding sequences in 1 kb intervals failed to reject neutrality, although local peaks of up to 4%
nucleotide diversity exist (Figure 3C). However, we were unable to identify bona fide transcription factor binding sites using the program ExPlain 3.1 (BIOBASE). Taken together, analyses of genetic variation at the eud-1 locus provide strong evidence for purifying selection in the coding region of eud-1 but do not indicate a role for specific cis-regulatory elements.

**Reciprocal Hemizygosity Results in Contrasting Mouth-Form Frequencies in Males**

Given the evidence against positive selection and functional differences of eud-1, we next tested for interspecific divergence of eud-1 alleles by performing reciprocal hemizygosity experiments with *P. pacificus* and *P. exspectatus*. When we analyzed hybrid
namely of a *P. pacificus* to this observation, the male offspring from the reciprocal cross, the same cross were all St (0% Eu; Figure 4 E). In strong contrast, males were 69% Eu (Figure 4 C), whereas male offspring of the reciprocal cross of *P. pacificus* were 97% Eu (Figure 4 E). Hybrid males thus expressed a high Eu frequency if the X chromosome was provided by *P. pacificus*, and not *P. exspectatus*. These results allow two major conclusions. First, the X chromosome, including the *eud-1* locus, differs between the two species. Given that hybrid females or *P. pacificus* hermaphrodites that overexpress the *eud-1* allele of *P. exspectatus* are highly Eu, the differences between the species are most likely either located in regulatory elements of *eud-1* or in other X-linked trans-acting factors, rather than in the coding region of *eud-1*. Second, additional, autosomal trans-acting factors might influence the expressivity of the Eu mouth form. Together, these findings suggest that factors in the *eud-1* regulatory network are important for evolutionary changes in mouth-form regulation. Although we cannot rule out the existence of cis-regulatory elements in the *eud-1* gene itself, there is no direct evidence clearly supporting their involvement.

Because reciprocal hemizygosity experiments cannot distinguish between *P. pacificus*-specific, trans-acting, X-linked activators and autosomal, dosage-dependent suppressors, we compared the phenotype of F1 hybrid males with *P. pacificus* mothers to males from crosses within *P. pacificus*. The predominantly St (20% Eu) phenotype of *P. pacificus* males (Figure 2), in contrast to the highly Eu phenotype of the former cross, suggests the presence of autosomal suppressors. We hypothesize that two copies of an autosomal dosage-dependent suppressor repress *eud-1* expression in *P. pacificus* males, whereas one copy of the suppressor in male interspecies hybrids is insufficient to repress *eud-1* expression. Furthermore, the haploinsufficiency of *eud-1* is consistent with the hypothesis of an autosomal dosage-dependent suppressor: whereas the Eu-biased hermaphrodites of *P. pacificus* (RS2333) would mostly escape suppression, mutant heterozygotes, which show a highly St phenotype, would not.

**EUD-1 Acts as a Sulfatase and Is Expressed in Neurons**

The experiments described above provide strong evidence that *eud-1* is necessary and sufficient to control the mouth-form switch and that it operates in different wild populations and species of *Pristionchus*. To assay the functional activity of *Ppa-EUD-1*, we first tested whether competitive inhibitors of arylsulfatases, sulfate ions (Dodgson and Spencer, 1953; Glössl et al., 1979), could mimic a *eud* phenotype. Indeed, application of such salts resulted in a significant reduction in the Eu frequency in the wild-type California strain, indicating that sulfates act by product inhibition (Figure 5 A). Similarly, application of phosphate ions, also known to inhibit sulfatases, significantly reduced the frequency of Eu animals (Figure 5 A), supporting the action of EUD-1 as a sulfatase. It is interesting to note that this inhibition was not strong enough to alter the all-Eu phenotype of the transgenic animals, which show massive overexpression of the sulfatase.

To determine the cells that express EUD-1, we generated a transcripational reporter of a 7 kb *eud-1* promoter element, the genomic region that was sufficient for the rescue of the mutant phenotype, to drive RFP expression. This reporter was

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**Figure 5. Activity and Epistasis of *P. pacificus* EUD-1**

(A) Competitive inhibition of sulfatases by sulfate and phosphate ions is shown. Application of inhibitors at 120 mM mimics a *eud* phenotype in the wild-type California strain (“CA”), but a strain overexpressing *eud-1*, *tu442; Ex[eud-1*CA*] (“Tg”) does not respond to nonlethal concentrations of salts.

(B) Epistasis of EUD-1 to pheromone signaling is shown. Whereas the pheromone dasc#1 strongly induces the Eu form in wild-type strains of *P. pacificus*, *eud-1* mutants are completely unresponsive to this signal.

(C) Epistasis of EUD-1 to the receptor of Δ7-DA is presented. A line of *P. pacificus* overexpressing EUD-1 completely inhibits induction of the St form by treatment with Δ7-DA.

Data are represented as the total Eu frequency ± a 95% confidence interval, estimated using a binomial test on the total count data pooled across replicate treatments. ***p < 10−6, Fisher’s exact test.**
expressed in several classes of somatic and pharyngeal neurons (Figure 3E), and RFP expression was seen consistently in all postembryonic stages and all independently generated transgenic lines. The observed RFP expression suggests a role for Ppa-eud-1 in sensory transduction or neuroendocrine signaling in development. Therefore, we next constructed tests to determine the position of EUD-1 with respect to the known cascade of mouth-form development.

**Pheromone Signaling Acts through the EUD-1 Switch**

Individual pheromone molecules were previously found to influence the mouth-form decision, the strongest effect resulting from the diascaroside dasc#1 (Bose et al., 2012). This molecule is only known from *P. pacificus* and, in contrast to compounds that also induce dauer formation, is specific to the mouth dimorphism. To determine whether EUD-1 acts downstream of developmental cascades initiated by pheromone, we treated eud-1(tu445) mutants with diascaroside #1 (dasc#1). Whereas pheromone induced significantly higher Eu frequencies (p < 10^{-6}) in the highly St strain RS5200B (84% Eu) and in the wild-type California strain (98%), it had no effect on the mutant (0%; Figure 5B). This finding demonstrates that the EUD-1 switch controls the response of the mouth plasticity to external, pheromonal cues.

**EUD-1 Is Epistatic to Δ7-DA/DAF-12**

Because DAF-12 is the final common target of dauer regulatory pathways in *C. elegans* (Antebi et al., 2000), the epistasis of EUD-1 over the receptor of Δ7-DA is consistent with EUD-1 being an ultimate developmental switch for the mouth dimorphism.

**DISCUSSION**

This study has identified a developmental switch that controls a morphological plasticity in the nematode adult stage and that acts through the sulfatase EUD-1. Our findings result in four major conclusions. First, and most significantly, we show that a gene uncovered in an unbiased genetic screen is linked to patterns of micro- and macroevolution of an ecologically relevant trait (Figure 6). Laboratory approaches, in particular with genetic experimentation, have been powerful in many fields of biology, such as in developmental biology and neurobiology. In contrast, several aspects of evolutionary research usually escape laboratory studies, including functional analysis of genes controlling traits that mediate interaction with and directly respond to the environment. Testing the evolutionary significance of genetically identified regulatory mechanisms requires integrative approaches that link development with ecology, population genetics, and a well-resolved phylogenetic framework (Moczek et al., 2011; Sommer and McGaughran, 2013).

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**Figure 6. The Genetics of Developmental Plasticity Reflect Patterns of Microevolution and Macroevolution**

Forward genetics (left) uncovered a gene, eud-1, as part of a switch controlling the development of an ecologically significant trait, a morphology that directly determines feeding abilities. Identifying a similar phenotype in wild populations of *P. pacificus* (center) and other species (right), all in a resolved phylogenetic context, allowed testing the relevance of this gene in natural history. Functional analysis of EUD-1 in St-biased variants (red), with respect to strains with a Eu bias or intermediate phenotype (blue), empirically demonstrates how changes in the genetic network regulating EUD-1 underlie diversification of the dimorphism. Center tree inferred by Morgan et al. (2012); tree on right, by Kanzaki et al. (2013b).
Here, we show that the genetically identified eud-1 locus acts as a master regulator that is variably expressed among populations, and genetic transformation with this gene affects variation of the trait in wild isolates. The analysis of genetic variation at the eud-1 locus shows strong signs of purifying selection in the coding region of the gene, implying that the EUD-1 protein itself does not contribute to phenotypic evolution. Rather, the genetic network regulating eud-1, involving potential X-linked and autosomal trans-acting factors, seems to be responsible for evolutionary changes in the expressivity of the mouth forms. Together, our analyses show that developmental processes inferred experimentally through mutations can reflect those that have withstood the test of natural selection. Such a link can most easily be identified for genetic networks specifying divergent postembryonic or adult traits, with the effects of BMP4 signaling on beak shape in finches (Abzhanov et al., 2004) and those of insulin signaling on horn size in scarab beetles (Emlen et al., 2012) representing prominent analogous examples.

The second conclusion is that the regulation of developmental plasticity involves a gene that controls a developmental switch. Mutations in the eud-1 gene eliminate the Eu mouth form, whereas overexpression of this gene fixes it. Developmental switch genes are well known in signaling pathways that control multiple aspects of animal development, for example the GTPase RAS in Drosophila eye and C. elegans vulva development (Han and Sternberg, 1990; Simon et al., 1991). Most developmental switch genes have pleiotropic functions and are highly conserved throughout evolution. In contrast, the single known phenotype of eud-1 suggests that this gene acts specifically to control the mouth dimorphism. Furthermore, eud-1 has resulted from recent gene duplications, indicating that new genes can be recruited as master regulators of development. The phylogenetic history of Ppa-eud-1 shows the existence of multiple paralogs of sul-2 (Figure 3D). Duplications of the gene have occurred both since the split of Pristionchus from other sequenced species as well as within Pristionchus. This finding provides empirical evidence to complement recent discussions on the evolutionary origin and function of orphan and other novel genes (Long et al., 2003; Tautz and Domazet-Loso, 2011). The assumption of a recently duplicated switch gene as a downstream target for mouth-form development is striking, considering that the addition of genetic elements to regulatory cascades during evolution is thought to occur primarily upstream (Wilkins, 2002). This was demonstrated in the well-characterized animal sex-determination pathway: the final target Doublesex/mab-3 is broadly conserved (Raymond et al., 1998; Matsuda et al., 2002; Miller et al., 2003), whereas divergent components have been recruited upstream. However, in nematode mouth-form development, the epistasis of EUD-1 to phenomone signaling and the Δ7-DA/DAF-12 module shows that regulatory pathways can evolve by terminal addition of novel genes.

Mitsunaga-Nakatsubo et al. (2009). It is thus possible that EUD-1 modifies structural molecules in P. pacificus mouth-form development, although the expression of EUD-1 in the nervous system does not support such a role. Instead, we speculate that a steroid hormone might be the EUD-1 target. The observed expression pattern of Ppa-eud-1 would be consistent with the previously described role of DAF-12/NHR in mouth-form regulation and the predicted presence of sulfated steroids in nematodes (Carroll et al., 2006; Hattori et al., 2003). Finally, this study elucidates a mechanism that generates a morphological novelty of the adult stage. Developmental plasticity has been increasingly discussed as a facilitator of phenotypic diversity and the generation of ecologically relevant traits. Several authors have argued that plasticity is essential both for the interaction between development and the environment and for driving evolution and divergence (Brakefield et al., 1998; Pigliucci, 2001; Nijhout, 2003; Schlichting, 2003; West-Eberhard, 2003). However, two major challenges result from the “facilitator” hypothesis. First, the genetic and molecular mechanisms underlying developmental plasticity need to be linked to micro- and macroevolutionary divergence. The present study provides an important example by identifying a gene that is part of a genetic network linking genetic regulatory processes to hormone signaling, a known target of developmental plasticity (Nijhout, 2003).

The second challenge concerns the long-term evolutionary success and influence of developmental plasticity. Do traits that show two or multiple morphs evolve faster than other traits, and do they contribute to phenotypic evolution in general? Although relevant studies in nematodes are still in their infancy, the Pristionchus mouth dimorphism supports this idea. Pristionchus nematodes are uniform in most morphological traits, and the majority of the 30 confirmed Pristionchus species were previously only diagnosed by their molecular profiles (Hermann et al., 2006; Kanzaki et al., 2012a). In contrast, the recent description and morphological analysis of 14 new Pristionchus species have shown the mouth structures of these species to be highly diverse, supporting the link between plasticity and diversity (Kanzaki et al., 2012b, 2013a, 2013b, 2013c). Most interesting among these is a clade of three Pristionchus species that have added a novel type of plasticity to the already existing mouth dimorphism, and through phylogenetically supported intermediates, this plasticity has culminated in new mouth morphology (Ragsdale et al., 2013). Comparative analysis of Pristionchus mouthparts thus strongly supports the hypothesis...
that developmental plasticity facilitates phenotypic diversity. The identification of EUD-1 as part of a developmental switch brings this area of evolution and ecology into the realm of genetics and molecular biology.

**EXPERIMENTAL PROCEDURES**

**Culture Conditions**

Except when used in crosses or assays, all *Pristionchus* strains were kept on 6 cm plates with nematode growth medium (NGM) agar and were fed with a lawn of *Escherichia coli* OP50 grown in 400 μL-L-Broth. For crosses, plates were seeded with a lawn grown from 25 μL OP50 in L-Broth. Cultures were maintained at 20°C–25°C. Because the mouth-form ratio is sensitive to unknown environmental and putative epigenetic effects (Serobyan et al., 2013), all experiments include their own controls for the wild-type Eu frequency. Consequently, the mouth-form ratio is not comparable across all experiments, which necessarily introduce different variables. Also, to minimize the potential for laboratory evolution of the trait, a new culture of the California strain was revived annually from a frozen voucher.

**Phenotype Scoring**

The mouth-form phenotype was scored as previously described by Serobyan et al. (2013). In short, characters used to discriminate between Eu and St individuals, respectively, were (1) the presence versus absence of a subventral tooth, (2) a claw-like versus flattish or triangular dorsal tooth, and (3) a wide versus narrow stoma (mouth). Characters 1 and 2 were discrete, nonoverlapping, and sufficient to distinguish the two forms. Apparent intermediates between the two forms were rare (<0.1%) and were not included in counts. In most cases, phenotypes could be scored using Zeiss Discovery V12 and V20 stereomicroscopes and were supplemented where necessary with differential interference contrast (DIC) microscopy on a Zeiss Axioskop. Transgenic lines, *P. exspectatus*, hybrids, and individuals in the pheromone and Δ7-DA assays were scored exclusively by DIC microscopy. For sample sizes of animals scored, see Tables S2, S3, and S4.

**Forward Genetics**

Techniques used for forward genetics in *P. pacificus* have been previously described by Pires da Silva (2006). Detailed protocols for the forward genetics screen, genetic mapping, whole-genome sequencing for mutant identification, and gene cloning are provided in the Extended Experimental Procedures.

**Genetic Transformation**

Extrachromosomal arrays were generated as described by Schlager et al. (2009). The germlines of adult hermaphrodites were injected with a *Ppa*-eud-1 construct (15 ng/μl), the marker *Ppa*-egl-20::TurboRFP (10 ng/μl), and genomic carrier DNA (60 ng/μl) from the recipient strain. Transgenic lines were scored for their mouth-dimorphism phenotype over multiple generations. Sample sizes of analyzed animals are given in Table S3. All *Ppa*-eud-1 constructs consisted of a 13 kb fragment containing a 7 kb promoter element, the 4.6 kb coding region including all introns, and 1.6 kb 3’ UTR fragment. The *Pex-eud-1* locus is of similar size, and the constructs used for transformation experiments consisted of promoter fragments, the coding region including introns, and the 3’ UTR.

To rescue more than one allele with the same extrachromosomal array, a line transformed by microinjection, *eud-1(tu450) Ex[eud-1(tu450)]*, was crossed to the mutant line *eud-1(tu442)*. Because *Ppa*-eud-1 is on the X chromosome, transgenic males were crossed to mutant hermaphrodites to eliminate the X chromosome of the transgenic line from F1 females. F1 males reporting the array were crossed to the mutant line. F2 hermaphrodites, which carried the X chromosome from the mutant *eud-1(tu442) as well as the array, were screened for rescue of the mouth-dimorphism phenotype.

To generate transgenic males, wild-type California males or *eud-1(tu450) males were crossed to *eud-1(tu450) Ex[eud-1(tu450)]* hermaphrodites, such that, in either type of cross, F1 males inherited both the X-linked locus and the extrachromosomal array from the transgenic line.

For transgenic experiments including other *P. pacificus* strains, control transformations of RSS200B were performed with a presumptive null *eud-1* allele, tu445.

**Natural Variation in *P. pacificus***

To test for the presence of natural variation in the mouth-form frequency in wild populations of *P. pacificus*, phenotypes were scored for at least three culture populations spanning at least as many generations. Strains that were highly St (<20% Eu) were screened similarly over at least six culture populations.

**Whole-Genomic Sequencing and Diversity Analysis**

Methods for preparation and analysis of whole-genomic and transcriptomic sequences and for analysis of diversity patterns at the eud-1 locus are provided in the Extended Experimental Procedures.

**Phenotypic Conversion of Interspecific Hybrids**

An extrachromosomal array carrying *eud-1* was introduced into interspecific hybrids through reciprocal crosses. To ensure that all F1 offspring of *P. exspectatus* males and *P. pacificus* hermaphrodites were hybrids, a recessive morphological marker, *Ppa-unc-1*, was used. A transgenic double mutant, *unc-1; eud-1(tu450); Ex[eud-1(Ca)]*, was generated as a marker for transformation of hybrids. Offspring of *P. pacificus* males crossed to *P. exspectatus* females were considered hybrids. Crosses in the latter direction were performed with males of *eud-1(tu450); Ex[eud-1(Ca)]*, whereas control crosses in the same direction were performed with males of strain RS2333. Crosses each consisted of three to five females or hermaphrodites and of five *P. exspectatus* males or eight to ten *P. pacificus* males. Hybrid-cross experiments to introduce an array including *Pex-eud-1* were performed with *P. pacificus* RS2333 males and *P. exspectatus* females.

**Reporter Constructs**

The transcriptional reporter of *Ppa*-eud-1::TurboRFP contained the same 7 kb fragment as that of the rescue construct. The *Ppa*-eud-1::TurboRFP promoter element was fused with a fragment containing a nuclear localization signal, the coding region of the fluorophore TurboRFP (Evrogen), and the 3’ UTR sequence of the gene *Ppa*-pdi-23Ca (Schlager et al., 2009). Fragments were then fused and amplified by overlapping extension PCR. All amplified fragments were verified by sequencing. Primers for amplification of the final reporter construct contained Xmal restriction sites for subsequent digestion of *Ppa*-eud-1::TurboRFP. The expression construct (15 ng/μl) was co-injected with 60 ng/μl genomic carrier DNA, cut with Xmal and PstI, and 10 ng/μl of the injection marker *Ppa-egl-20::TurboRFP* cut with PstI. Three independent lines were generated. Reporting individuals were imaged using a Zeiss ApoTome wide-field microscope.

**Sulfatase Inhibition and Epistasis Tests**

Details of assays for sulfatase inhibition and for responses to pheromone and Δ7-DA are provided in the Extended Experimental Procedures. See also Tables S5 and S6.

**Phylogenetic Analysis**

Methods for the identification of eud-1 homologs and for phylogenetic analysis are provided in the Extended Experimental Procedures. See also Tables S5 and S6.

**Statistical Analyses of Phenotypic Data**

In sulfatase inhibition, pheromone, and Δ7-DA assays (Figure 5), in which individuals were screened for their unique developmental responses to treatment molecules, samples were pooled across replicate treatments. In the survey for natural variation of the mouth phenotype in wild isolates of *P. pacificus* (Figure 4A), each sample was an entire culture population for which the Eu frequency was recorded. All other bar charts (Figures 2 and 4B–4E) show the Eu frequency calculated from the total individuals screened, for which sample sizes are given in Tables S2, S3, and S4. All significant differences were tested by a two-sided Fisher’s exact test, as implemented in the program R. Tests were performed pairwise and with each variable including the total count data, i.e., the number of Eu individuals of all individuals scored. Tests on the phenotypic conversion of *P. pacificus* isolates RS200B and RS5410, which
involved multiple independently transformed lines (Figures 4B and 4D), were performed as pairwise comparisons with a Bonferroni correction of z.

**ACCESSION NUMBERS**

The GenBank accession numbers for *P. elegans* sul-2.1 are reported in this paper are KF466323–KF466325.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, three figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.09.054.

**AUTHOR CONTRIBUTIONS**

E.J.R. and R.J.S. designed the research, except for sulfatase inhibition experiments, which were designed by M.R.M. E.J.R. and M.R.M. performed the research, except for the analysis of whole-genomic sequence data, which was performed by C.R. E.J.R. and R.J.S. wrote the manuscript.

**ACKNOWLEDGMENTS**

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**REFERENCES**


EXTENDED EXPERIMENTAL PROCEDURES

Forward Genetics Screen
Mutants of \textit{P. pacificus} were produced by previously described protocols (Pires da Silva, 2006). Mutagenized mid-J4 stage hermaphrodites \( \text{P0} \) were picked individually onto new plates, from which cultures F2 hermaphrodites were cloned. To isolate \textit{eud} mutants, the F3 broods of cloned F2 were screened for a phenotype of < 5% Eu hermaphrodites. 10 St F3 individuals from each presumptive \textit{eud} line were again cloned, after which one F4 brood with a low (<5%) Eu frequency of hermaphrodites was kept. Mutants were backcrossed twice to the wild-type strain as follows. First, mutant hermaphrodites were crossed to California males. Next, F1 males were crossed to California hermaphrodites with a recessive dumpy-like marker, \textit{Ppa-pdf-2} (Kenning et al., 2004). Finally, the F2 were cloned to recover the mutant phenotype in the F3, cloned again in multiple as F3, and then screened in the F4 to confirm homozygosity of putatively dominant alleles.

Mapping by SSLP and SSCP Detection
For genetic mapping, mutants starting with a California background were crossed twice to the Washington strain (PS1843). In the first cross, mutant hermaphrodites were crossed to Washington males. F1 males were then crossed to Washington hermaphrodites with a recessive dumpy-like marker \( \text{tu406} \). The F2 were cloned and screened for two generations to confirm the mutant phenotype and the homozygosity of mutations. Genomic DNA of outcrossed mutant lines was extracted for genetic mapping. Simple-sequence length (SSLP) or conformation (SSCP) polymorphism markers were tested against 30-40 outcrossed mutant lines and detected as previously described (Srinivasan et al., 2002, 2003).

Whole-Genome Sequencing for Mutant Identification
To prepare samples for whole-genomic sequencing, DNA was extracted and purified using the MasterPure DNA purification kit (Epizentech). DNA was quantified and genomic libraries were prepared as described (C.R., G. Bartelmes, A. Weller, H. Witte, W. Röseler, W.E. Mayer, C. Dieterich, and R.J.S., unpublished data). All libraries were diluted to a concentration of 10 nM in 0.1% EB-Tween and pooled as 4-plex. The resulting libraries were sequenced as 100-bp paired ends on an Illumina Genome Analyzer W. Roessler, W.E. Mayer, C. Dieterich, and R.J.S., unpublished data). All libraries were diluted to a concentration of 10 nM in 0.1% EB-Tween and pooled as 4-plex. The resulting libraries were sequenced as 100-bp paired ends on an Illumina Genome Analyzer II to a theoretical coverage of 9X. Raw sequencing data were processed as described in Rae et al. (2012).

Gene Cloning
Following preparation of mixed-stage RNA libraries for the California strain of \textit{P. pacificus}, coding DNA (cDNA) was amplified by reverse transcriptase PCR and sequenced. 5’ and 3’ RACE-PCR was also performed to confirm ends of transcript sequences. Lesions in mutant alleles were confirmed by PCR and Sanger sequencing of genomic DNA. Gene-specific primers were designed according to the available genomic sequence for \textit{Ppa-eud-1}.

RNA-Sequencing Experiments
Presence and levels of gene expression were measured by whole-transcriptome sequencing (RNA-Seq) of individual lines of mutants, wild isolates of \textit{P. pacificus}, and \textit{P. exspectatus}. For experiments, culture populations were allowed to grow until their food was exhausted, immediately after which the cultures were processed for sequencing. Five mixed-stage plates were washed with 40 ml M9, centrifuged immediately at 1,300 g for 4 min, rinsed with 40 ml 0.9% NaCl treated with 40 ml ampicillin and 40 ml chloramphenicol and shaken gently for 2 hr, and finally concentrated into a pellet by centrifugation and immediately frozen in liquid nitrogen. RNA-Seq libraries were sequenced as 2 × 100-bp paired-end reads on an Illumina HiSeq 2000, yielding 11-45 million paired-end reads per sample. Raw reads were aligned to the reference genomes of \textit{P. pacificus} (Hybrid1) and \textit{P. exspectatus}, respectively (http://www.pristionchus.org), using the software Tophat v.2.0.3 (Trapnell et al., 2012). Transcriptomes of \textit{P. elegans}, which are also original in this study, were assembled using the Oases assembler (Schulz et al., 2012). Expression levels were estimated and compared using the programs Cufflinks and Cuffdiff v.2.0.1 (Trapnell et al., 2012).

Analysis of Diversity Patterns at the \textit{eud-1} Locus
dN/dS ratios were calculated by counting nonsynonymous and synonymous substitutions between \textit{P. pacificus} and \textit{P. exspectatus} orthologs with normalization by the number of nonsynonymous and synonymous sites, respectively. For intraspecies comparisons, we calculated \( \tau \) values as the average pairwise distance in nucleotides across 104 natural isolates with available genome sequencing data (C.R., G. Bartelmes, A. Weller, H. Witte, W. Röseler, W.E. Mayer, C. Dieterich, and R.J.S., unpublished data) and used the corresponding nonsynonymous and silent-site diversity measures to calculate intraspecies dN/dS ratio. For the analysis of the \textit{eud-1} promoter sequence, we applied frequency spectrum neutrality tests based on Tajima’s D and Achaz’s Y in 1-kb windows at the \textit{eud-1} locus (Achaz, 2009).

Sulfatase Inhibition
To test the enzymatic activity of EUD-1, the wild-type strain and a strain overexpressing \textit{eud-1, eud-1(tu442); Ex[eud-1\text{CA}]}, were treated with the known sulfatase inhibitors Na\(_2\)SO\(_4\) decahydrate, K\(_2\)SO\(_4\), and K\(_2\)HPO\(_4\) trihydrate. Salts were dissolved in water and thereafter mixed with melted NGM agar to bring salts to a final concentration of 120 mM. NGM agar was prepared with...
MgCl₂ as a replacement for MgSO₄ so that the final concentration of sulfate ions could be precisely established. Control treatments consisted of agar mixed with the corresponding volumes of water. 3.5-cm plates containing 3 ml agar were seeded with 75 μl OP50 and 120 mM of the test salt in L-Broth and were then incubated overnight at room temperature to allow bacterial growth. Two J4 hermaphrodites were picked to each plate from the same well-fed source plate. Plates were kept at 20°C. Experiments were conducted in at least four replicates for each treatment type.

Epistasis with Pheromone Signaling

To test whether EUD-1 acts downstream of pheromone signaling, dasc#1 was applied to the presumptive null mutant eud-1(tu445), the highly St strain RS5200B, and the wild-type California strain. The assay was performed as described in Bose et al. (2012), with pheromone administered at 1 μM and with the following modifications: each plate was seeded with two J4 hermaphrodites, and a random sample of 100 individuals per plate was screened. Experiments were conducted in triplicate for each treatment.

Epistasis with ∆7-DA/DAF-12

An assay for the response of eud-1(tu442); Ex[eud-1CD] to ∆7-DA was used to test the epistasis of eud-1 to the receptor of ∆7-DA. The response was assayed in parallel with the California strain. In the assay, a solution of ∆7-DA in ethanol was diluted to 10 mM and applied in 3.0-μl aliquots to 3-cm NGM plates containing 3 ml agar and previously seeded with 50 μl OP50, such that the final starting concentration of ∆7-DA was 10 μM. Control plates were treated with the corresponding volumes of ethanol. After letting plates dry for 2 hr, two J4 hermaphrodites were picked onto each plate. Plates were kept at 20°C for 7 days, with 3.0-μl aliquots of ∆7-DA or ethanol applied twice more at two-day intervals. The experiment was performed in triplicate per treatment per strain. At the end of the treatment, broods consisted completely of adults, at which time all hermaphrodite progeny (n ≥ 60 per plate) were scored for their mouth phenotype.

Identification of eud-1 Homologs

Putative orthologs across nematodes were identified by amino-acid sequence similarity using reciprocal best BLASTp against the WormBase database. In all species where a clear homolog was found, reciprocal similarity in P. pacificus always identified Ppa-eud-1 and two other predicted protein-coding genes, herein named Ppa-sul-2.1 and Ppa-sul-2.2.1 (Table S5). The closest paralogs of sul-2 were, where annotated, orthologs of C. elegans sulfatase 3 (sul-3) (Table S6), except for in Loa loa, in which the closest paralog was the presumptive 6-O-endosulfatase Llo-sul-1 and could not be aligned to sul-2 or sul-3. Other sequences included in the analysis were homologs of sul-2 and sul-3 that could be identified in P. elegans.

Phylogenetic Analysis

Predicted amino-acid sequences of sul-2 and sul-3 were aligned automatically using MUSCLE (Edgar, 2004) and then manually in MEGAS.05 (Tamura et al., 2011), where ambiguous alignment positions were removed. The gene tree of putative sul-2 and sul-3 amino acid sequences was inferred under the maximum likelihood (ML) criterion, as implemented in RAxML (Stamatakis, 2006). The analysis invoked a Whelan and Goldman model with a gamma-shaped distribution of rates across sites. Forty independent runs were performed. Bootstrap support for the most likely tree among all runs was estimated by 500 pseudoreplicates.

SUPPLEMENTAL REFERENCES

Figure S1. Genomic Sequence and Conceptual Translation of *Ppa-eud-1*, Related to Figure 3A
Gray bars represent exons as confirmed by RACE-PCR. Genomic sequence is available at Pristionchus.org.
**Figure S2. Expression Values for eud-1 from RNA-Seq Experiments, Related to Figures 2 and 4**

Expression values were measured as fragments per kilobase of transcript per million (FPKM) sequenced fragments estimated by a Bayesian inference method (Trapnell et al., 2012). Data are represented as FPKM +/- 95% confidence interval. Gene expression levels were quantified as relative expression with respect to all genes. Strong correlations in pairwise comparisons (Spearman’s $r > 0.85$, Figure S3) indicated comparability of the data sets. Relative to the *P. pacificus* wild-type hermaphrodite of the California strain (RS2333), males (RS2333M), *eud-1* mutant alleles (tu442, tu445, tu450, tu451), and highly St wild isolates (RS5200B, RS5410) exhibit a pronounced downregulation of *eud-1*. The presumptive null allele of *eud-1*, tu445, showed zero expression. The expression level in *P. exspectatus* (RS5522B) was estimated after alignment and quantification against the *P. exspectatus* genome and annotation. Although expression levels are not directly comparable across different wild isolates and species borders, log$_2$ FPKM values for orthologous genes (best reciprocal BLAST hits) show a strong correlation ($r = 0.81$, Pearson), suggesting very similar transcriptome profiles between the two species. In contrast, the ortholog of *eud-1* in *P. exspectatus* shows downregulation comparable to the *eud-1* mutant alleles (no test for differential expression across species borders was performed).
Expression levels for all samples were quantified independently as relative expression with respect to all genes (FPKM) using the program Cufflinks (Trapnell et al., 2012). Comparisons of *P. pacificus* mutant, male, and wild-isolate samples with the mixed-stage culture of the reference strain (RS2333) showed strong correlations (Spearman’s $\rho > 0.85$). Expression levels of one-to-one orthologs across species also showed a high correlation in all comparisons (Spearman’s $\rho = 0.76$). Correlations indicate that normalization with respect to all genes is valid and that the data sets are indeed comparable.
Table S1. Summary of Mutations in *eud-1* Alleles, Related to Figure 3B

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutation</th>
<th>Location</th>
<th>Predicted target or effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>tu442</td>
<td>P97L</td>
<td>Exon 5</td>
<td>Catalytic site</td>
</tr>
<tr>
<td>tu445</td>
<td>R80stop</td>
<td>Exon 5</td>
<td>Null</td>
</tr>
<tr>
<td>tu450</td>
<td>A to T</td>
<td>2 bp upstream of exon 2</td>
<td>Splice acceptor</td>
</tr>
<tr>
<td>tu451</td>
<td>R99Q</td>
<td>Exon 5</td>
<td>Catalytic site</td>
</tr>
</tbody>
</table>
Table S2. Frequency of Eu Animals in Wild-Type and Mutant Lines of *P. pacificus*, Related to Figure 2

<table>
<thead>
<tr>
<th><em>P. pacificus</em> strain</th>
<th>% Eu</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS2333 (+/+)</td>
<td>90</td>
<td>5894</td>
</tr>
<tr>
<td>RS2333, males</td>
<td>25</td>
<td>160</td>
</tr>
<tr>
<td><em>eud-1(tu442)/+</em></td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td><em>eud-1(tu442)/eud-1(tu442)</em></td>
<td>0</td>
<td>715</td>
</tr>
<tr>
<td><em>eud-1(tu445)/+</em></td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td><em>eud-1(tu445)/eud-1(tu445)</em></td>
<td>0</td>
<td>550</td>
</tr>
<tr>
<td><em>eud-1(tu450)/+</em></td>
<td>9</td>
<td>104</td>
</tr>
<tr>
<td><em>eud-1(tu450)/eud-1(tu450)</em></td>
<td>0</td>
<td>778</td>
</tr>
<tr>
<td><em>eud-1(tu451)/+</em></td>
<td>14</td>
<td>91</td>
</tr>
<tr>
<td><em>eud-1(tu451)/eud-1(tu451)</em></td>
<td>0</td>
<td>601</td>
</tr>
</tbody>
</table>

Phenotypes are of hermaphrodites/females unless otherwise indicated.
Table S3. Frequency of Eu Animals in Genetically Transformed Lines of *P. pacificus*, Related to Figures 2, 4B, and 4D

<table>
<thead>
<tr>
<th>Transformed line</th>
<th>RFP+</th>
<th>RFP-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Eu</td>
<td>n</td>
</tr>
<tr>
<td>RS2333; Ex[tu450] #1</td>
<td>89</td>
<td>157</td>
</tr>
<tr>
<td>RS2333; Ex[tu450] #2</td>
<td>96</td>
<td>52</td>
</tr>
<tr>
<td>eud-1(tu450); Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #1</td>
<td>100</td>
<td>320</td>
</tr>
<tr>
<td>eud-1(tu450); Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #2</td>
<td>100</td>
<td>162</td>
</tr>
<tr>
<td>eud-1(tu450); Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #3</td>
<td>99</td>
<td>451</td>
</tr>
<tr>
<td>eud-1(tu450); Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #4*</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>eud-1(tu445); Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #1</td>
<td>100</td>
<td>178</td>
</tr>
<tr>
<td>eud-1(tu445); Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #2</td>
<td>100</td>
<td>184</td>
</tr>
<tr>
<td>eud-1(tu442); Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #4, by crossing</td>
<td>100</td>
<td>178</td>
</tr>
<tr>
<td>eud-1(tu450); Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #1, males</td>
<td>100</td>
<td>130</td>
</tr>
<tr>
<td>RS5200B; Ex[tu445] #1</td>
<td>3</td>
<td>61</td>
</tr>
<tr>
<td>RS5200B; Ex[tu445] #2</td>
<td>3</td>
<td>101</td>
</tr>
<tr>
<td>RS5200B; Ex[tu445] #3</td>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td>RS5200B; Ex[tu445] #4</td>
<td>6</td>
<td>62</td>
</tr>
<tr>
<td>RS5200B; Ex[tu445] #5</td>
<td>0</td>
<td>57</td>
</tr>
<tr>
<td>RS5410; Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #1</td>
<td>55</td>
<td>110</td>
</tr>
<tr>
<td>RS5410; Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #2</td>
<td>51</td>
<td>216</td>
</tr>
<tr>
<td>RS5410; Ex[eud-1&lt;sup&gt;CA&lt;/sup&gt;] #3*</td>
<td>99</td>
<td>469</td>
</tr>
<tr>
<td>RS5200B; Ex[eud-1&lt;sup&gt;5200B&lt;/sup&gt;] #1</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>RS5200B; Ex[eud-1&lt;sup&gt;5200B&lt;/sup&gt;] #2</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>RS5200B; Ex[eud-1&lt;sup&gt;5200B&lt;/sup&gt;] #3</td>
<td>98</td>
<td>60</td>
</tr>
<tr>
<td>RS5410; Ex[eud-1&lt;sup&gt;5410&lt;/sup&gt;]</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>eud-1(tu450); Ex[Pex-eud-1]</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>eud-1(tu450); Ex[Pex-eud-1] x RS5522B</td>
<td>100</td>
<td>31</td>
</tr>
</tbody>
</table>

*Only RFP+ animals observed.

Phenotypes are of hermaphrodites/females unless otherwise indicated.
Table S4. Frequency of the Eu Form in Male and Female F1 Hybrids between *P. pacificus* Strains and *P. exspectatus* (RS5522B), Related to Figures 4C and 4E

<table>
<thead>
<tr>
<th>Cross type</th>
<th>crosses (N)</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS5522B ♀ x RS5522B ♂</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RS5522B ♀ x RS2333 ♂</td>
<td>14</td>
<td>69</td>
<td>327</td>
</tr>
<tr>
<td>RS5522B ♀ x eud-1(tu450); Ex[eud-1CA] ♂</td>
<td>17</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Ppa-unc-1 x RS5522B ♂</td>
<td>8</td>
<td>52</td>
<td>67</td>
</tr>
<tr>
<td>Ppa-unc-1; eud-1(tu450); Ex[eud-1CA] x RS5522B ♂</td>
<td>7</td>
<td>100</td>
<td>74</td>
</tr>
</tbody>
</table>
Table S5. Putative Orthologs and Paralogs of *sul-2* in 10 Nematode Species, Related to Figure 3D

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence identifier</th>
<th>Gene or putative gene</th>
<th>Transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bursaphelenchus xylophilus</em></td>
<td>BUX.s01143.356</td>
<td>Bxy-sul-2</td>
<td>Predicted</td>
</tr>
<tr>
<td><em>Caenorhabditis brenneri</em></td>
<td>CBN13791</td>
<td>Cbn-sul-2-A</td>
<td>Predicted</td>
</tr>
<tr>
<td></td>
<td>CBN06224</td>
<td>Cbn-sul-2-B</td>
<td>Predicted</td>
</tr>
<tr>
<td><em>Caenorhabditis briggsae</em></td>
<td>CG11339</td>
<td>Cbr-sul-2</td>
<td>Predicted</td>
</tr>
<tr>
<td><em>Caenorhabditis elegans</em></td>
<td>D1014.1</td>
<td>Cel-sul-2</td>
<td>Confirmed by cDNA&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Caenorhabditis japonica</em></td>
<td>CJA12246</td>
<td>Cjp-sul-2</td>
<td>Predicted</td>
</tr>
<tr>
<td><em>Caenorhabditis remanei</em></td>
<td>CRE05490</td>
<td>Cre-sul-2</td>
<td>Predicted</td>
</tr>
<tr>
<td><em>Loa loa</em></td>
<td>LOAG_05452</td>
<td>Llo-sul-2</td>
<td>Predicted</td>
</tr>
<tr>
<td><em>Pristionchus elegans</em></td>
<td>18569</td>
<td>Pel-sul-2.1</td>
<td>Confirmed by cDNA&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>12959+21596</td>
<td>Pel-sul-2.2</td>
<td>Confirmed by cDNA&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Pristionchus exspectatus</em></td>
<td>scaffold51-snap.28</td>
<td>Pex-sul-2.1-A</td>
<td>Predicted</td>
</tr>
<tr>
<td></td>
<td>scaffold51-snap.29</td>
<td>Pex-sul-2.1-B</td>
<td>Predicted</td>
</tr>
<tr>
<td></td>
<td>scaffold22-snap.17</td>
<td>Pex-sul-2.2.1</td>
<td>Confirmed by cDNA&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>scaffold22-snap.18</td>
<td>Pex-eud-1</td>
<td>Confirmed by cDNA&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Pristionchus pacificus</em></td>
<td>PPA21290</td>
<td>Ppa-sul-2.1</td>
<td>Predicted</td>
</tr>
<tr>
<td></td>
<td>PPA06135</td>
<td>Ppa-sul-2.2.1</td>
<td>Confirmed by cDNA&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PPA06136</td>
<td>Ppa-eud-1</td>
<td>Confirmed by cDNA&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Information available at WormBase.org.
<sup>2</sup>Present study.
<sup>3</sup>Rödelsperger et al. (submitted).
Table S6. Putative Orthologs of sul-3 in Eight Nematode Species, Related to Figure 3D

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence identifier</th>
<th>Gene or putative gene</th>
<th>Transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bursaphelenchus xylophilus</td>
<td>BUX.s01109.101</td>
<td>Bxy-sul-3</td>
<td>Predicted</td>
</tr>
<tr>
<td>Caenorhabditis brenneri</td>
<td>CBN18180</td>
<td>Cbn-sul-3</td>
<td>Predicted</td>
</tr>
<tr>
<td>Caenorhabditis briggsae</td>
<td>CBG16830</td>
<td>Cbr-sul-3</td>
<td>Predicted</td>
</tr>
<tr>
<td>Caenorhabditis elegans</td>
<td>C54D2.4</td>
<td>Cel-sul-3a</td>
<td>Confirmed by cDNA¹</td>
</tr>
<tr>
<td>Caenorhabditis japonica</td>
<td>CJA13201</td>
<td>Cjp-sul-3*</td>
<td>Predicted</td>
</tr>
<tr>
<td>CJA29064</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caenorhabditis remanei</td>
<td>CRE00019</td>
<td>Cre-sul-3</td>
<td>Predicted</td>
</tr>
<tr>
<td>Pristionchus exspectatus</td>
<td>scaffold279-snap.12</td>
<td>Pex-sul-3*</td>
<td>Confirmed by cDNA²</td>
</tr>
<tr>
<td>Pristionchus pacificus</td>
<td>PPA23475</td>
<td>Ppa-sul-3</td>
<td>Confirmed by cDNA³</td>
</tr>
</tbody>
</table>

*Indexed as two sequences but are orthologous with the 5' and 3' ends, respectively, of Cel-sul-3.
¹Information available at WormBase.org.
²Rödelsperger et al. (submitted).
³Present study.