Current Biology *17*, 1925–1937, November 20, 2007 DOI 10.1016/j.cub.2007.10.061

Article

Trends, Stasis, and Drift in the Evolution of Nematode Vulva Development

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Summary

Background: A surprising amount of developmental variation has been observed for otherwise highly conserved features, a phenomenon known as developmental system drift. Either stochastic processes (e.g., drift and absence of selection-independent constraints) or deterministic processes (e.g., selection or constraints) could be the predominate mechanism for the evolution of such variation. We tested whether evolutionary patterns of change were unbiased or biased, as predicted by the stochastic or deterministic hypotheses, respectively. As a model, we used the nematode vulva, a highly conserved, essential organ, the development of which has been intensively studied in the model systems *Caenorhabditis elegans* and *Pristionchus pacificus*.
Results: For 51 rhabditid species, we analyzed more than 40 characteristics of vulva development, including cell fates, fate induction, cell competence, division patterns, morphogenesis, and related aspects of gonad development. We then defined individual characters and plotted their evolution on a phylogeny inferred for 65 species from three nuclear gene sequences. This taxon-dense phylogeny provides for the first time a highly resolved picture of rhabditid evolution and allows the reconstruction of the number and directionality of changes in the vulva development characters. We found an astonishing amount of variation and an even larger number of evolutionary changes, suggesting a high degree of homoplasy (convergences and reversals). Surprisingly, only two characters showed unbiased evolution. Evolution of all other characters was biased.
Conclusions: We propose that developmental evolution is primarily governed by selection and/or selection-independent constraints, not stochastic processes such as drift in unconstrained phenotypic space.

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Introduction

Much of research in evolutionary developmental biology is concerned with elucidating how divergent and novel features have evolved or how the same type of feature has evolved convergently [1–5]. However, there are many features that have remained largely static, even over vast evolutionary distances between species [2]. It might be expected that purifying selection would also prevent change to the developmental mechanisms that give rise to such features. Indeed, one of the main architects of molecular evolution, Emile Zuckerkandl, predicted that stabilizing selection on the phenotype would be reflected in stability of the underlying molecular features [6]. Nevertheless, several examples from a variety of different systems demonstrate that a large amount of variation has evolved in the development of homologous, highly conserved features [2].

Theoretically, such variation, which has been called "developmental system drift" (DSD) [2] or "phenogenetic drift" [7], could have accumulated by either stochastic or deterministic mechanisms or some combination of both [1, 2, 8, 9]. For example, purifying selection could have been relaxed at the level of the developmental mechanisms (e.g., because of redundancy or canalization), thus allowing developmental variation to accumulate stochastically [9, 10]. DSD demonstrates that there are often many ways to produce the same structure, suggesting that constraints other than selection (i.e., "generative constraints" or "developmental constraints") might not be very limiting either. On the other hand, it is also possible that the deterministic processes of directional ("positive") selection or other constraints predominate and bias or "channel" the path of developmental evolution, with stochastic processes playing a minor role [1, 11, 12].

Here, using a phylogenetic approach, we determine the pattern of evolutionary change in a system undergoing DSD and infer which processes play the predominant role in the evolution of DSD. This approach combines a detailed comparative analysis of development with a well-resolved phylogeny. This method allows us to infer the number, directionality, and phylogenetic distribution of the evolutionary changes leading to DSD. Two different patterns of evolutionary change are expected. (1) Unbiased changes would produce a variety of character states, as well as reversals back to an ancestral state. (2) If changes are biased, only a subset of several possible character states may be found, and reversals would be rare. Two kinds of processes are likely to be responsible for these patterns. (1) Unbiased patterns are expected if the predominant process is stochastic, i.e., if the evolution of a character is limited

neither by selection nor by other constraints. (2) Biased patterns are likely if deterministic processes (either selection or selection-independent constraints) predominate. Note that evolution under selection or other constraints may have stochastic aspects, like mutation or genetic drift, but is still considered deterministic and is likely to produce biases, such as irreversibility.

As an experimental system, we use the rhabditid nematode vulva, a major model for investigating the mechanisms of organogenesis. The vulva is an essential organ for copulation as well as egg-laying and is highly conserved. However, significant developmental differences have been uncovered in comparative studies of just a few nematode species (e.g., [13–17], reviewed by [18]), making the vulva a primary example of DSD [2].

Vulva development in the two nematode model systems *Caenorhabditis elegans* and *Pristionchus pacificus* [18, 19] is described in Figure 1. We distinguish five steps of vulva development that vary between species. There is also variation in other features related to vulva development, including vulva position along the body axis, division timing of the somatic gonad precursor cells, and number of gonad arms. To determine the range of variation in vulva development across rhabditid species, we compiled published information (see comprehensive list in the Supplemental Data available with this article online) or characterized vulval development for 51 species and classified the variation into different states of 52 well-defined, discrete characters.

Relationships among rhabditid nematodes have classically been difficult to resolve. Few morphological characters have been considered to be phylogenetically informative, and of those, many are homoplastic [20, 21]. To date, the published molecular phylogenies of rhabditids rely on the sequence of only SSU rRNA. Also, they either include few rhabditid species [22–24] or resolution of rhabditid relationships is low [21, 25]. Here, we present a well-resolved phylogeny of 65 rhabditid species, based on nearly complete sequences of SSU and LSU rRNA genes and part of the gene for the largest subunit of RNA polymerase II.

By combining this phylogeny (Figure 2) with our dataset of vulva development characters, we identified the direction of evolutionary changes, the phylogenetic lineage in which the changes happened, the number of times that convergent or parallel changes occurred, and where changes in different characters are correlated. We then used this information to investigate whether vulva developmental evolution is unbiased or biased. First, we find that, whereas some developmental characters do not change, many characters show a surprising amount of evolutionary change, consistent with a high level of developmental system drift. Second, we find unbiased evolutionary changes in only two characters; the changes affecting most characters are biased, sometimes highly biased. We therefore propose that developmental evolution, at least in the vulva model, is primarily influenced by deterministic processes, not stochastic ones.

Results

Molecular Phylogeny of Rhabditids

We obtained DNA sequences from small and large subunit ribosomal RNA genes and a portion of the RNA polymerase II gene for 65 species, with six representatives of the outgroup. Phylogenetic analysis using weighted maximum parsimony (MP) and Bayesian likelihood resolved most relationships (Figure 2 and Figures S1 and S2). The two reconstruction algorithms resulted in topologies that were identical in all but three branches (see Supplemental Data).

From this tree, the following new conclusions for the phylogeny of rhabditids can be drawn. (1) Rhabditids, including strongylids and diplogastrids, form a monophyletic group. (2) Diplogastrids are clearly part of rhabditids. (3) The first branch of the rhabditid clade is *Poikilolaimus*. (4) There are three major clades within rhabditids. The first clade "Eurhabditis," includes *Caenorhabditis* and its sister group, the "*Protorhabditis* group." The other Eurhabditis species form the "*Rhabditis* group," which includes *Oscheius*, the vertebrate-parasitic strongylids and the insect-pathogenetic *Heterorhabditis*, among others. The first branch of Eurhabditis appears to be *Choriorhabditis*. A second clade consists of *Rhabditoides inermis* and diplogastrids, which include *Pristionchus pacificus*. The third major clade, Pleiorhabditis, comprises *Rhabditoides inermiformis*, *R. regina*, the genus *Pelodera*, and a clade of species with a posterior vulva, the *Mesorhabditis* group. Of the three major clades, diplogastrids + *R. inermis* are most closely related to Eurhabditis; Pleiorhabditis branches off first.

Evolutionary Changes in Vulva Development

The study of vulva development in the 51 species allowed us to define 55 characters with one to nine different character states. Based on our molecular phylogeny, we then evaluated the evolutionary changes within rhabditids. For a detailed discussion of each character and a character-state matrix, see the Supplemental Data. Here we concisely describe the evolution of the relevant characters at five stages of vulva development.

Pn.p Cell Patterning

The number of Pn.p cells was found to be 12 in all studied species, as in Cephalobina [16].

The midbody Pn.p cells P(3-8).p remain unfused in the L2 stage in all species except diplogastrids and *Poikilolaimus oxycercus*, in which P(1-4).p and P(9-11).p undergo programmed cell death (PCD). In all species, the vulva develops from a subset of these midbody Pn.p cells. Genetic data from *C. elegans*, *Oscheius tipulae*, and *P. pacificus* have shown that the Hox gene *lin-39* is required to prevent Pn.p fusion and cell death [26–28]. In *P. pacificus*, *lin-39* is suppressed by Ppa-Groucho and Ppa-Hairy for P3.p and P4.p to undergo PCD [29].

The size of the competence group (all cells competent to form a vulva [30]) is assessed by ablation experiments. If a Pn.p cell is able to replace an ablated cell and become vulval, it is part of the competence group. In *C. elegans*, six Pn.p cells, P(3-8).p, are competent to form vulval tissue, whereas only three of them, P(5-7).p, normally adopt vulval fates. In all rhabditid species, P(5-7).p were competent, whereas the other cells experienced frequent changes in competence. Remarkably, different cellular mechanisms account for noncompetence. One mechanism is programmed cell death, another might be an early fusion to the epidermal syncytium hyp7. In at least one species, *Pelodera strongyloides*

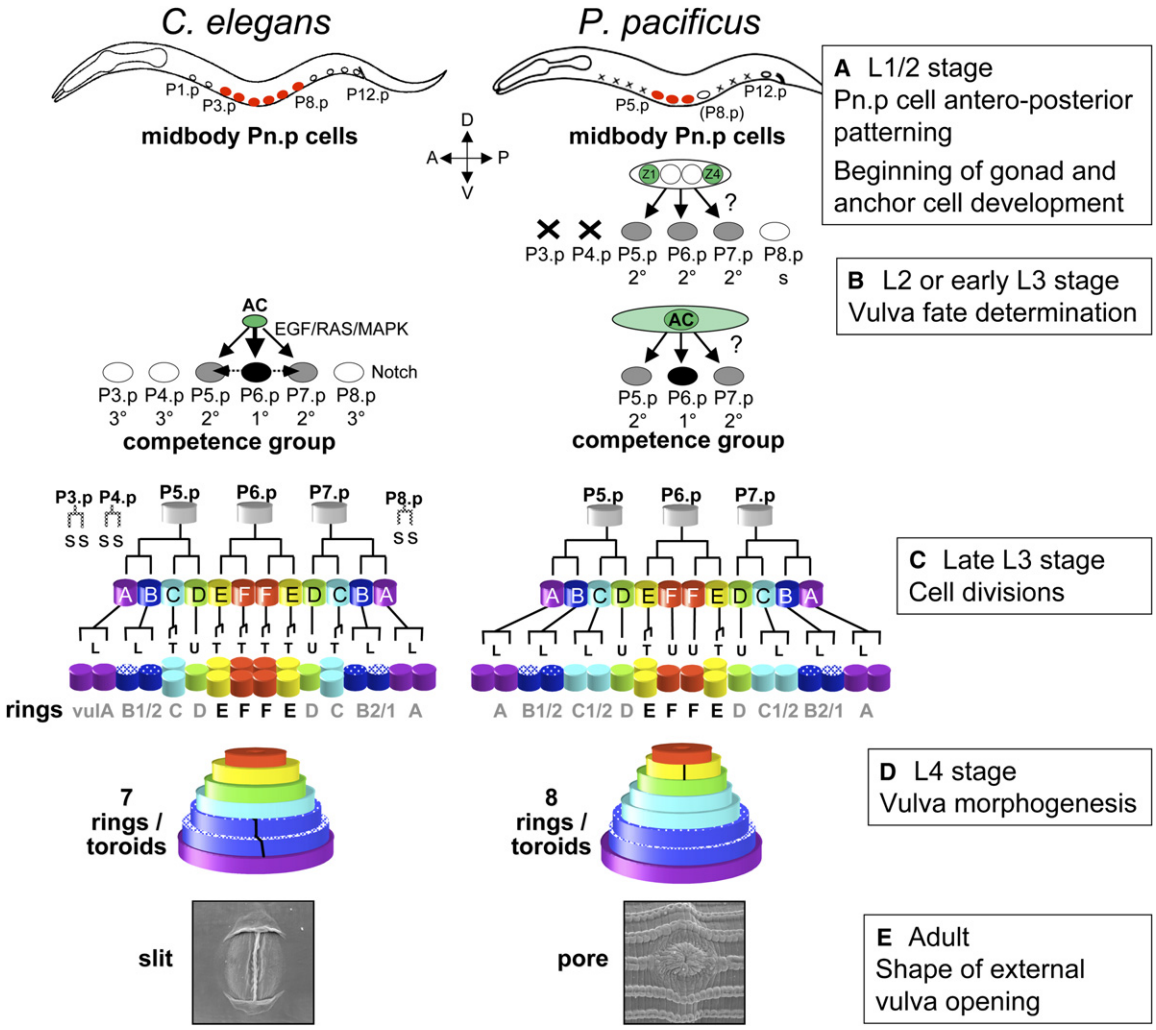

Figure 1. Vulval Development of *Caenorhabditis elegans* and *Pristionchus pacificus* in Comparison

(A) Midbody Pn.p cells [P(3-8).p in *C. elegans*, P(5-8).p in *P. pacificus*] are set aside for vulva formation. In *C. elegans*, other Pn.p cells fuse to the epidermis. In *P. pacificus*, they die in L1 and P8.p loses competence.

(B) Pn.p cells competent to form the vulva [competence group; P(3-8).p in *C. elegans* and P(5-7).p in *P. pacificus*] can adopt one of three fates. P6.p adopts the inner vulval fate (1°, black), P(5,7).p the outer vulval fate (2°, gray), and P(3,4,8).p a non-vulval fate (3°, white). In *C. elegans*, this spatial pattern of cell fates relies on an induction of vulval fates by the anchor cell (AC) of the gonad through EGF/Ras/Map kinase signaling (full arrows, [60]) and lateral signaling between P6.p and its neighbors through a Delta-Notch pathway (dashed arrows), which inhibits the 1° fate and activates the 2° fate in P(5,7).p [61–64]. In *P. pacificus*, the inductive signal comes from several cells of the somatic gonad. First, the 2° fate is induced in all three vulval cells. Further signaling is required for induction of 1° fates in P6.p. The nature of the signal is currently unknown. There is also signaling between Pn.p cells (not depicted, [48]).

(C) Each fate corresponds to a specific cell division pattern that is executed in the late L3 stage [65]. In *C. elegans,* the 3° Pn.p cells (dotted) undergo one division and fusion to the hyp7 epidermal syncytium (s). The 2° Pn.p lineage results in seven progeny, the 1° fate lineage in eight progeny, with characteristic orientations of the third round of division: T = transverse division (left-right), L = longitudinal (antero-posterior division), U = undivided. In *P. pacificus*, P8.p never divides and fuses with hyp7 early. The orientation of the VulC division is longitudinal and not transverse as in *C. elegans*. P6.p has only six progeny [31].

(D) In the L4 stage, the symmetric cells of the P5.p and P7.p lineages, and of the two daughters of P6.p, migrate toward each other, fuse, and form seven (in *C. elegans*) or eight (in *P. pacificus*) superposed syncytial rings around a vulval invagination [17, 46, 66]. In *C. elegans*, the two sisters of the B granddaughter form two rings, vulB1 and vulB2; the progeny of all other granddaughters form a single ring. In *P. pacificus*, the C cell also forms two rings.

(E) The external vulval opening has the shape of a transverse slit in *C. elegans* and that of a round pore in *P. pacificus.*

PS1129, P3.p is noncompetent by a third mechanism because neither early fusion nor cell death occur.

In *Poikilolaimus oxycercus* and diplogastrids, P(1-4).p and P(9-11).p die by PCD [31]. The small competence group of diplogastrids is a derived character, and the similarity with *Poikilolaimus oxycercus* is a convergence, both in competence group reduction and in the cellular mechanism involved (apoptosis) (Figures S4 and S5).

P8.p is competent in *P. oxycercus* [31] but rendered variably noncompetent in different diplogastrids through fusion to hyp7 [32]. For P3.p, three changes in competence occurred: one gain of competence after the divergence of Pleiorhabditis and two losses in *C. briggsae* and within the *Rhabditis* group (Figure S5).

The division pattern of P3.p is often variable within a species. However, the frequency at which divisions

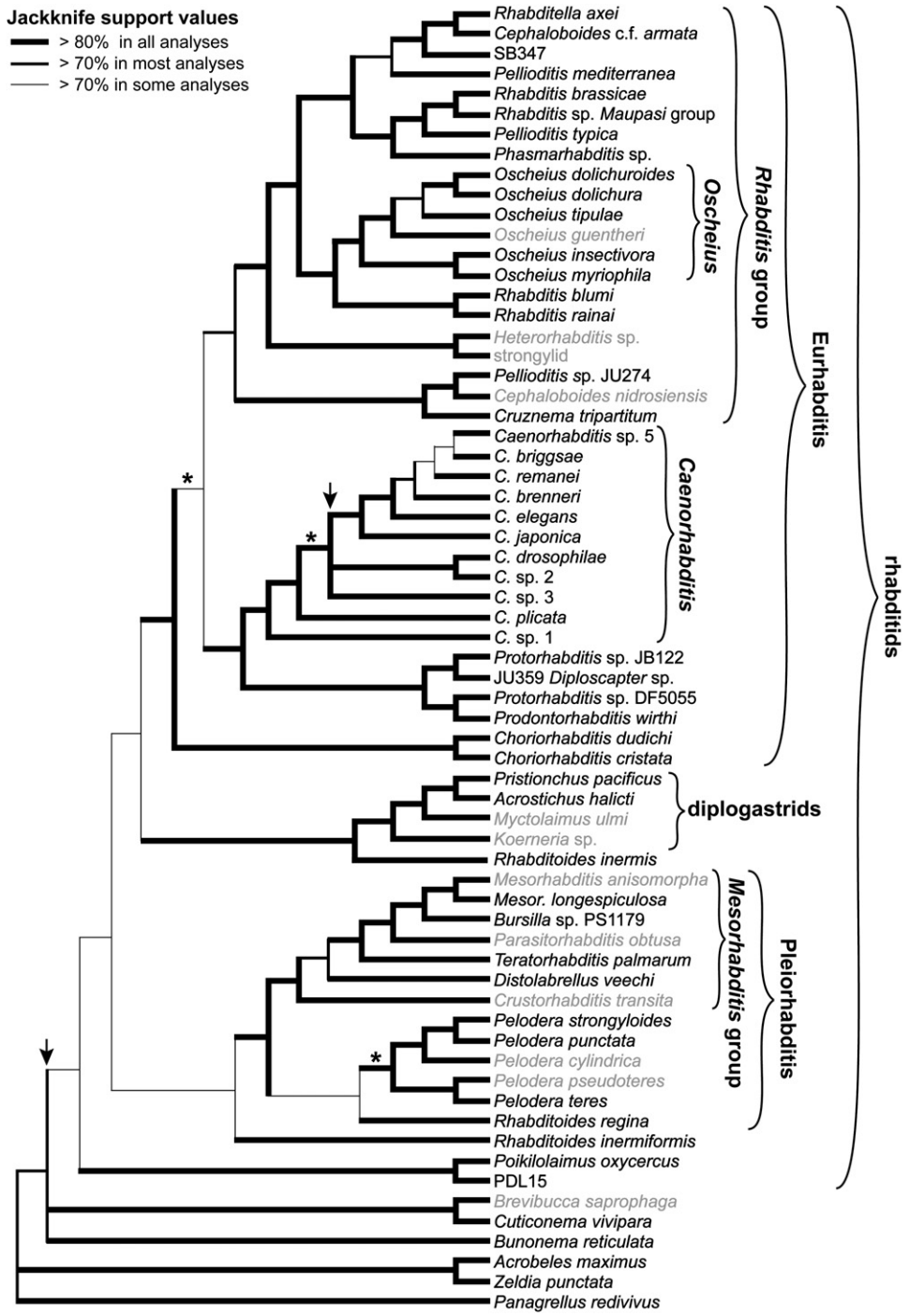

Figure 2. Rhabditid Phylogeny

Our best hypothesis for the phylogenetic relationships of rhabditids as derived from the six different weighted maximum parsimony jackknife analyses performed with concatenated sequences of genes for SSU and LSU rRNA and the largest subunit of RNA polymerase II. The relationships of the *Elegans* group within *Caenorhabditis* are resolved after an additional phylogenetic analysis (see Supplemental Data). Thickness of branches denotes the level of jackknife support. Only two branches (arrows) had less than 60% jackknife support in all analyses and were collapsed to reflect this ambiguity of resolution. Taxa in gray were absent from three of the six analyses and from the Bayesian analyses; the Bayesian analyses recovered the same tree with the exception of three branches marked with asterisks (*). All other branches have posterior probabilities ≥ 0.99.

occur differs between species (Figure S11). There is a relationship between division of P3.p and its competence. In species in which P3.p divides in most animals, it is also competent, whereas in species in which P3.p remains undivided, it is not competent. Exceptions are: *Caenorhabditis* sp. 1 SB341, *Rhabditoides inermis*, and *Panagrellus redivivus*; P3.p remains undivided—like P4.p in these species—but is competent; in *Pelodera*

strongyloides (PS1129), P3.p divides in more than 50% of the animals, but it is not competent.

Midbody Pn.p Cell Migration. In most species, the midbody Pn.p cells remain approximately in the same position until their divisions. However, they migrate posteriorly in (1) *Cruznema tripartitum*, (2) all members of the *Mesorhabditis* group, (3) at least one diplogastrid, *Diplogastrellus gracilis* [18], (4) some outgroup species, such as *Brevibucca saprophaga* and, to a lesser extent, *Panagrellus redivivus* [16]. In these species, the cells form a vulva posterior to the middle of the animal. Posterior migration evolved several times in rhabditids and was never reversed (Figure S7B).

Gonad and Anchor Cell Development

Because vulva development is influenced by the somatic gonad, we also looked at relevant aspects of gonad development. We found a strong heterochrony in gonad development among rhabditids. Whereas the precursors of the somatic gonad Z1 and Z4 divide at the L1 stage in *C. elegans*, in many other species, these divisions occur at the L2 stage, and the anchor cell (AC) is not born until the late L2 stage. Late development of the somatic gonad is ancestral, and early development in L1 evolved four times independently, within Pleiorhabditis, *Rhabditoides inermis*, within the *Caenorhabditis* genus, and within the *Rhabditis* group of Eurhabditis (Figure S6). There is one reversal in *Rhabditis* sp. SB347 in which this character is dimorphic.

A morphological variation in gonad development concerns the number of ovary arms. Some species have only one anterior arm (monodelphy), whereas others have two symmetric gonad arms (didelphy). The rhabditid stem species was didelphic (Figure S7). Posterior ovary reduction occurred at least six times independently: (1) in the stem species of the *Mesorhabditis* group, (2 and 3) at least twice within diplogastrids (independently in *Myctolaimus ulmi* and *Diplogastrellus gracilis* following the phylogeny of [33]), (4) in *Cruznema*, (5) in *Oscheius guentheri*, (6) in *Caenorhabditis* sp. 1. The two latter species are monodelphic exceptions within otherwise didelphic genera. Overall, posterior arm reduction occurred frequently, whereas neither reduction of the anterior arm nor reversal to a didelphic gonad occurred within rhabditids.

Vulva Fate Determination

The spatial pattern of vulval precursor cell fates is an invariant character within rhabditids. In all studied species, P6.p adopts a central vulval fate (1°) and P(5,7).p outer vulval fates (2°). In Cephalobina, the vulva is formed from four cells, P(5-8).p, and the AC and vulva pattern are centered between P6.p and P7.p [16].

The role of the gonad and the AC in vulval cell fate specification was determined by ablating them at different time points during development (see Supplemental Data). In contrast with the invariance of the "2°1°2°" spatial fate pattern, cell fate specification mechanisms vary extensively (Figure 3). Complete gonad-independence of vulval differentiation evolved in the *Mesorhabditis* group of Pleiorhabditis, and convergently in *Diplogastrellus gracilis* [18]. Among the other species, we found that inductive signaling can be restricted to the AC (i.e., the earliest AC ablations abolished vulval differentiation, as in *C. elegans*) or that more cells of the somatic gonad participate in vulval induction. The latter situation must have been present in the rhabditid stem species. Induction of vulval fates by only the AC evolved twice within rhabditids. There were two independent reversals to induction by more cells of the somatic gonad: in *Rhabditis* sp. SB347 and in *Caenorhabditis* sp. 1. In species in which the induction signal is not focused on the AC, induction starts before the AC is determined or even before its precursors are born [34, 35]. The evolutionary change is therefore best described as a heterochronic change.

The number of induction steps required to specify the 2°1°2° fate pattern of P(5-7).p also changed during evolution. In *C. elegans* and some other *Caenorhabditis* species, AC ablation at the time of P6.p division usually results in a normal pattern [36, 37]. In most other species, however, P6.p daughters adopt a 2°-like fate after AC ablation in the mid-L3 stage (see Supplemental Data, Table S14), as in Cephalobina [16]. Although this was not noted previously, published ablation data in *P. pacificus* clearly conform to this pattern [34]. Thus, in most species, proper 1° fate specification requires late signaling from the AC. Strikingly, the phylogenetic analysis showed that early 1° fate specification evolved only within the *Caenorhabditis* genus (Figure S8). Thus, the early ("one-step") 1° fate determination and probably the state of the molecular network of intercellular signaling events of *C. elegans* are highly derived.

Midbody Pn.p Cell Divisions

Midbody Pn.p cell divisions occur at the end of the L3 stage until right after the molt to the L4 stage in all species but *Rhabditoides inermis*, where the first round of divisions occurs in early L3. This almost complete invariance is remarkable when compared with the extensive heterochronies in other developmental events, such as gonad development or hatching (data from this study and [13, 16, 17, 38, 39]).

The P6.p lineage varied infrequently within rhabditids (Figure S9). In the rhabditid stem species, the P6.p cell lineage comprised three division rounds, the third being transverse ("TTTT"). The outer granddaughter division is absent in the *Mesorhabditis* group ("UTTU" lineage). Loss of the division of the inner granddaughters occurred twice independently, in diplogastrids and in *Rhabditis* sp. SB347 ("TUUT" lineage). There are no longitudinal divisions of the P6.p granddaughters.

The lineage of the outer vulval cells P5.p and P7.p is more variable (Figure S10). Among their four granddaughters (A, B, C, D), the innermost (D) cell is exceptional in that it never divides. Our analysis indicates that the other three granddaughter cells (A, B, C) divided longitudinally ("L") in the rhabditid stem species. These divisions were lost several times (at least four times for A, six times for B, and five times for C). A lost division was never regained. The C granddaughter division changed its orientation from longitudinal to transverse within Eurhabditis after the *Choriorhabditis* branch. From this transverse division, there were three losses of the division and one reversal to a longitudinal division. A second change from a longitudinal to a transverse division of the C cell took place in the *Pelodera* stem species, and the orientation of this division changed back to longitudinal within *Pelodera strongyloides*, where two strains show different lineage patterns for these cells.

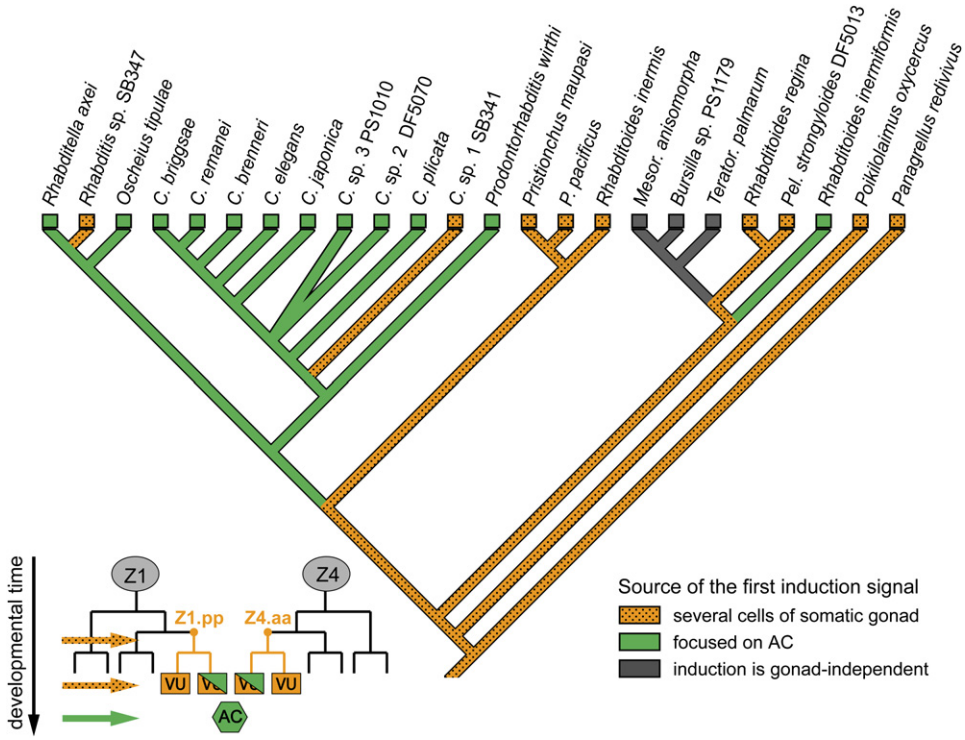

Figure 3. Frequent Evolutionary Changes in the Source of the First Signal for Vulva Induction

The source of the first inductive signal changed four times between two states: (orange) a group of cells of the developing somatic gonad (Z1.pp and Z4.aa or their daughters, of which one becomes the AC and three become ventral uterus precursor cells VU), and (green) the AC alone. These changes are largely heterochronic in nature because induction occurs either (1) only after the AC is specified (green arrow in inset) or (2) earlier, sometimes before the AC precursors are born (orange arrows in inset). In *C. elegans*, the inductive signal LIN-3 is expressed in the preanchor cells and in the ventral uterine cells, and it is confined to the AC only later during development [67]. If the EGF-Ras-MAP kinase pathway is involved in vulva induction in all species (currently only known for *Oscheius tipulae* [68] and *C. elegans*), slight quantitative changes, such as the timing of expression of the EGF receptor in the vulval cells, could explain the changes in this character. In the species of the *Mesorhabditis* group, vulva induction is gonad independent (gray). Gonad independence is also reported for *Diplogastrellus gracilis*, a diplogastrid [18].

The 3° lineage is the most variable (Figure 4). It even varies within an isogenic strain, most strikingly in *Oscheius tipulae*, where zero, one, or two divisions occur with intraspecific variations in their frequencies [40]. We found a similar variability in *Choriorhabditis dudichi* and in *Pelodera strongyloides* DF5013, with all patterns from zero division to four division rounds (Tables S7 and S8). Among the species in our analysis, we found variations from zero to nine divisions. There were at least 11 evolutionary changes, including one clear transition from no division to one division at the base of *Caenorhabditis*. No transverse divisions occur in these cells.

P3.p Divisions. We recorded whether P3.p divided or not. This feature is variable within a species. However, the fraction of animals in which divisions occur differs between species. Our analysis suggests that P3.p divided rarely in the rhabditid stem species. At least four changes to more frequent divisions occurred (Figure S11).

Vulval Morphogenesis and Ring Formation

The formation of syncytial rings from P(5-7).p progeny is an invariant trait in all rhabditids. In the outgroup species *Panagrellus redivivus*, however, syncytial rings do not form [41].

Variation is found mostly in the number of rings formed by the B and C cell progeny (one or two), except in *Rhabditoides inermiformis*, *R. regina*, and *P. strongyloides*, where the innermost granddaughters of P4.p and P8.p seem to participate in ring formation, and in *R. inermis*, where, in contrast, the A cell seems to fuse with hyp7 and not form a ring. Kolotuev and Podbilewicz [17, 41] suggested a correlation between the orientation of the B and C cell divisions with the number of rings produced: longitudinal divisions lead to two rings; transverse, oblique, or no division lead to one ring. The A cell never forms more than one ring. Using this rule, we can derive predictions for ring numbers from the P(5-7).p division patterns of all species (Figure 5). Visualization of rings by antibody staining of adherens junctions confirmed this prediction in all 13 cases tested [41]. The distribution of the predicted ring numbers on the phylogeny suggests that the rhabditid stem species had eight rings. This number was reduced seven times to seven or six rings, with only one reversal from seven to eight rings within Eurhabditis.

The Adult Vulva

A posterior vulva evolved at least three times independently from a mid-body vulva with no reversal. In addition, a posterior vulva evolved repeatedly in diplogastrids (e.g., in *Diplogastrellus* and within *Butlerius* and *Diplogasteroides* [33]) and also in the outgroup, e.g., in the lineage to *Brevibucca*. The vulva opening

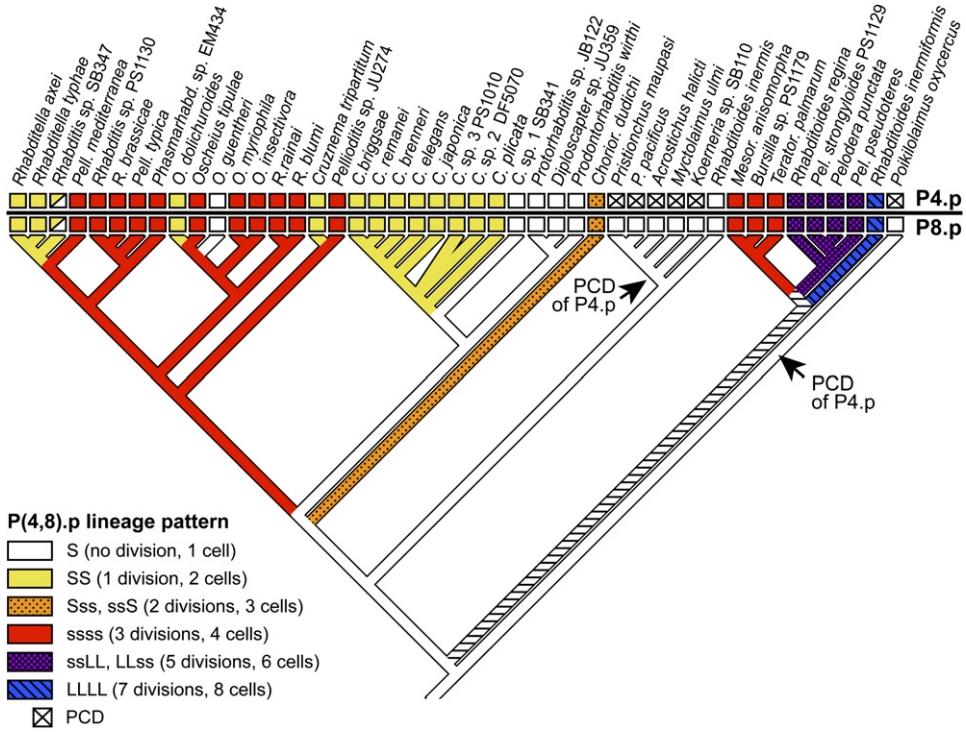

Figure 4. Stochastic Changes in the P4.p and P8.p Lineage Pattern

From undivided P(4,8).p in the stem species of rhabditids, many changes to multiple divisions and some reversals to fewer or no divisions occurred within this clade. We propose that the changes in this character are due to stochastic evolution because there is no clear trend. P4.p undergoes programmed cell death (PCD) in *Poikilolaimus oxycercus* and in diplogastrids. Hatched branches indicate that no unequivocal assignment of character states could be made.

morphology changed from a pore to a transverse slit in the stem species of Eurhabditis.

In summary, we found that of 41 characters for which there was enough information, 15 did not experience any change within rhabditids. Among the other 26 characters, 92 changes were inferred by parsimony. These results are summarized in Table 1.

Discussion

The vulva is an essential structure of the nematode body plan, and its overall adult morphology is conserved. However, in different species, the developmental processes leading to this structure show extensive variation, a signature of developmental system drift (DSD) [2]. There are two kinds of hypotheses about how DSD evolves. One hypothesis is that stochastic processes, e.g., genetic drift or mutation, can be sufficient to give rise to DSD [7, 10, 42, 43]. According to this null hypothesis, evolutionary changes between character states should be unbiased; for example, unordered patterns of changes, including reversals, would be expected. Alternatively, DSD could result from deterministic processes like selection or other constraints [1, 8]. In this case, we would observe biased changes such as unidirectional evolutionary trends in independent lineages. To discriminate between these hypotheses, we reconstructed the phylogeny for species representing the major groups of rhabditids and used it to trace the numbers and directions of changes in vulva development.

A Phylogeny for Rhabditids

For the first time, because of the use of several genetic loci and dense taxon representation, almost all of the relationships among the major taxa within rhabditids are resolved (Figure 2), thus providing the necessary foundation for analyzing character evolution. The phylogeny also offers an evolutionary context for the model organisms *C. elegans* and *P. pacificus*. Most importantly, we found that diplogastrids are not only part of rhabditids, but are (together with *Rhabditoides inermis*; see also [21]) the sister clade of Eurhabditis, placing them even more clearly within rhabditids. This somewhat surprising finding, although consistent with an early study [22], contradicts several recent phylogenies based on SSU rRNA sequences or morphological data [21, 23, 24, 44].

Evolutionary Stasis of Adult Vulva Morphology

Gross morphology of the adult vulva has remained constant in rhabditids, except for three clear changes. First, a change in the shape of the vulval opening from a round pore to a transverse slit occurred in the stem species of Eurhabditis. This character does not correlate with any of our vulva development characters. The change to a slit-like opening might instead be the consequence of a change in cell-cell contacts: in *C. elegans*, the vulE ring is attached to the lateral seam [45, 46]; such a connection might be absent or its symmetry different in species with a round vulva opening. Also, two independent changes to an anteriorly tilted vulva occurred in posterior-vulva species (*Cruznema* and the *Mesorhabditis*

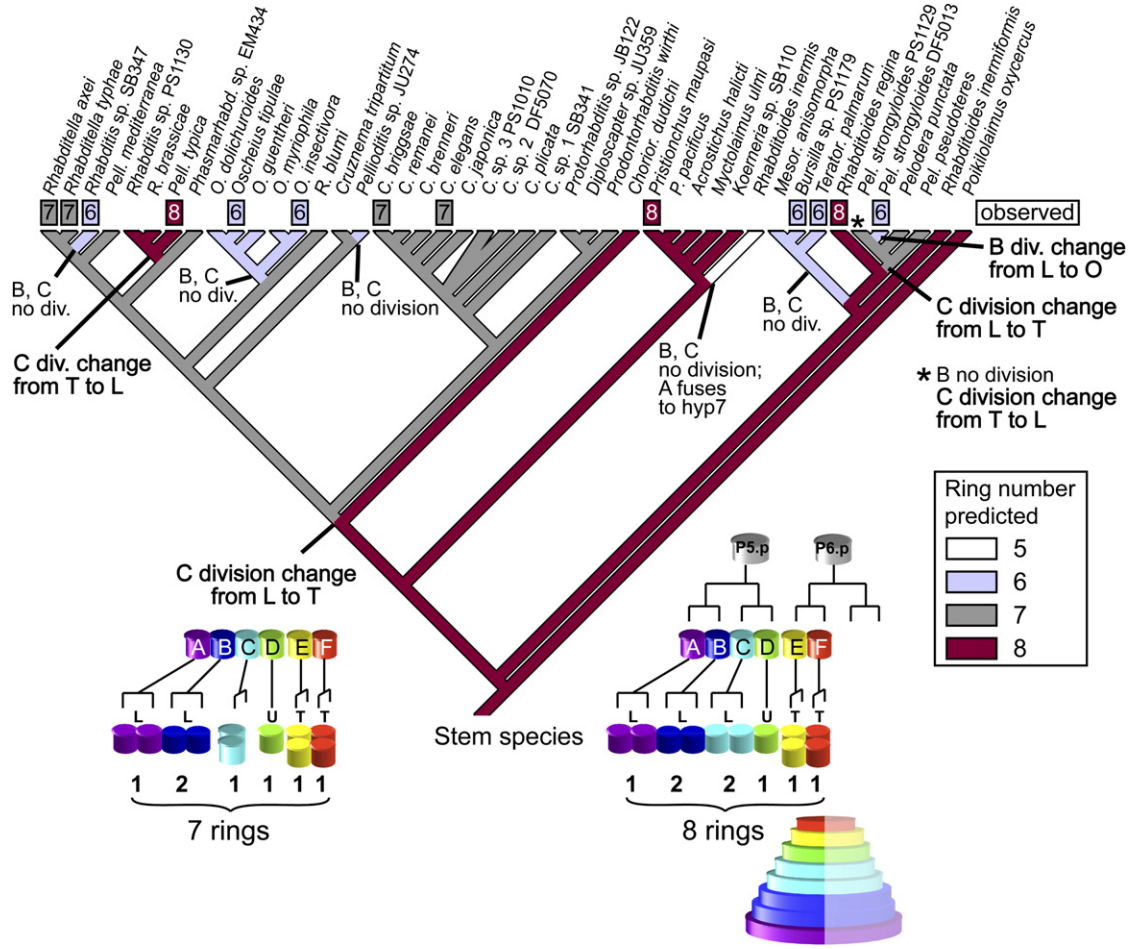

Figure 5. Evolutionary Changes in Vulval Ring Numbers as Predicted from Vulval Cell Lineage Patterns

A correlation between division pattern of the vulval granddaughter cells and ring number was previously proposed [17]: longitudinally dividing cells produce two rings, transversely and obliquely dividing cells produce one ring; the A cells nearly always produce a single vulA ring. Based on this rule, it is predicted that the rhabditid stem species had eight rings (B and C cells divided longitudinally). Changes to fewer rings occurred by loss of C and B divisions, or by changes in the orientation of the C division from longitudinal to transverse. There are two predicted gains of a ring through a reverse change in the orientation of the C division: within Eurhabditis and in *Pelodera strongyloides* PS1129. The predicted ring number was confirmed in 13 species (see also [41]).

group). In rhabditids, this character is also not correlated with any other developmental character aside from those associated with the posterior vulva position. That changes in these characters are rare is consistent with strong stabilizing selection on the adult vulva.

Vulva Development Shows Extensive DSD

As expected from previous studies (reviewed in [18]), the amount of variation in vulva development is substantial. Moreover, mapping this variation on the phylogeny reveals that there are even more changes than expected if the diversity in character states alone were considered. That is, if there were only one change per character state, 37 changes would account for all the different character states across 26 variable characters and 45 rhabditid species. However, we find more than 2.5 times as many (i.e., 92) changes. Thus, independent changes to the same character state or reversals to an ancestral character state happened frequently, and homoplasy is extensive. We analyzed the pattern of this homoplasy for the signature of stochastic evolution, i.e., for frequent changes to many different character states and frequent reversals.

Despite Extensive Change, Few Characters May Evolve Stochastically

One character that appears to evolve stochastically is the division pattern of the 3° cells P(4, 8).p where we find six different character states and 11 changes, of which at least two are reversals (Figure 4). Consistent with neutrality, these cells do not contribute to the vulva, but to an already large syncytium for which a small change in the number of nuclei may not matter much. However, even for this character, there may be a developmental bias in some lineages: there is no change in the P(4,8).p division in *Caenorhabditis*, whereas there are changes within *Oscheius*. Correspondingly, Delattre and Félix [40]) and Dichtel et al. ([47]) observed many 3°-lineage mutants and a large amount of variation in the 3° division pattern in *Oscheius*, but not in *Caenorhabditis*. This suggests that the mutability of this character changed within rhabditids. A second example for

Table 1. Vulva Development Characters and Their Evolutionary Changes within Rhabditids

Characters	No. of States	States	No. and Direction of Changes within Rhabditids
Number of Pn.p cells	1	12	no change
PCD of Pn.p cells	2	no yes	2 changes: 2x no PCD → PCD (including outgroup: 5 changes: 4x no PCD → PCD; 1x PCD → no PCD)
Number of midbody Pn.p cells[a]	2	6 cells 4 cells	2 changes: 2x 6 cells to 4 cells; no reversal
P3.p competence	2	not competent competent	5 changes: 1x non-comp. → comp.; 4x comp. → non-comp.
Size of competence group[a]	4	3–6 cells	6 changes: 5x size reduction; 1x size enlargement
Migration of midbody Pn.p cells	2	no yes	3 changes: 3x no migr. → migr. (including outgroup: 5 x no migr. → migr.); no reversal
Z1/Z4 division time	2	in L2 stage in L1 stage	5 changes: 4x L2 → L1; 1x L1 → L2
Number of ovaries	2	two one	6 changes:[b] 6x two → one; no reversal
Spatial pattern of vulval cell fates	1	2° 1° 2°	no change
Vulva induction by	3	several cells of somatic gonad AC gonad independent	6 changes: 2x several cells → AC; 2x several cells → indep.[b]; 2x AC → several cells
Number of induction steps	2	more than one step one step	1 change: 1x more steps → one step
P6.p lineage	3	TTTT TUUT UTTU	3 changes: 1x TTTT → TUUT; 2x TTTT → UTTU
A cell divisions	2	L (longitudinal) U (undivided)	4 changes: 4x L → U; no reversal
B cell divisions	3	L U O (oblique)	7 changes: 6x L → U; no reversal; 1x L → O
C cell divisions	3	L U T (transverse)	9 changes: 5x L → U; no reversal; 2x L → T; 2x T → L
D cell divisions	1	U	no change
P(4, 8).p divisions	6	S, SS, Sss, ssss, ssLL, LLLL	11 changes: many changes to more and fewer divisions
P3.p divisions	2	in < 20% of animals in > 20% of animals	7 changes: 5 (6)x < 20% → > 20%; (1) 2x > 20% → < 20%
vulA ring number (predicted)[a]	2	one none	1 change: 1x one ring → no ring
vulB ring number (predicted)[a]	2	two one	7 changes: 7x two rings → one ring; no reversal
vulC ring number (predicted)[a]	2	two one	6 changes: 4x two ring → one ring; 2x one ring → two rings
vulD ring number (predicted)[a]	1	one	no change
vulE ring number (predicted)[a]	1	one	no change
vulF ring number (predicted)[a]	1	one	no change
Total ring number (predicted)[a]	4	five six seven eight	9 changes: 2x eight → seven rings; 1x seven → eight rings; 1x eight → six rings; 4x seven → six rings; 1x eight → five rings
Vulva position[a]	2	midbody posterior	3 changes: 3x midbody → posterior; more changes in outgroup
Vulva opening	2	pore slit	1 change: 1x pore → slit

PCD, programmed cell death; L, longitudinal division; T, transverse division; U, no division; S, fusion with hypoderm; ss, fusion with hypoderm after division.
[a] Dependent on other characters in this matrix.
[b] Considering *Diplogastrellus gracilis*, which was not part of the original study.

changed evolvability might be found in P8.p of diplogastrids. This cell never divides, probably in connection with a reduced competence or a unique role in vulva induction [48]. Changes in the lineage pattern of P3.p may also be neutral. Consistent with this assumption, intraspecific variation in this character is common (see also [40]).

The source of the inductive signal for vulval development is another character where multiple changes including reversals occurred (Figure 3). Ancestrally, this signal was produced by several cells of the somatic gonad. Twice within rhabditids, the source of the signal became restricted to the anchor cell (AC). However, two

Table 2. Characters Proposed to Be under Stochastic or Biased Evolution

		Biased	
Developmental Level	Stochastic/Unbiased	Evolutionary Trend	No Change
Pn.p cell patterning		posterior migration of midbody Pn.p cells	midbody Pn.p cells form vulva
Fate determination	source of inductive signal	reduction of size of competence group PCD induction gonad independent in posterior-vulva species	vulva is made from P(5-7).p, centered on P6.p
Vulva cell divisions	P3.p divisions? P(4,8).p divisions	reduction in division number of B and C cells	no division of D cell
Vulva morphogenesis			only one vulA, vulD, vulE, vulF ring
Adult morphology			(only one change in shape of vulva opening)
Gonad development		time Z1 and Z4 divide loss of posterior ovary	

reversals occurred. These changes seem to be heterochronic (see Figure 3).

There Is an Extensive Evolutionary Bias in Most Vulva Development Characters

For the remaining vulva developmental characters, we find evidence for two types of bias: either there are multiple changes to the same character state in independent lineages, or there are changes to different states, but reversals are absent.

Our analysis indicates that the size of the competence group was reduced several times convergently, even though assessment of the state in the rhabditid stem species is problematic (see Supplemental Data). If the competence group in the stem species included six cells (P3.p-P8.p), its size was reduced six times independently. If the ancestral competence group did not include P3.p, there were five reductions in its size and one enlargement. Thus, in either scenario, there is a bias toward a reduction of the competence group (Figure S5).

Removal of non-vulval cells by programmed cell death (PCD) evolved independently in diplogastrids and *Poikilolaimus oxycercus* within rhabditids and again twice within the outgroup. Our new phylogenetic analysis of the outgroup (see Supplemental Data) suggests that PCD was only reversed once within Cephalobina (Figure S4). Thus, there is a bias toward the evolution of PCD. Permanent removal of cells that might differentiate ectopically could be favored by selection.

In all granddaughters of the vulval cells, except for D, 15 changes from division of the cell to no division occurred within rhabditids, but there is no evidence for a reversal to division of an undividing vulva cell. Thus, there is an evolutionary trend toward reducing these divisions.

Changes in the orientation of cell divisions correlate with changes in ring number [41]. Specifically, except for the A cell, we observe that a longitudinal division leads to the formation of two rings; a transverse division or the absence of a division leads to only one ring. Using this rule, we can make predictions about the evolutionary changes in ring numbers (Figure 5). No species has more than one vulD, vulE, and vulF ring, because no longitudinal divisions occur in these cells. The stem species of rhabditids had eight rings. Ring number was then reduced by one, two, or perhaps three rings in eight separate lineages. Thus, there is a strong bias toward the evolution of fewer rings. However, there is one reversal from seven to eight rings due to the change in the orientation of the C cell division from transverse to longitudinal. Thus, it is possible for the ring number to increase. The evolutionary bias toward fewer rings is perhaps due to the irreversible loss of divisions in the vulval cells and not to a selective advantage of a vulva consisting of fewer rings.

The developmental time at which the precursors of the somatic gonad Z1 and Z4 first divide changed four times from the L2 to the L1 stage. The reverse heterochronic change from an early division to a later division occurred only once within the dimorphic *Rhabditis* sp. SB347, where Z1 and Z4 divide in L1 in the female morph, but in L2 in the hermaphroditic morph.

In our group of species, five losses of the posterior ovary occurred (six including *Diplogastrellus gracilis)*, but the ovary was never regained. There is also no loss of the anterior ovary.

There are several changes from a vulva in the middle of the animal to a vulva in the posterior body region, which results from posteriad migration of the midbody Pn.p cells. In rhabditids, an anterior vulva is not observed (but is known from many "adenophorean" nematodes [49]). There is a correlation between the presence of a posterior vulva and the absence of the posterior ovary. There is no rhabditid species with a posterior vulva and two ovaries. However, *Oscheius guentheri* has a central vulva, and its posterior gonad arm is often vestigial [50]. This suggests that monodelphy channels the evolution of a posterior vulva. A posterior vulva in the *Mesorhabditis* group and in *Diplogastrellus gracilis* [18] concurs with gonad-independent vulva induction. The same is true for *Brevibucca* in the outgroup [16]. This suggests that a posterior vulva channels the evolution of gonad-independent vulva induction or vice versa. In species in which cells of the somatic gonad induce vulva development, these signaling cells must be positioned close to the competent cells to induce the proper vulval fates. Migration of the midbody Pn.p cells away from the gonad primordium disrupts this spatial arrangement.

In summary, we found two or three characters that might evolve stochastically and 12 characters for which

an evolutionary bias was observed (Table 2). We thus conclude that much or most of the variation that has been described as developmental system drift is determined predominantly by biased evolution. For binary characters, we explored the use of statistical tests. However, we found these tests to be unreliable for a number of reasons (see Supplemental Data).

Processes Underlying a Biased Evolutionary Pattern

Biases can have two causes: (1) selection and (2) selection-independent constraints that might result from the relationship between genotype and phenotype (often called "developmental constraints," "developmental bias" [51], "genetic/epigenetic constraints" [52], or "reproductive constraints" [53]) or from a bias in the introduction of variation [54]. The conceptual as well as epistemological distinction between selection and selection-independent constraints remains difficult (see e.g., [51–53, 55–57]). An operational definition of selection-independent constraints is: "a factor that reduces the amount of variation on which selection can act." This kind of constraint might be shown most clearly through experimental and population genetic approaches, e.g., by measuring the amount of variation obtained by random mutagenesis and mutation-accumulation experiments and by surveying variation in natural populations [58]. Because of their genetic capabilities, nematodes are ideal for these kinds of studies [58].

Here, we derived evidence for a bias from comparative data. This approach does not allow a similarly stringent distinction between bias by selection and by selection-independent causes. However, it paves the way for follow-up investigations using other methods, like genetic and population genetic approaches. One test for bias by positive selection is to study the mechanisms that underlie convergent changes. If convergent changes to the same character state occurred in two lineages but by different mechanisms, a bias by selection is indicated. In our dataset, one character may satisfy this condition: the reduction of the size of the competence group in rhabditids occurred by at least two different mechanisms, early fusion with hyp7 (e.g., P3.p in *C. briggsae*) and programmed cell death (in diplogastrids and *Poikilolaimus oxycercus*). A bias can also be caused by purifying selection. In this case, no changes occur, even though enough evolutionary time elapsed. For rhabditids, this condition is met, because genetic divergence among these species is exceptionally large [59]. Examples of vulva development characters likely to be under purifying selection include the following. (1) The vulva is always made from Pn.p cells in the middle of the animal. If the adult vulva is posterior, the midbody Pn.p cells must migrate posteriad. Which Pn.p cells can form the vulva is probably constrained by the expression domain of the Hox gene *lin-39* [14]. Formation of a vulva from posterior Pn.p cells would require a homeotic transformation likely to have deleterious pleiotropic effects. (2) There is no evidence for a change in the number of vulD, vulE, and vulF rings. The number of these rings might be under purifying selection because the vulva muscles, required for egg-laying, are attached between them (vm1 between vulC and vulD rings, vm2 between vulF ring and uterus [46]).

In conclusion, our study shows that evolution of vulva development is strongly biased and only few aspects are likely to change in an unconstrained stochastic fashion. Our phylogeny for rhabditid species, including the model systems *C. elegans* and *P. pacificus*, provides a foundation for evolutionary analyses of other characters as well. If the patterns that we observed in the vulva system are found more generally, then most of developmental system drift is driven by deterministic and not stochastic processes.

Supplemental Data

The Supplemental Data for this article (14 figures, 16 tables, Results and Discussion, and Experimental Procedures) can be found online at http://www.current-biology.com/cgi/content/full/17/22/1925/DC1/.

Acknowledgments

We are very grateful to those who provided us with samples and strains, especially W. Sudhaus and the *Caenorhabditis* Genetics Center. We also thank M.-L. Dichtel for *O. myriophila* AC ablations; L. Bonnaud for some *Caenorhabditis* Pn.p ablations; and M. Zheng for AC ablations in *Pelodera strongyloides* DF5013. We thank four anonymous reviewers for valuable comments. This work was supported by a collaborative HFSP grant (RGP0016/2001-M) and NSF grant 0228692 to D.H.A.F.

Received: July 27, 2007
Revised: October 19, 2007
Accepted: October 22, 2007
Published online: November 15, 2007

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