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Regulators of an ancient polyphenism evolved through episodic protein divergence and parallel gene radiations

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Polyphenism is a form of developmental plasticity that transduces environmental cues into discontinuous, often disparate phenotypes. In some cases, polyphenism has been attributed to facilitating morphological diversification and even the evolution of novel traits. However, this process is predicated on the origins and evolutionary maintenance of genetic mechanisms that specify alternate developmental networks. When and how regulatory loci arise and change, specifically before and throughout the history of a polyphenism, is little understood. Here, we establish a phylogenetic and comparative molecular context for two dynamically evolving genes, *eud-1* and *seud-1*, which regulate polyphenism in the nematode *Pristionchus pacificus*. This species is dimorphic in its adult feeding-structures, allowing individuals to become microbivores or facultative predators depending on the environment. Although polyphenism regulation is increasingly well understood in *P. pacificus*, the polyphenism is far older than this species and has diversified morphologically to enable an array of ecological functions across polyphenic lineages. To bring this taxonomic diversity into a comparative context, we reconstructed the histories of *eud-1* and *seud-1* relative to the origin and diversification of polyphenism, finding that homologues of both genes have undergone lineage-specific radiations across polyphenic taxa. Further, we detected signatures of episodic diversifying selection on *eud-1*, particularly in early diplogastrid lineages. Lastly, transgenic rescue experiments suggest that the gene's product has functionally diverged from its orthologue's in a non-polyphenic outgroup. In summary, we provide a comparative framework for the molecular components of a plasticity switch, enabling studies of how polyphenism, its regulation, and ultimately its targets evolve.

1. Introduction

Developmental plasticity, a ubiquitous feature of growing organisms, has been proposed as a major catalyst for morphological diversification [1–4]. In particular, polyphenism—the production of alternative, discontinuous and sometimes disparate phenotypes to match expected environments—exemplifies the ability of genotypes to cross morphological space and thus potentially promote evolutionary change and even novelty [5]. For instance, novel alternative morphs might permit the exploration of adaptive landscapes without requiring changes to previously adaptive morphs, and multiple hypotheses predict that this process allows rapid diversifying selection on the new morph [1,6,7]. However, this process is contingent on the evolutionary maintenance of both morphs through multiple lineage splits, which itself would depend on a heritable programme for the morphs' reliable, conditional appearance [8]. To understand the role of polyphenism in generating phenotypic variation and how it is subsequently selected, the genetic components underlying polyphenism and its change must first be described. Specifically, a comparative molecular analysis of polyphenism is needed to understand how its regulatory components arise and how those components are constrained or free to change.

Given its multimodal nature, polyphenism may often involve a switch-like genetic mechanism that channels environmental responses [9,10]. Indeed, the alternative transcriptional outputs and, in some cases, the regulatory factors underlying polyphenism have begun to be defined in numerous systems [11–15]. However, most molecular studies of polyphenism have focused on instances from single species, so it is still unknown how plasticity control mechanisms vary with or affect macroevolutionary patterns. For example, evolutionary changes to these control mechanisms can influence environmental sensitivity to a given set of external cues [16]. Consequently, such changes influence the frequency with which alternative morphs are expressed within lineages, thereby controlling their conditional exposure to selection [17]. By gating the selection pressure on alternative phenotypes, polyphenism control mediates the tempo of evolution of the molecular effectors—i.e. the targets of the developmental switch—that produce them.

An example of polyphenism that has been maintained through several phylogenetic splits is feeding-structure dimorphism in the nematode family Diplogastridae (figure 1). In this group, polyphenism evolved once and has persisted through several daughter lineages [18]. In *Pristionchus pacificus*, where the diplogastrid polyphenism has been best studied, alternative morphs enable individuals to be either strict microbivores or facultative predators based on resource availability [19]. However, in other diplogastrid species, the polyphenism takes on an array of putative ecological functions, using resources in both generalist and specialist associations with dung, rotting plant tissue and a range of insects from wood-boring beetles to fig wasps [20,21]. Reflecting the diversity of this ancestral polyphenism's functions, plastic mouth morphologies of Diplogastridae have rapidly diversified, both in morphometric terms and in the appearance of qualitatively new structures [18]. Furthermore, cue sensitivity varies within and among species, indicating divergence in plasticity itself [18,22,23]. In principle, the diversification of form and function of polyphenism may be reflected by changes in the machinery that regulates it.

The model *P. pacificus*, in particular, offers an entry point into the comparative developmental genetics of polyphenism. In this species, several genes fully influencing the polyphenism switch have been identified, including those for several enzymes [9,16,24] and a nuclear receptor (NHR-40) [25]. Two of these genes, which encode the sulphatase EUD-1 and sulfotransferase SEUD-1, are dosage-dependent and antagonistic in their phenotypic effects [16]. Further, in contrast to the receptor gene *nhr-40*, which is present as a single orthologue between polyphenic and outgroup species, these two genes have numerous paralogues in *Pristionchus* species. Both genes have specialized as polyphenism regulators relative to their paralogues [26,27], and for *eud-1*, in particular, this specialization extends to its apparently high connectivity: it is directly modified by multiple factors, including a chromatin remodeller and antisense RNAs [28]. Nevertheless, the events giving rise to *eud-1* and *seud-1* as polyphenism regulators are still ambiguous. Because comparative studies of polyphenism regulatory loci have been mostly limited to *Pristionchus* and a close sister group (*Micoletzkya*), the timing of the genes' duplications relative to the origin of the polyphenism is unknown. However, building on recent advances in the *Pristionchus* model, comparative analyses of regulatory loci are now poised to infer the ancestral genetic contexts in which a morphological polyphenism evolved.

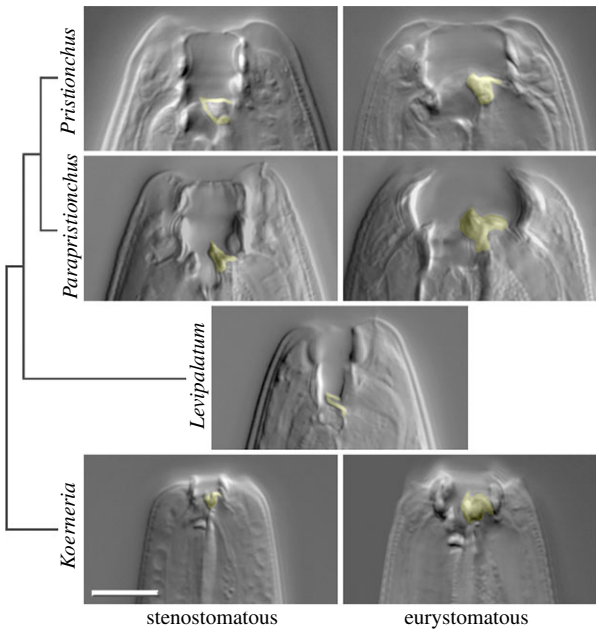

Figure 1. Diversifcation of mouthpart polyphenism among diplogastrid nematodes. Since its origin, which coincided with the evolution of movable teeth (false-coloured yellow), polyphenism has diversified in its morphology, environmental sensitivity and ecological function, shown here by representatives of *Koerneria*, *Parapristionchus* and *Pristionchus*. In other lineages, a single morph, together with some novel structures such as the dorsal tooth, has become fixed (e.g. *Levipalatum*). Tree is simplified from one previously inferred using wider taxon sampling [18]. Mouthparts shown in sagittal plane, with dorsal to right. Scale bar, 5 µm; all images to same scale. (Online version in colour.)

Here, we combine phylogenetic and functional genetic approaches to reconstruct the evolutionary history of components of an extant polyphenism switch mechanism. First, we reconstruct the history of two polyphenism switch genes, *eud-1* and *seud-1*, by determining how and when their ancestral copies diversified with respect to the earliest resolved divergences among polyphenic nematodes. Second, we track the phylogenetic timing of when these copies changed at a sequence level. Third, we provide a functional genetic context to this reconstructed history, transgenically assaying the function of a switch-gene protein representing the ancestral state prior to the evolution of polyphenism. We thus provide, to our knowledge, a first historical account for how incipient polyphenism regulators arose and changed during the origin and evolutionary maintenance of a polyphenism.

2. Material and methods

(a) Detection of switch-gene homologues

To access the histories of switch-gene homologues in Diplogastridae, we explored previously unpublished genome assemblies for key representatives across the family (electronic supplementary material, tables S1, S2). To infer events spanning the deepest resolved splits among mouth-polyphenic lineages, we used sequences for *Koerneria luziae* and *Allodiplogaster sudhausi*. Further, we used new sequences for two species (*Diplogasteroides magnus*, *Levipalatum texanum*) from a lineage that had lost polyphenism. To improve character polarization, we also used a new genome assembly for *Bunonema* sp. (RGD898), the nearest

sequenced outgroup to both Diplogastridae and its sister lineage (Rhabditidae) [18,29]. Genomes of the above species will be detailed elsewhere, but in brief, our sequencing strategy was as described previously, as were assembly and annotation for *A. sudhausi*, *D. magnus* and *L. texanum* [30]; for *Bunonema* sp. and *K. luziae*, gene predictions were made with Augustus [31] using protein sequences from *P. pacificus* and *Caenorhabditis elegans* as a training set. With these and published resources, we identified all annotated *eud-1* and *seud-1* homologues by reciprocal best BLASTp with the genes' single *C. elegans* orthologues (*sul-2* and *ssu-1*, respectively). In only *K. luziae*, independent queries of its transcriptome revealed additional putative homologues of *eud-1/sul-2* and *seud-1/ssu-1*, which were also included in downstream analyses. In the case of *Pristionchus*, which drew on several recently published genomes [32], we identified *ssu-1* homologues that were highly divergent (i.e. BLAST *e*-values ≥ 0.001) and could not be objectively aligned to other homologues. Consequently, we excluded all *Pristionchus seud-1/ssu-1* sequences from our analyses, although the presence of obvious *seud-1/ssu-1* homologues in *Parapristionchus giblindavisi* allowed us to reconstruct ancestral states older than *Parapristionchus* + *Pristionchus*.

(b) Reconstruction of switch-gene histories

To reconstruct the evolutionary histories of *eud-1/sul-2* and *seud-1/ssu-1*, we analysed both DNA and protein-sequence alignments. The automated alignment was performed in MAFFT v. 7 [33]. Alignment invoked the L-INS-i algorithm, as informed by the enzymes' domain structures [34,35], and was run otherwise under default settings. The resulting alignments were then manually edited to remove ambiguous sites, particularly at sequence termini.

We inferred gene trees using the CIPRES Science Gateway [36]. In a first approach, we inferred trees from DNA under maximum likelihood (ML) as implemented in RAxML v. 8 [37]. For these inferences, alignments for *eud-1/sul-2* and *seud-1/ssu-1* were drawn into four and two partitions, respectively: for *eud-1/sul-2*, partitions included the first and second codon position for both the predicted catalytic region of the sulphatase domain and the rest of the predicted sulphatase domain ('non-catalytic' region); for *seud-1/ssu-1*, the sulfotransferase domain was likewise partitioned in two. 5′ and 3′ sequences terminal to the enzymatic domains were excluded. Under a general time-reversible (GTR) model, designating *Loa loa* as outgroup *a priori* [38,39], and setting the number of rate categories that maximized the likelihood of the tree *a posteriori*, we performed 200 independent inferences from each matrix, with node support on the final trees estimated from 1000 bootstrap pseudoreplicates. In a second approach, we performed Bayesian inference from DNA sequences of enzymatic domains, including third codon positions, and specifying a codon model of substitution, as implemented in MrBayes v. 3.2.6 [40]. For both matrices, which also specified the outgroup, the analysis consisted of two runs, with four chains each, for 16 million generations sampled every 1000 generations. Analyses were run under a GTR + gamma model and default priors. Burn-in was confirmed to have followed run convergence and included the first 25% of generations. Our third approach inferred gene histories from amino acid sequences by ML. For this analysis, we partitioned alignments as for ML analyses above and included an additional partition for confidently aligned terminal sequences. We estimated the best-fitting models of protein evolution for each data partition using ModelTest-NG v. 0.1.5 [41], using a constraint tree inferred in RAxML under a WAG model and default settings. These models were then used for a second ML inference of 200 independent runs. The most likely tree from all runs was then used to iteratively estimate model fit, converging on models that were used for the final inferences, which were otherwise performed as for ML analyses of DNA above. All resulting alignments and gene trees have been deposited in the Dryad Digital Repository [42].

Following our inference of gene trees, we reconciled them with a well-resolved species tree to correct for possible gene-tree error and incomplete lineage sorting [43,44]. For reconciliation, we used a species-tree topology supported by multiple datasets and analyses [18,29,38,45] and gene trees inferred from DNA sequences under ML (electronic supplementary material, figure S1). Nodes with less than 75% bootstrap support were rearranged to minimize the number of duplication and loss events, with duplications costing 1.5× losses, as implemented in Notung [46]. Nodes in reconciled trees were then assigned support values from, or incongruence with (i.e. non-presence or less than 50% support in), trees from other inference methods.

(c) Signal-peptide detection

To query possible functional changes in *eud-1/sul-2*, we surveyed for signal-peptide coding sequences in *eud-1/sul-2* homologues across taxa using SignalP v. 5.0 [47]. Phylogenetic history of signal-peptide presence was reconstructed by ML under a Mk1 model [48], as implemented in Mesquite v. 3.51 [49].

(d) Selection analyses

With the reconstructed history of *eud-1/sul-2* homologues, we tested for episodic selection on them along individual branches of the tree. Specifically, we used the adaptive branch-site random effects likelihood (aBSREL) model, which offers a sensitive test for diversifying (positive) selection at sites per branch by optimizing branch-specific rate categories [50]. We performed selection analyses using the Datamonkey webserver [51]. We analysed sequences separately for each of the two sulphatase subdomains as described above. The analysis assumed the reconciled gene trees, on which test branches included those in Diplogastridae + Rhabditidae. Significance ($p < 0.001$) was determined according to a likelihood ratio test and adjusting for multiple comparisons.

(e) Transgenic assay

We interrogated the function of *sul-2*, the ancestral homologue of *eud-1*, using a transgenic, ancestral-proxy approach. Specifically, we cloned the coding sequence for *C. elegans* (*Cel*) *sul-2*, to which we ligated *P. pacificus* regulatory elements, and then we attempted heterologous rescue of a *P. pacificus* (*Ppa*) *eud-1* null mutant, *eud-1(tu445)* (electronic supplementary material, table S3). The 1.4 kb coding sequence for *Cel-sul-2* was cloned from a *C. elegans* (N2) cDNA library prepared as previously described [16]. Regulatory sequences included a 2.6 kb *Ppa-eud-1* promoter and the 415 bp *Ppa-eud-1* 3′ untranslated region (UTR), which are together sufficient for the gene's expression and translation [9]. Fragments were ligated into a single construct using restriction digestion and cloning into a pGEM-T Easy vector (Promega). The construct was then delivered together with a fluorescent reporter gene (*Ppa-egl-20p::TurboRFP*) and digested genomic carrier DNA into the gonads of *eud-1(tu445)* hermaphrodites. Reporting F_1 hermaphrodites were selfed to F_2 to establish two independently transformed lines. Heterologous expression of the full *Cel-sul-2* coding sequence was confirmed by polymerase chain reaction using the above primers. cDNA libraries, which were each constructed from pools of 30 mixed-stage, reporting individuals, were prepared as above. Mouth-morph phenotypes of transformed *P. pacificus* individuals (at least 80 per line) were scored as previously described [52].

(f) Statistical analyses

All statistical analyses were performed in R v. 3.3.0 [53]. To test whether the presence of polyphenism could be predicted by

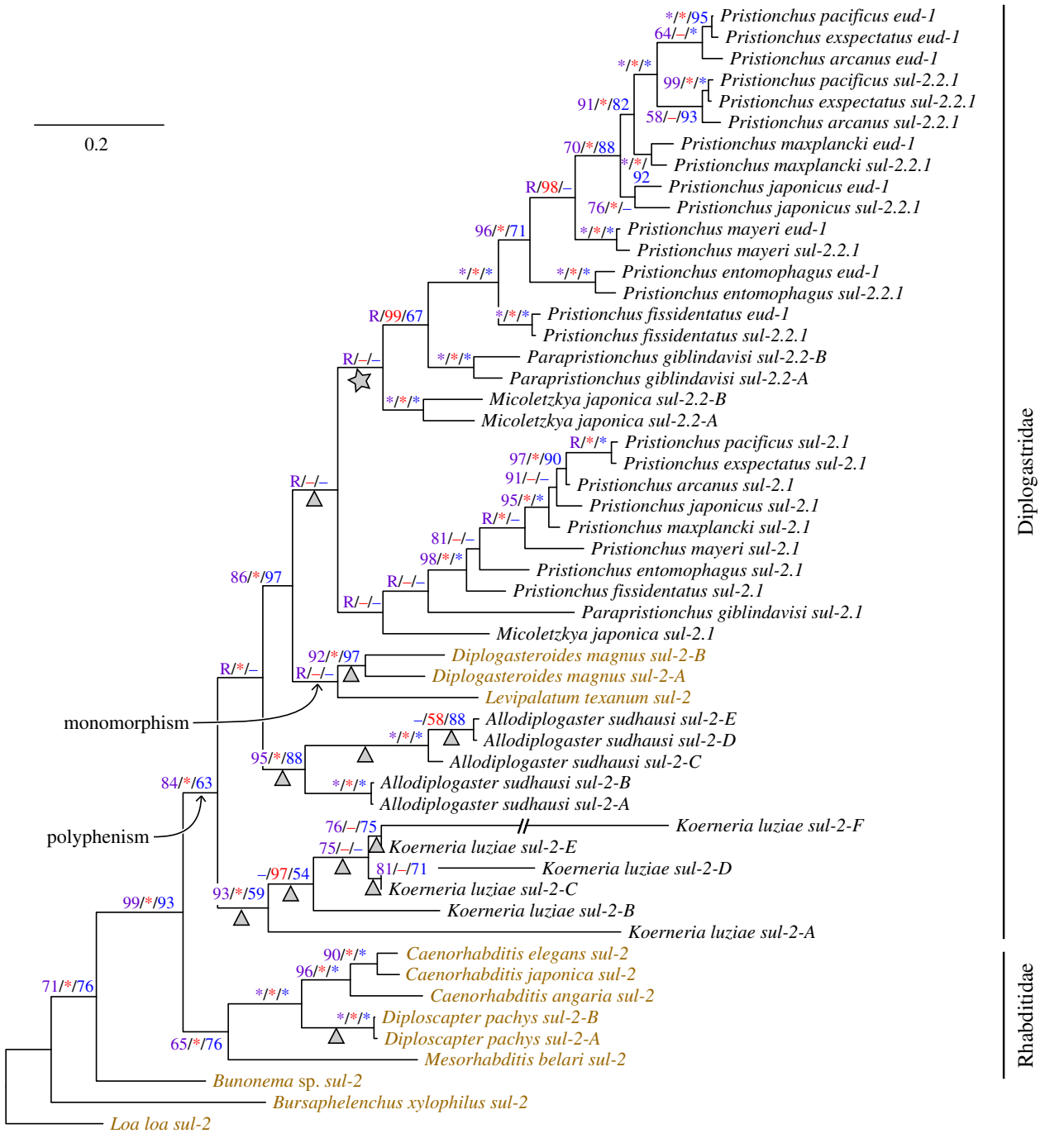

Figure 2. Reconstructed history of *eud-1/sul-2* switch-gene homologues in Diplogastridae and outgroups. Genes from monomorphic taxa shown in brown. Node support values are given as: bootstrap support (BS) from maximum likelihood (ML) inference of sulphatase-domain coding sequences (left, purple values); posterior probabilities following Bayesian inference (middle, red values); BS of ML inference of amino acid sequences (right, blue values). Triangles mark inferred gene duplication events; a five-pointed star indicates single inferred duplication of *sul-2.2* into supergene loci *eud-1* and *sul-2.2.1*. Asterisks indicate 100% support; dashes indicate less than 50% support or node absence. Reconstructed nodes (R) subtended by branches of arbitrary length. Letters suffixed to gene names are arbitrary by species. (Online version in colour.)

homologue number, we performed a logistic regression using a generalized linear model with a Poisson distribution and log function. To distinguish the effects of phylogenetic correlation, the taxonomic family was added as an additional variable to the model.

3. Results

(a) Polyphenic species have increased numbers of switch-gene homologues

Newly sequenced nematode genomes revealed multiple homologues of *eud-1/sul-2* (3–6 per species) and *seud-1/ssu-1* (≥4 per species) for polyphenic Diplogastridae (figures 2 and 3). By contrast, most examined outgroup (Rhabditidae) species showed one orthologue or, at most, two recently duplicated copies of either gene, and one of the two non-polyphenic (i.e. secondarily monomorphic) diplogastrid species (*L. texanum*) likewise showed a single homologue of each gene. Polyphenism presence thus correlated both with *eud-1/sul-2* homologue number ($z = 3.027$; $p = 0.00247$) and with *seud-1/ssu-1* homologue number ($z = 5.611$, $p = 2.01 \times 10^{-8}$). Because polyphenism evolved once in Diplogastridae, we also sought to tease apart the effects of phylogeny from those of polyphenism *per se*. When we added taxonomic

family as a covariate in our statistical model, correlation between polyphenism and homologue number and polyphenism was no longer significant for *sul-2* ($z = 1.376$; $p = 0.169$) yet still so for *ssu-1* ($z = 2.37$; $p = 0.0205$), although statistical power was reduced by the inclusion of only two monomorphic diplogastrids in our comparisons. Consistent with this caveat, we found that polyphenism was indeed the most important variable in our model, with taxonomic family having a negligible effect (for *eud-1/sul-2*: $z = 0.402$; $p = 0.688$). In summary, our detection of *eud-1/sul-2* and *seud-1/ssu-1* homologues showed an expansion of these genes in polyphenic relative to monomorphic taxa.

(b) *sul-2* underwent parallel radiations following polyphenism's origin

Having recovered multiple switch-gene homologues among Diplogastridae, we reconstructed their phylogenetic history relative to the evolution of polyphenism. Because *eud-1*, in particular, has specialized in *P. pacificus* relative to its paralogs [26], we inferred the timing and hence possible roles of gene duplication in the origin of this switch gene. Despite multiple *eud-1/sul-2* homologues being found in multiple diplogastrid taxa, we inferred a single orthologue of *sul-2* as present in the family's common ancestor (figure 2) as well as several daughter lineages giving rise to *Pristionchus*. By this inference, the multiple *sul-2* homologues present in several polyphenic lineages—namely, *Koerneria*, *Allodiplogaster*, and the ancestor of all other examined diplogastrids—were the result of independent radiations of this single orthologue. We also recovered the signature of concerted gene evolution between *sul-2.2.1* and *eud-1*, a pattern previously reported for *Pristionchus* and *Micoletzkya* [24], and we found the same pattern in *Parapristionchus*. Further, our results show that these two genes probably arose as a supergene, also as previously described, independently of duplications in other nematode lineages. Because the inferred split of multiple *A. sudhausi sul-2* homologues predated that between the *sul-2.2* supergene and *sul-2.1*, our results specifically support the origin of the supergene and its subsequent gene-conversion events to be restricted to the lineage containing *Pristionchus*, *Parapristionchus* and *Micoletzkya*. Indeed, because *A. sudhausi sul-2* homologues are not closely physically linked [24], gene-conversion events among other *sul-2* homologues prior to the duplication of *sul-2.2* are not likely to explain taxon-specific gene clusters outside of *sul-2.2*. Thus, the multiple *eud-1* homologues distinguishing polyphenic lineages were probably the result of independent, taxon-specific radiations of a single, ancestral sulphatase gene.

(c) Multiple homologues of *ssu-1* were probably present at the origin of polyphenism

We also reconstructed the evolutionary history of *seud-1/ssu-1*, which like *eud-1/sul-2* is represented by multiple homologues across polyphenic species. As in *eud-1*, diplogastrid *seud-1/ssu-1* genes have experienced lineage-specific radiations across Diplogastridae (figure 3). However, all optimal reconciled trees suggest that two homologues were also already present in the oldest inferred polyphenic ancestor, although the ancestry of several daughter genes was not resolved. It is thus possible that the order of early duplications in the family was even more complex then we infer, reminiscent of the especially dynamic histories of *seud-1/ssu-1* homologues in *Pristionchus* [16]. Additionally, our results suggest that the history of *seud-1/ssu-1* must have included at least one loss since the origin of polyphenism, based on the presence of a single *ssu-1* homologue in *L. texanum*. If so, this event would represent a retraction of *seud-1/ssu-1* genes in a non-polyphenic lineage, although multiple *ssu-1* homologues are present in the non-polyphenic *D. magnus*. Together, our findings indicate that two homologues of *seud-1/ssu-1* were probably present early in the evolution of polyphenism, suggesting that duplication and functional specialization of *seud-1* would have been possible prior to either the origin or genetic accommodation of polyphenism regulation.

(d) Stem diplogastrid lineages report strong diversifying selection on *eud-1/sul-2*

Because *sul-2* was probably present as a single orthologue when polyphenism evolved, we hypothesized that this gene or one of its daughters changed while assuming the new role of polyphenism regulation. To test this idea, we first examined whether the capacity to be secreted may have changed, based on the presence of a signal peptide in *Ppa-eud-1* [9]. Predicted signal-peptides were inferred for most ancestral states, suggesting that obvious differences are not in the enzymes' secretion (electronic supplementary material, figure S2). We then examined other domains of the enzyme. Because *P. pacificus* EUD-1 and SUL-2.2.1 apparently differ in protein function [24], we searched for specific differences that may have persisted between these duplicates across *Pristionchus* species despite ongoing gene conversion. We did not identify residues consistently distinguishing *eud-1* orthologues from other *sul-2* duplicates, so we next assayed for signatures of episodic selection across the coding sequence throughout the history of *eud-1/sul-2*. We found that both the catalytic region and the rest ('non-catalytic' region) of the sulphatase domain show strong, branch-specific signatures of diversifying selection (i.e. $K_a/K_s \gg 1.00$ for one of two rate categories across sites), all restricted to the diplogastrid lineage (table 1). In the non-catalytic sulphatase subdomain, we found diversifying selection in the ancestors of Diplogastridae, *Pristionchus*, and a *Pristionchus* subclade including *P. pacificus*. Further, both sulphatase subdomains independently reported signatures of diversifying selection in the ancestor of diplogastrids excluding *Koerneria* and *Allodiplogaster*. Other lineages reporting episodic selection were terminal genes as well as the lineage that lost polyphenism, with the caveat that the branch representing the latter lineage was present only in the reconciled *sul-2* tree. Curiously, few sites (approx. 2%) in the latter case drove this signal, while the rest report strong purifying selection (i.e. $K_a/K_s \approx 0$) in this lineage, suggesting the gene's maintenance for a function other than polyphenism regulation. Together, our results suggest that stem lineages of both Diplogastridae and *Pristionchus* experienced episodic diversifying selection on *eud-1/sul-2*, possibly for a new or alternative function in polyphenic lineages.

(e) An outgroup SUL-2 sequence does not rescue polyphenism in *Pristionchus pacificus*

Because selection patterns distinguished *eud-1/sul-2* in polyphenic lineages from *sul-2* in outgroups, we asked whether

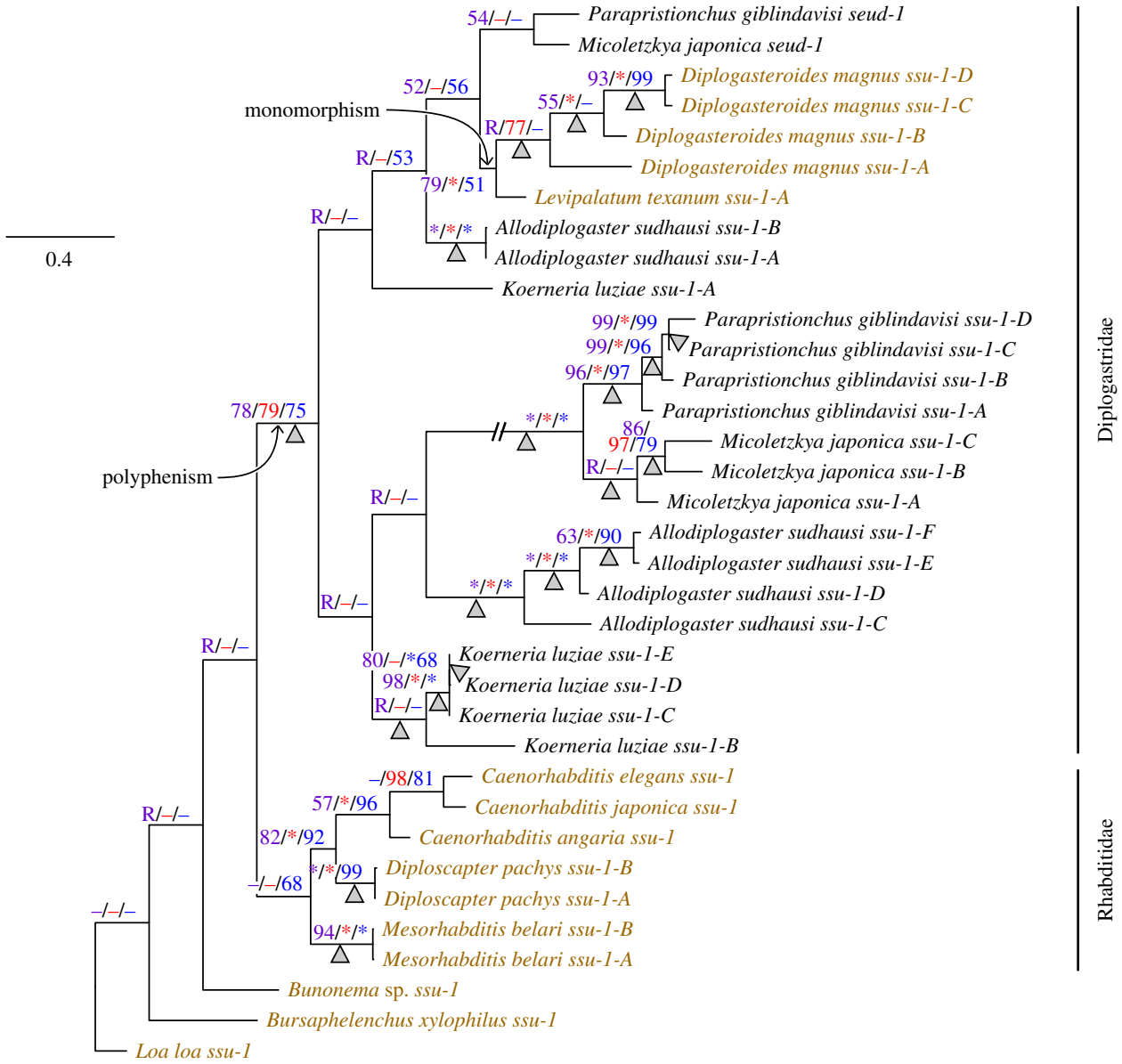

Figure 3. Reconstructed history of *seud-1/ssu-1* switch-gene homologues in Diplogastridae and outgroups. Genes from monomorphic taxa shown in brown. Node support values are given as: bootstrap support (BS) from maximum likelihood (ML) inference of sulphatase-domain coding sequences (left, purple values); posterior probabilities following Bayesian inference (middle, red values); BS of ML inference of amino acid sequences (right, blue values). Triangles mark inferred gene duplication events. Asterisks indicate 100% support; dashes indicate less than 50% support or node absence. Reconstructed nodes (R) subtended by branches of arbitrary length. Letters suffixed to gene names are arbitrary by species. (Online version in colour.)

Table 1. Branches of *eud-1/sul-2* and *seud-1/ssu-1* showing significant, site-specific diversifying selection ($K_a/K_s \gg 1$) among Diplogastridae and Rhabdtidae. ('Branch' refers to that subtending listed node; '+' indicates most recent common ancestor of genera listed; percentage of sites is that in the maximum rate class; 'LRT', likelihood ratio test. Boldface font indicates non-terminal gene lineages.)

sulphatase subdomain	branch	max. K_a/K_s	% sites	LRT value	P_{adj}
catalytic	*Koerneria luziae sul-2-D*	1670	16	98.69	$<1\times10^{-5}$
catalytic	***Pristionchus* + *Levipalatum***	491	4.1	21.00	0.0009
catalytic	***Diplogasteroides* + *Levipalatum***	17.9	2.2	17.93	0.004
rest of domain	*P. japonicus eud-1*	221	6.4	20.90	$<1\times10^{-5}$
rest of domain	***Pristionchus eud-1/sul-2.2.1***	128	12	20.21	$<1\times10^{-5}$
rest of domain	***P. mayeri* + *P. pacificus eud-1/sul-2.2.1***	561	3.7	18.12	$<1\times10^{-5}$
rest of domain	**Diplogastridae**	47.3	12	15.35	0.0002
rest of domain	*P. maxplancki eud-1*	833	9.1	14.87	0.0002
rest of domain	***Pristionchus* + *Levipalatum***	966	7.7	13.07	0.0005

functional differences supported this distinction. Specifically, we tested whether the SUL-2 enzyme from a non-polyphenic outgroup could regulate plasticity in a polyphenic species, *P. pacificus*. For this assay, we expressed the full coding sequence of *C. elegans sul-2*, together with functional *P. pacificus eud-1* regulatory elements, in *P. pacificus eud-1* mutants. These regulatory elements drive over-expression of *eud-1* in *P. pacificus*, resulting in a complete conversion of otherwise all-stenostomatous *eud-1* mutants to an all-eurystomatous phenotype [9]. Therefore, a strong or complete conversion of the mouth-polyphenism phenotype by *Cel-sul-2* would fail to show an obvious distinction between *Cel-sul-2* and *Ppa-eud-1* function, whereas a weaker or absent phenotypic conversion would suggest functional differences between the two genes. When we expressed *Cel-sul-2* in *P. pacificus eud-1* mutants, the heterologous gene completely failed to rescue the mutant phenotype (figure 4; electronic supplementary material, figure S3). While it is possible that this outcome might have been owing to non-production of a functional *Cel*-SUL-2 protein from the expressed transcript, the previous success of synthetic rescue constructs in *P. pacificus* suggests that protein differences between SUL-2 and EUD-1 most simply explain this result. In summary, the function of *eud-1/sul-2* suggests lineage-specific functional differences, particularly between a nematode with polyphenism and a non-polyphenic outgroup species.

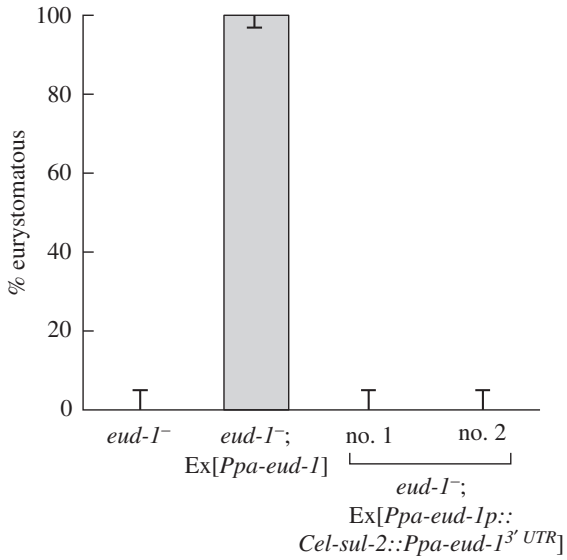

Figure 4. Transgenic, functional assay of *sul-2* from a non-polyphenic outgroup to Diplogastridae. Mouth-polyphenism phenotypes of *Pristionchus pacificus* (*Ppa*) *eud-1* mutants over-expressing *eud-1/sul-2* sequences. In lines expressing the heterologous rescue construct (right two bars), a full *sul-2* coding sequence from *Caenorhabditis elegans* (*Cel*) is flanked by endogenous *Ppa-eud-1* regulatory sequences. Mouth-phenotypes are given as proportions per line, with confidence intervals (whiskers) calculated by a binomial test.

4. Discussion

The evolutionary maintenance of developmental polyphenism relies on the presence of a heritable mechanism to regulate it. Despite a growing molecular understanding of such mechanisms, little was previously known about the evolutionary processes that give rise to their components nor how labile these components are over evolutionary time. Here, we have placed polyphenism regulators described for a nematode model, *P. pacificus*, into a phylogenetic context. We have traced the history of two genes whose products compete to control a resource polyphenism, reconstructing the events surrounding their origin, molecular change and functional specialization. Our findings have revealed that the evolutionary context for polyphenism regulation involved a series of duplications and putative specialization in protein function. Consequently, our study establishes a comparative framework for the genetic origins of plasticity regulation, which is needed to determine how and the degree to which they lead or follow the evolution of plasticity itself [54,55].

This comparative framework supports two major themes. The first is that the evolution of switch-gene homologues have undergone extensive duplications throughout the phylogenetic history of the polyphenism. While transcriptional regulators of polyphenism genes, NHR-40 and NHR-1, are conserved in number and putatively function [25,56,57], studies within *Pristionchus* have indicated the enzymatic factors gating their activity to be dynamic in number [9,16,26,27]. However, *Pristionchus* is just one of several polyphenic lineages, and how general this feature might be among polyphenic nematodes more broadly was previously unclear. Our findings show that duplications of both *sul-2* and *ssu-1* have occurred repeatedly across even the deepest divergences in Diplogastridae, and more frequently in polyphenic species. While duplicate genes have long been thought to provide a substrate for the evolution of novel gene functions and thus novel traits [58–60], the fixation of any given duplicate is normally highly unlikely [61], warranting explanations for the higher copy numbers among polyphenic diplogastrids. One explanation may be the additive function of some duplicates: duplications resulting in a phenotypic change might protect daughter genes from relaxed selection and ultimately pseudogenization, thereby promoting the fixation of new genes under the appropriate selective regimes [62,63]. Indeed, in at least one *P. pacificus* polyphenism switch gene, *seud-1*, duplicates apparently amplify the phenotypic effects of the gene [16]. Because the parallel radiations we infer statistically correlate with the presence of polyphenism, it is easy to hypothesize a functional connection as well. However, the regulation of polyphenism by *sul-2* duplicates in other diplogastrid lineages remains to be tested. The feasibility of reverse genetics in *Pristionchus* [24,26,57] suggests that functional assays of multiple switch-gene homologues might be readily expanded to other species *in situ*. Our detection of lineage-specific clusters of switch-gene homologues in this system provides a map to determining the importance of gene duplication more broadly in polyphenism evolution.

A second theme our study supports is that both regulatory and protein-coding evolution probably contributed to the evolution of polyphenism switch-gene function. Whereas many examples have highlighted the former in the regulation of novel phenotypes, especially in macroevolution [64–66], the role of the latter may be at least as important [67]. In the case of *seud-1/ssu-1*, previous studies have shown that regulatory differences can explain at least some divergence in function between *C. elegans* and *P. pacificus*, as the expression of the sulfotransferase genes has clearly diverged between these species. Whereas *ssu-1* is expressed in a pair of

sensory (amphid) neurons in *C. elegans* [68], *seud-1* in *P. pacificus* is localized to pharyngeal and adjacent cells producing the animal's dimorphic mouthparts [16]. By contrast, the protein's activity may be conserved in at least one feature: as part of a stress response in *C. elegans*, *ssu-1* promotes the activity of a nuclear receptor, NHR-1 [69]; in *P. pacificus*, *seud-1* promotes the activity of NHR-40 and, at least indirectly, NHR-1 as well [16,57]. Therefore, an interaction between this sulfotransferase and a conserved nuclear receptor may have already been in place before the evolution of polyphenism.

However, strongly selected coding-sequence differences also underlie the evolution of polyphenism regulation, as our results show for *eud-1/sul-2* in Diplogastridae. Patterns of diversifying selection were specifically detected in early lineages probably possessing a single copy of the gene as well as in later lineages, namely in *Pristionchus*, in which both copies making up a supergene have experienced positive selection through concerted evolution. Further, our findings here suggest that protein function has diverged between *P. pacificus* and a non-polyphenic outgroup. This difference is similar to a previous finding that even recent duplicates have probably diverged in protein function, specifically *P. pacificus* EUD-1 and SUL-2.2.1, which differ in their ability to rescue *eud-1* mutants when expressed under a common promoter [24]. Although our results do not distinguish between a loss of incipient polyphenism function in the *Caenorhabditis* lineage from a gain in the *Pristionchus* lineage, the coding-sequence divergence of diplogastrid *eud-1/sul-2* homologues lead us to hypothesize that functional differences occurred immediately prior to or during the evolution of Diplogastridae. While the identity and timing of the relevant changes are still to be determined, we have found this putative functional divergence to be reflected by protein-coding differences that have episodically accumulated between lineages.

In summary, our findings provide a comparative framework for the molecular basis of polyphenism, specifically the components of a plasticity switch. This framework has revealed two features—extensive gene duplication and rapid coding-sequence evolution—that characterize plasticity switch-gene homologues during the early evolution of a polyphenism. As the functional importance of these homologues is explored, this framework will inform what is needed to accommodate or maintain alternative morphs over evolutionary time. Because polyphenism is an exemplar of how developmental plasticity may facilitate the evolution of new traits [1], molecular studies of polyphenism's control and persistence should reveal the genetic parameters for its evolutionary potential.

Data accessibility. The data supporting this article have been either uploaded as part of the electronic supplementary material or deposited in the Dryad Digital Repository: https://doi.org/dryad.3tx95×6bk [42].

Authors' contributions. J.F.B. and E.J.R. designed the research, performed the research, analysed the data and wrote the paper.

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References

1. West-Eberhard MJ. 2003 *Developmental plasticity and evolution*. Oxford, UK: Oxford University Press.
2. Moczek AP, Sultan S, Foster S, Ledón-Rettig C, Dworkin I, Nijhout HF, Abouheif E, Pfennig DW. 2011 The role of developmental plasticity in evolutionary innovation. *Proc. R. Soc. B* **278**, 2705–2713. (doi:10.1098/rspb.2011.0971)
3. Levis NA, Pfennig DW. 2016 Evaluating 'plasticity-first' evolution in nature: key criteria and empirical approaches. *Trends Ecol. Evol.* **31**, 563–574. (doi:10.1016/j.tree.2016.03.012)
4. Parsons KJ, McWhinnie K, Pilakouta N, Walker L. 2019 Does phenotypic plasticity initiate developmental bias? *Evol. Dev.* **19**, e12305. (doi:10.1111/ede.12304)
5. Pfennig DW, Wund MA, Snell-Rood EC, Cruickshank T, Schlichting CD, Moczek AP. 2010 Phenotypic plasticity's impacts on diversification and speciation. *Trends Ecol. Evol.* **25**, 459–467. (doi:10.1016/j.tree.2010.05.006)
6. West-Eberhard MJ. 1986 Alternative adaptations, speciation, and phylogeny (a review). *Proc. Natl Acad. Sci. USA* **83**, 1388–1392. (doi:10.1073/pnas.83.5.1388)
7. Nijhout HF. 2015 To plasticity and back again. *Elife* **4**, e05463. (doi:10.7554/eLife.06995)
8. Moran NA. 1992 The evolutionary maintenance of alternative phenotypes. *Am. Nat.* **139**, 971–989. (doi:10.1086/285369)
9. Ragsdale EJ, Müller MR, Rödelsperger C, Sommer RJ. 2013 A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**, 922–933. (doi:10.1016/j.cell.2013.09.054)
10. Xu HJ *et al.* 2015 Two insulin receptors determine alternative wing morphs in planthoppers. *Nature* **519**, 464–467. (doi:10.1038/nature14286)
11. Beldade P, Mateus ARA, Keller RA. 2011 Evolution and molecular mechanisms of adaptive developmental plasticity. *Mol. Ecol.* **20**, 1347–1363. (doi:10.1111/J.1365-294x.2011.05016.X)
12. Lafuente E, Beldade P. 2019 Genomics of developmental plasticity in animals. *Front. Genet.* **10**, 720. (doi:10.3389/fgene.2019.00720)
13. Projecto-Garcia J, Biddle JF, Ragsdale EJ. 2017 Decoding the architecture and origins of mechanisms for developmental polyphenism. *Curr. Opin. Genet. Dev.* **47**, 1–8. (doi:10.1016/j.gde.2017.07.015)
14. Serobyan V, Sommer RJ. 2017 Developmental systems of plasticity and trans-generational epigenetic inheritance in nematodes. *Curr. Opin. Genet. Dev.* **45**, 51–57. (doi:10.1016/j.gde.2017.03.001)
15. Richard G, Le Trionnaire G, Danchin E, Sentis A. 2019 Epigenetics and insect polyphenism: mechanisms and climate change impacts. *Curr. Opin. Insect Sci.* **35**, 138–145. (doi:10.1016/j.cois.2019.06.013)
16. Bui LT, Ivers NA, Ragsdale EJ. 2018 A sulfotransferase dosage-dependently regualtes mouthpart dimorphism in the nematode *Pristionchus pacificus*. *Nat. Commun.* **9**, 4119. (doi:10.1038/s41467-018-05612-8)
17. Van Dyken JD, Wade MJ. 2010 The genetic signature of conditional expression. *Genetics* **184**, 557–570. (doi:10.1534/Genetics.109.110163)
18. Susoy V, Ragsdale EJ, Kanzaki N, Sommer RJ. 2015 Rapid diversification associated with a macroevolutionary pulse of developmental plasticity. *Elife* **2015**, 1–39. (doi:10.7554/eLife.05463)
19. Serobyan V, Ragsdale EJ, Sommer RJ. 2014 Adaptive value of a predatory mouth-form in a dimorphic nematode. *Proc. R. Soc. B* **281**, 20141334. (doi:10.1098/rspb.2014.1334)
20. Sudhaus W, Fürst von Lieven A. 2003 A phylogenetic classification and catalogue of the Diplogastridae (Secernentea, Nematoda). *J. Nematode Morphol. Syst.* **6**, 43–90.
21. Susoy V *et al.* 2016 Large-scale diversification without genetic isolation in nematode symbionts of figs. *Sci. Adv.* **2**, e1501031. (doi:10.1126/sciadv.1501031)
22. Kanzaki N, Ragsdale EJ, Herrmann M, Röseler W, Sommer RJ. 2013 Two new species of *Pristionchus* (Nematoda: Diplogastridae) support the biogeographic importance of Japan for the

evolution of the genus *Pristionchus* and the model system *P. pacificus*. *Zool. Sci.* **30**, 680–692. (doi:10.2108/zsj.30.680)

23. Kanzaki N, Ragsdale EJ, Herrmann M, Susoy V, Sommer RJ. 2013 Two androdioecious and one dioecious new species of *Pristionchus* (Nematoda: Diplogastridae): new reference points for the evolution of reproductive mode. *J. Nematol.* **45**, 172–194.
24. Sieriebriennikov B, Prabh N, Dardiry M, Witte H, Röseler W, Kieninger MR, Rödelsperger C, Sommer RJ. 2018 A developmental switch generating phenotypic plasticity is part of a conserved multi-gene locus. *Cell Rep.* **23**, 2835–2843. (doi:10.1016/j.celrep.2018.05.008)
25. Kieninger MR, Ivers NA, Rödelsperger C, Markov GV, Sommer RJ, Ragsdale EJ. 2016 The nuclear hormone receptor NHR-40 acts downstream of the sulfatase EUD-1 as part of a developmental plasticity switch in *Pristionchus*. *Curr. Biol.* **26**, 2174–2179. (doi:10.1016/j.cub.2016.06.018)
26. Ragsdale EJ, Ivers NA. 2016 Specialization of a polyphenism switch gene following serial duplications in *Pristionchus* nematodes. *Evolution (NY).* **70**, 2155–2166. (doi:10.1111/evo.13011)
27. Namdeo S, Moreno E, Rödelsperger C, Baskaran P, Witte H, Sommer RJ. 2018 Two independent sulfation processes regulate mouth-form plasticity in the nematode *Pristionchus pacificus*. *Development* **145**, dev66272. (doi:10.1242/dev.166272)
28. Serobyan V, Xiao H, Namdeo S, Rödelsperger C, Sieriebriennikov B, Witte H, Röseler W, Sommer RJ. 2016 Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene eud-1 control predatory feeding plasticity. *Nat. Commun.* **7**, 12337. (doi:10.1038/ncomms12337)
29. Kiontke K, Barriere A, Kolotuev I, Podbilewicz B, Sommer R, Fitch DH, Felix MA. 2007 Trends, stasis, and drift in the evolution of nematode vulva development. *Curr. Biol.* **17**, 1925–1937. (doi:10.1016/j.cub.2007.10.061)
30. Ragsdale EJ, Koutsovoulos G, Biddle JF. 2019 A draft genome for a species of *Halicephalobus* (Panagrolaimidae). *J. Nematol.* **51**, 1–4. (doi:10.21307/jofnem-2019-068).
31. Stanke M, Morgenstern B. 2005 AUGUSTUS: a web server for gene prediction in eukaryotes that allows user-defined constraints. *Nucleic Acids Res.* **33**, W465–W467. (doi:10.1093/nar/gki458)
32. Prabh N, Roeseler W, Witte H, Eberhardt G, Sommer RJ, Rödelsperger C. 2018 Deep taxon sampling reveals the evolutionary dynamics of novel gene families in *Pristionchus* nematodes. *Genome Res.* **28**, 1664–1674. (doi:10.1101/gr.234971.118)
33. Katoh K, Standley DM. 2013 MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772–780. (doi:10.1093/molbev/mst010)
34. Lukatela G, Krauss N, Theis K, Selmer T, Gieseimann V, Von Figura K, Saenger W. 1998 Crystal structure of human arylsulfatase A: the aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis. *Biochemistry* **37**, 3654–3664. (doi:10.1021/bi9714924)
35. Dajani R *et al*. 1999 X-ray crystal structure of human dopamine sulfotransferase, SULT1A3. *J. Biol. Chem.* **274**, 37 862–37 868. (doi:10.1074/jbc.274.53.37862)
36. Miller MA, Pfeiffer W, Schwartz T. 2010 *Creating the CIPRES Science Gateway for inference of large phylogenetic trees*. In *2010 Gateway Computing Environments Workshop, GCE 2010*, pp. 1–8. New Orleans, LA. (doi:10.1109/GCE.2010.5676129)
37. Stamatakis A. 2014 RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313. (doi:10.1093/bioinformatics/btu033)
38. van Megen H, van den Elsen S, Holterman M, Karssen G, Mooyman P, Bongers T, Holovachov O, Bakker J, Helder J. 2009 A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. *Nematology* **11**, 927–950. (doi:10.1163/156854109X456862)
39. Smythe AB, Holovachov O, Kocot KM. 2019 Improved phylogenomic sampling of free-living nematodes enhances resolution of higher-level nematode phylogeny. *BMC Evol. Biol.* **191**, 121. (doi:10.1186/s12862-019-1444-x)
40. Ronquist F *et al*. 2012 Mrbayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **61**, 539–542. (doi:10.1093/sysbio/sys029)
41. Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T. 2019 ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Mol. Biol. Evol.* **37**, 291–294. (doi:10.1093/molbev/msz189)
42. Biddle JF, Ragsdale EJ. 2020 Data from: Regulators of an ancient polyphenism evolved through episodic protein divergence and parallel gene radiations. Dryad Digital Repository. See https://doi.org/10.5061/dryad.3tx95x6bk.
43. Goodman M, Czelusniak J, Moore GW, Romero-Herrera AE, Matsuda G. 1979 Fitting the gene lineage into its species lineage, a parsimony strategy illustrated by cladograms constructed from globin sequences. *Syst. Zool.* **28**, 132–163. (doi:10.2307/2412519)
44. Maddison WP. 1997 Gene trees in species trees. *Syst. Biol.* **46**, 523–536. (doi:10.1093/sysbio/46.3.523)
45. Rödelsperger C, Röseler W, Prabh N, Yoshida K, Weiler C, Herrmann M, Sommer RJ. 2018 Phylotranscriptomics of *Pristionchus* nematodes reveals parallel gene loss in six hermaphroditic lineages. *Curr. Biol.* **28**, 3123–3127. (doi:10.1016/j.cub.2018.07.041)
46. Chen K, Durand D, Farach-Colton M. 2000 NOTUNG: a program for dating gene duplications and optimizing gene family trees. *J. Comput. Biol.* **7**, 429–447. (doi:10.1089/106652700750050871)
47. Almagro Armenteros JJ, Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H. 2019 SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat. Biotechnol.* **37**, 420–423. (doi:10.1038/s41587-019-0036-z)
48. Lewis PO. 2001 A likelihood approach to estimating phylogeny from discrete morphological character data. *Syst. Biol.* **50**, 913–925. (doi:10.1080/106351501753462876)
49. Maddison WP, Maddison DR. 2018 Mesquite: a modular system for evolutionary analysis. Version 3.51. See http://www.mesquiteproject.org. (doi:10.1017/CBO9781107415324.004)
50. Smith MD, Wertheim JO, Weaver S, Murrell B, Scheffler K, Kosakovsky Pond SL. 2015 Less is more: an adaptive branch-site random effects model for efficient detection of episodic diversifying selection. *Mol. Biol. Evol.* **32**, 1342–1353. (doi:10.1093/molbev/msv022)
51. Weaver S, Shank SD, Spielman SJ, Li M, Muse SV, Kosakovsky Pond SL. 2018 Datamonkey 2.0: a modern web application for characterizing selective and other evolutionary processes. *Mol. Biol. Evol.* **35**, 773–777. (doi:10.1093/molbev/msx335)
52. Serobyan V, Ragsdale EJ, Müller MR, Sommer RJ. 2013 Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed. *Evol. Dev.* **15**, 161–170. (doi:10.1111/ede.12030)
53. R Core Team. 2015 *R: a language and environment for statistical computing*. *R Found. Stat. Comput. Vienna, Austria* **55**, 275–286.
54. Schwander T, Leimar O. 2011 Genes as leaders and followers in evolution. *Trends Ecol. Evol.* **26**, 143–151. (doi:10.1016/J.Tree.2010.12.010)
55. Ehrenreich IM, Pfennig DW. 2015 Genetic assimilation: a review of its potential proximate causes and evolutionary consequences. *Ann. Bot.* **117**, 769–779. (doi:10.1093/aob/mcv130)
56. Bui LT, Ragsdale EJ. 2019 Multiple plasticity regulators reveal targets specifying an induced predatory form in nematodes. *Mol. Biol. Evol.* **36**, 2387–2399. (doi:10.1093/molbev/msz171)
57. Sieriebriennikov B, Sun S, Lightfoot JW, Witte H, Moreno E, Rödelsperger C, Sommer RJ. 2019 Conserved nuclear receptors controlling a novel trait target fast-evolving genes expressed in a single cell. *bioRxiv*, 809350. (doi:10.1101/809350)
58. Wagner GP, Lynch VJ. 2010 Evolutionary novelties. *Curr. Biol.* **20**, R48–R52. (doi:10.1016/j.cub.2009.11.010)
59. Khalturin K, Hemmrich G, Fraune S, Augustin R, Bosch TCG. 2009 More than just orphans: are taxonomically-restricted genes important in evolution? *Trends Genet.* **25**, 404–413. (doi:10.1016/j.tig.2009.07.006)
60. Kaessmann H. 2010 Origins, evolution, and phenotypic impact of new genes. *Genome Res.* **20**, 1313–1326. (doi:10.1101/gr.101386.109)
61. Lynch M, Conery JS. 2000 The evolutionary fate and consequences of duplicate genes. *Science* **290**, 1151–1155. (doi:10.1126/science.290.5494.1151)

62. Kondrashov FA, Kondrashov AS. 2006 Role of selection in fixation of gene duplications. *J. Theor. Biol.* **239**, 141–151. (doi:10.1016/j.jtbi.2005.08.033)
63. Bergthorsson U, Andersson DI, Roth JR. 2007 Ohno's dilemma: evolution of new genes under continuous selection. *Proc. Natl Acad. Sci. USA* **104**, 17 004–17 009. (doi:10.1073/pnas.0707158104)
64. Wittkopp PJ, Kalay G. 2012 *Cis*-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. *Nat. Rev. Genet.* **13**, 59–69. (doi:10.1038/nrg3095)
65. Wray GA. 2007 The evolutionary significance of *cis*-regulatory mutations. *Nat. Rev. Genet.* **8**, 206–216. (doi:10.1038/nrg2063)
66. Stern DL, Orgogozo V. 2008 The loci of evolution: how predictable is genetic evolution? *Evolution* (*NY*) **62**, 2155–2177. (doi:10.1111/j.1558-5646.2008.00450.x)
67. Gruber JD, Vogel K, Kalay G, Wittkopp PJ. 2012 Contrasting properties of gene-specific regulatory, coding, and copy number mutations in *Saccharomyces cerevisiae*: frequency, effects, and dominance. *PLoS Genet.* **8**, e1002497. (doi:10.1371/journal.pgen.1002497)
68. Carroll BT, Dubyak GR, Sedensky MM, Morgan PG. 2006 Sulfated signal from ASJ sensory neurons modulates stomatin-dependent coordination in *Caenorhabditis elegans*. *J. Biol. Chem.* **281**, 35 989–35 996. (doi:10.1074/Jbc.M606086200)
69. Burton NO, Dwivedi VK, Burkhart KB, Kaplan REW, Baugh LR, Horvitz HR. 2018 Neurohormonal signaling via a sulfotransferase antagonizes insulin-like signaling to regulate a *Caenorhabditis elegans* stress response. *Nat. Commun.* **9**, 5152. (doi:10.1038/s41467-018-07640-w)