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Evolution of ''Rhabditidae'' and the Male Tail

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Abstract: Evolution of diverse male tail epidermal features of representative species in the family Rhabditidae (Nematoda:Rhabditida) was mapped by parsimony on a molecular phylogeny inferred with nearly complete DNA sequences of small subunit ribosomal RNA genes. Although the molecular phylogeny is consistent with some previously proposed relationships, there are also some major differences, suggesting a revision of rhabditid taxonomy is required. To reconstruct male tail evolution, character states and homologies were determined with the aid of developmental profiling at the level of single cells. Because the model genetic system *Caenorhabditis elegans* is a member of Rhabditidae and allows the genetic and developmental mechanisms of morphogenesis to be elucidated, candidate genes and pathways can be proposed for several of the reconstructed evolutionary changes in male tail morphology.

Key words: bursa, *Caenorhabditis elegans,* development, homology, male tail, molecular systematics, morphology nematode, phylogeny, rDNA, Rhabditidae.

As a model to identify mechanisms in the evolution of morphological diversity, we are studying the male tail (copulatory bursa) of nematodes in the family Rhabditidae. Our first steps toward this goal, briefly reviewed here, have been to: (i) elucidate phylogenetic relationships in this family using molecular characters (small subunit RNA genes, SSU rDNA), (ii) identify male tail character homologies and states using both developmental and phylogenetic methods, and (iii) trace the evolution of these homologous characters using the molecular phylogeny.

''Rhabditidae'' is a variably defined taxon that has classically encompassed a variety of mostly free-living bacteriophagic nematodes, split into many subfamilies and genera by some systematists (e.g., Andrássy, 1983) or lumped into a subfamily (Rhabditinae) with few genera by others (e.g., Sudhaus, 1976). With a few exceptions (e.g., Sudhaus, 1993), systematists generally have not included parasitic taxa in considering the composition of ''Rhabditidae.'' In this review, I consider ''Rhabditidae'' a paraphyletic taxon (thus the quotation marks) that includes all the groups considered under Rhabditinae by Sudhaus (1976) but including *Diploscapter*. Because the objective of this review is only to demonstrate how our molecular systematic results can be used to develop hypotheses for the evolution of male tail characters, and because a complete phylogenetic analysis will be presented elsewhere (Fitch et al., unpub.), a full comparison between our systematic results and those of previous authors is not attempted (cf. Sudhaus and Fitch, in press). However, major differences between our phylogeny and the previous systems are pointed out, and it is clear that there is a need for dramatic revision of rhabditid taxonomy.

However the taxon ''Rhabditidae'' is defined, these species and their male tails provide excellent models to uncover mechanisms of morphological evolution. Many diverse forms have derived from paraphyletic ''Rhabditidae,'' including some parasites. ''Rhabditidae'' also includes *Caenorhabditis elegans,* an important developmental genetic model that allows us to identify mechanisms governing morphogenesis. The male tail has several experimental advantages. It is a postembryonic structure, and genetic manipulation in *C. elegans* is usually straightforward (for review, see Emmons and Sternberg, 1997). Because the male tail is sexually dimorphic and *C. elegans* hermaphrodites are self-fertile, mutations can be propagated through homozygotes even if such muta-

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tions affect male fertility or viability. Because of the variety of male tail forms in ''Rhabditidae'' (and in other taxonomic groups derived from within ''Rhabditidae''), this system provides an excellent model to study the evolution of morphological diversity.

Since male tail characters also have been used for their informativeness in a taxonomic and systematic context (e.g., Sudhaus, 1976; Andrássy, 1983), understanding the evolution of this feature is important for determining which character states are uniquely apomorphic (derived) for particular clades (monophyletic taxa). Eventually identifying the genetic changes underlying these evolutionary changes in morphology will also help resolve long-standing controversies regarding the saltational or gradual nature of such changes (e.g., Gould and Eldredge, 1977; Barton and Turelli, 1989; Orr and Coyne, 1992).

Materials and Methods

Strains: All strains of rhabditid nematodes we have used have been assigned unique strain identifers according to a convention originally adopted by *C. elegans* researchers (Horvitz et al., 1979) and suggested for general use by Bird and Riddle (1994). We have compiled a database (Worm Systematics Resource Network, WSRN) for all such strains, available on the World Wide Web (http://www.nyu.edu/projects/fitch/WSRN/).

DNA sequencing: Dye-primer sequencing (PE Biosystems, Foster City, CA) was performed using primers described previously (Fitch et al., 1995). Small-subunit ribosomal DNA (SSU rDNA) templates for sequencing were amplified by the polymerase chain reaction (PCR) directly from nematode lysates as described (Williams et al., 1992), purified by electroelution. Nearly complete sequences of SSU rDNA were assembled from multiple sequencing runs (on an ABI 377 sequencer) from both strands using Sequencher 3.1.1 (GeneCodes Corporation, Ann Arbor, MI). Sequences deposited in GenBank have accession numbers AF082994-AF083028.

Immunofluorescence: To visualize apical cell boundaries, MH27 antibody (kindly provided by R. Waterston, Washington University, St. Louis, Mo.) was used as the primary antibody to mark adherens junctions with rhodamine-conjugated goat anti-mouse secondary antibody after fixing and permeabilizing nematodes as previously described (Fitch and Emmons, 1995).

Phylogenetic analysis: A taxon list and sequence alignment (based on secondary structure predictions by R. De Wachter, University of Antwerp) are available at: http://www.nyu.edu/projects/fitch/fresources.html. Details of the alignments and phylogenetic analyses will be published elsewhere (Fitch et al., unpub.). Likelihood and weighted-parsimony approaches were used in analyses similar to those previously described (Fitch et al., 1995; Sudhaus and Fitch, in press) using PAUP* 4.0b2a (Swofford, 1999). Analyses were performed with all taxa together (requiring heuristic tree searches) and with groups of 8–12 taxa (allowing exhaustive searches). Different evolutionary, character- or transformation-weighting models were compared with respect to phylogenetic results. For maximum likelihood analyses, parameters for the model were estimated by likelihood-ratio tests of different models (ModelTest 1.0; Posada and Crandall, 1998). Robustness of relationships was tested with jackknife (50% deletion, 2,000 replications), bootstrap resampling (2,000 replications), decay indices (Bremer, 1988), and tree comparison tests (Kishino and Hasegawa, 1989). Only significantly resolved branches are shown (i.e., $\geq$90% of jackknife and bootstrap replicates or significant resolution in tree comparisons). The phylogeny of ''Rhabditidae'' (ingroup) was rooted with SSU rDNA from particular species (outgroup representatives) belonging to Cephalobina, Ascaridida, and Plectida.

Plotting evolutionary changes: Male tail character states were mapped onto the molecular phylogeny consensus, and maximum likelihood trees and ancestral changes were inferred according to the (set of) most parsimonious solution(s) as implemented in

MacClade 3.05 (Maddison and Maddison, 1992; Fitch, 1997). Because the concentrated changes test (Maddison, 1990) requires a fully resolved tree, the maximum likelihood tree (see http://www.nyu.edu/projects/fitch/fresearch/fsystemat/) was used for estimating correlations.

Results and Discussion

Molecular phylogeny: In all the ''Rhabditidae'' we have analyzed so far, small-subunit ribosomal genes (SSU rDNA) are arranged in tandemly repeated units and undergo concerted evolution, but not all copies are identical. By directly sequencing PCR products with dye-primers, we have been able to detect substitution and insertion-deletion polymorphisms within the same strain and within the same individual. However, these polymorphisms are rare (0.03% of all bases) and the polymorphic condition of a base character is never shared between different species (although some polymorphisms are identical between different strains of the same species). Thus, the entire SSU rDNA array can be treated as a single evolutionary unit and SSU rDNA paralogy is not a problem in ''Rhabditidae.''

Phylogenetic analysis of the SSU rDNA allows several relationships to be significantly resolved (Fig. 1). Additional molecular and morphological data partitions are currently being mined for phylogenetic information that will hopefully allow significant resolution of additional relationships. For example, when male tail characters were combined with SSU rDNA characters, better resolution was obtained than for either data set alone (Fitch, 1997).

Our phylogenetic results are similar to the systems of Sudhaus (1976) and Andrássy (1983) in some ways but differ in other interesting respects (see Sudhaus and Fitch, in press, for a more complete comparison). For example, as Sudhaus (1993) proposed, insect parasites belonging to family Heterorhabditidae are derived from within ''Rhabditidae'' and are closely related to a monophyletic *''Eurhabditis''* species group with a composition very similar to that proposed by Sudhaus (1976) but excluding some species of his originally proposed

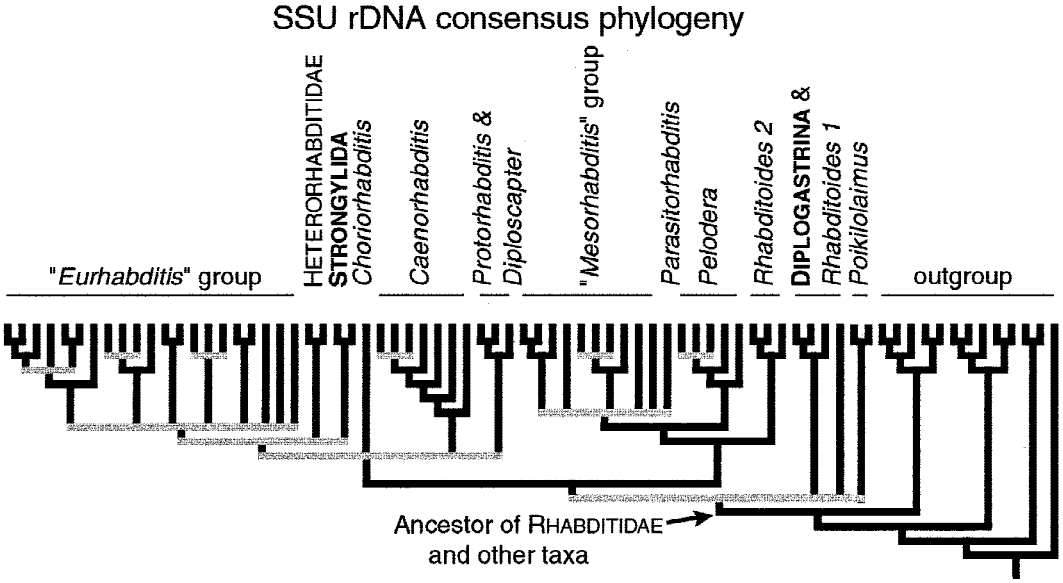

Fig. 1. Phylogenetic relationships among species groups of family ''Rhabditidae'' and with other taxa as inferred from SSU rDNA sequences. Only significantly resolved relationships are shown (black lines); polytomous branching (gray lines) represents uncertainty in the branching order. ''*Eurhabditis*'' includes species of subgenera *Cephaloboides, Oscheius, Pellioditis, Rhabditella,* and *Rhabditis* (Sudhaus, 1976, emended). *Caenorhabditis* relationships are identical to those proposed by Sudhaus and Kiontke (1996). ''*Rhabditoides* 2'' includes *Rhabditoides regina* and *Rhabditoides stammeri,* ''*Rhabditoides* 1'' includes *Rhabditoides inermis* and *Rhabditoides inermiformis.*

Cephaloboides group, which were later placed into subgenus *Poikilolaimus* (Sudhaus, 1980). Similar to the Mesorhabditinae group proposed by Andrássy (1983), a significantly supported monophyletic group (the ''*Mesorhabditis* group,'' Fig. 1) includes species of *Teratorhabditis, Mesorhabditis, Crustorhabditis,* and *Bursilla,* the *Monhystera* group of Sudhaus (1976). In a previous SSU rDNA phylogeny with fewer ''Rhabditidae'' represented (Blaxter et al., 1998), *Teratorhabditis* was less closely related to other ''Rhabditidae'' than were diplogasterids, although this was not statistically significant. Including more taxa presumably allows the significant resolution of *Teratorhabditis* to the ''*Mesorhabditis*'' group. Although Sudhaus (1976) believed that *Pelodera* was a sister taxon of *Teratorhabditis,* our phylogeny suggests that *Teratorhabditis* is most closely related to other members of the ''*Mesorhabditis*'' group and that *Pelodera* diverged earlier (Fig. 1). *Pelodera* does not share an immediate common ancestor with *Caenorhabditis* or other peloderan species (i.e., species without pointed tail tips extending posterior of the bursa velum or ''fan'' of the male tail) of the ''*Eurhabditis*'' group; Andrássy's (1983) subfamily Peloderinae is therefore polyphyletic. Consistent with recent molecular and morphological results (Sudhaus, 1993; Fitch and Thomas, 1997; Blaxter et al., 1998), parasites of order Strongylida and family Heterorhabditidae are actually derived from within ''Rhabditidae'' and are closely related to the ''*Eurhabditis*'' group (Fig. 1).

Some of our phylogenetic results have not been predicted by any prior systematic work. For example, *Protorhabditis* and *Diploscapter* species are closely related to each other (*Diploscapter* may be derived from within *Protorhabditis*) and are closely related to the *Caenorhabditis* and ''*Eurhabditis*'' groups (Fig. 1). Sudhaus (1976) did not consider *Diploscapter,* and Andrássy (1983) put the taxon into a separate family altogether. Both authors thought that *Protorhabditis* and *Parasitorhabditis* groups diverged early from the rest of the ''Rhabditidae.'' Our analysis suggests instead that *Protorhabditis* and *Parasitorhabditis* derived recently from different lineages (see Fig. 1). Although some authors (e.g., Maggenti, 1981) have placed diplogastrids as far away from rhabditids as a separate subclass, this monophyletic group is probably derived from within ''Rhabditidae'' and may be closely related to one or more species of *Rhabditoides* (i.e., of the ''*Rhabditoides* 1'' species group of Fig. 1). Finally, species of *Poikilolaimus* (*sensu* Sudhaus, 1980; *nec* Andrássy, 1983) may be the most anciently diverged of ''Rhabditidae,'' contrary to all other systems suggested.

Differences between our phylogeny and the systems of Andrássy (1983) and Sudhaus (1976) may arise from mistaken *a priori* assumptions about primitive character states and character homologies. For example, Sudhaus (1976) believed that the lack of a glottoid apparatus in *Protorhabditis* and *Parasitorhabditis* indicated a primitive state, and Andrássy (1983) kept these taxa in the same subfamily (Protorhabditinae) largely because they share the lack of this feature. However, the position of these lineages in our phylogeny indicates that a glottoid apparatus was independently lost at least twice. As another example, it is not possible to deduce homologies among the ray sensilla (genital papillae) in the male tail merely by looking at ray patterns in adults, because adult ray positions and numbers vary markedly. Instead, the assignment of ray homologies must be aided by information about common ancestral and developmental origins (discussed below).

Developmental analyses: Using MH27 (an antibody that allows the visualization of apical cell boundaries because it recognizes an epitope in nematode belt adherens junctions), we previously showed that the cells producing the rays originate in the same relative positions in all ''Rhabditidae'' analyzed so far (Fitch and Emmons, 1995; Fitch, unpub.). Because this pattern of relative cell origins (Fig. 2A) is identical (and thus ancestral, symplesiomorphic) for all of these species, homologies among the ray primordia (and associated hypodermal cells) can be identified at this developmental stage (Fitch and Emmons, 1995; Fitch, 1997). Differences in the ray patterns of adults of dif-

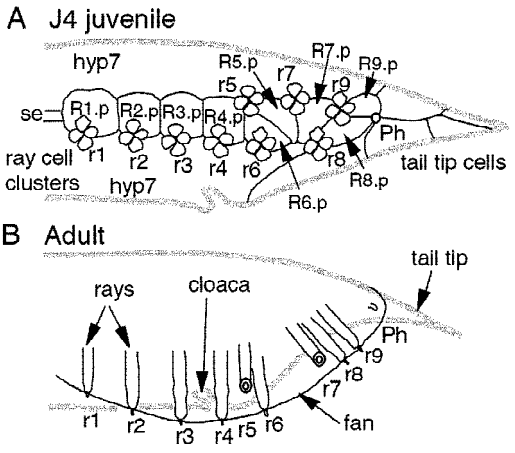

FIG. 2. Archetype for the external features of the rhabditid male tail, view of the left lateral surface. Thick gray lines represent the body outline; thin black lines represent apical cell boundaries, as visualized by MH27 immunofluorescent labeling. Ph = phasmid, se = seam, hyp7 = large hypodermal syncytium, r*n* = ray primordial cell clusters (A) or rays (B), R*n*.p = hypodermal cells related by cell lineage to each of the r*n* cell clusters. A) Schematic of relative cell positions just after the ray cells originate in the lateral hypodermis in the early J4 juvenile. One cell of each 4-cell cluster undergoes a programmed cell death, and the other cells differentiate into a ray. The phasmid has already been fixed in position by the end of the J1 stage, although it may be in a different position from that shown. Depending on the species, there may be 4 cells in the tail tip (as shown here) or 3 cells. B) Schematic of an adult tail with a hypothetical ''default'' pattern of rays predicted if cells did not change their relative positions during J4 morphogenesis (ray numbers are thus in the same anteroposterior order as were the ray primordia in the juvenile). There is no species in which some ray cells have not moved and the archetype is *not* representative of any ancestor (Fitch, 1997). All species share with the archetype the positioning of ray homologs r5 and r7, which open on the dorsal surface of the fan (Fitch and Emmons, 1995; Fitch, 1997). If the tail tip cells do not retract during J4 morphogenesis, a leptoderan tail results, as shown in the archetype (peloderan tails result from tail tip retraction; Nguyen et al., 1999).

ferent species result from morphogenetic differences (i.e., in cell migrations or cell sorting) that occur only after the ray cells are born (Fitch and Emmons, 1995; Fitch, 1997). Tracing the changes in the positions of these cells during development allows one to follow the positional fate of particular ray homologs into the adult (Fig. 3). In a hypothetical construction called an ''archetype'' (Fitch, 1997), a default adult ray pattern can be predicted if ray cells do not migrate from their points of origin (Fig. 2B). This archetype can be used as a basis for comparing ray development in different species (Fitch and Emmons, 1995) and setting character states for rays (Fitch, 1997). By this analysis, it is clear that ray homologies cannot be inferred merely from their anteroposterior order in the adult tail (Fig. 3). (Appendix 4 of Fitch, 1997, provides more details about the significance of homology errors in previous systematic studies using male tail characters.)

Other external features of the male tail that we have analyzed include the phasmids and the tail tip (Fitch and Emmons, 1995; Nguyen et al., 1999). Several descriptions of rhabditid species (especially those described with ''10 rays'') have mistaken the phasmid for a ray. As pointed out for species of *Teratorhabditis* and *Pelodera* in Figure 3, the 7th papilla (counting from the anterior) is not a ray, but the phasmid. Although superficially similar to the rays, MH27 staining and dye-filling can be used to differentiate phasmids from the rays (Fitch and Emmons, 1995). Scanning electron microscopy (SEM) also reveals external differences between phasmids and rays and has been used in an extensive survey of phasmid position relative to rays in male nematodes (Kiontke and Sudhaus, in press).

With regard to the tail tip, male tails have been described as ''peloderan'' or ''leptoderan'' (i.e., the male tail tip is blunt and does not protrude beyond the fan, or is pointy and can protrude ventral and/or posterior of the fan, respectively). Peloderan tail tips result from a change in shape and an anteriad ''retraction'' of the tail tip cells during J4 male tail morphogenesis, whereas leptoderan tail tips results from a failure of complete tail tip retraction (Nguyen et al., 1999). There does not appear to be any cellular basis for distinguishing between tails distinguished as ''leptoderan'' and ''pseudopeloderan'' by Andrássy (1983). MH27 immunofluorescent labeling of adherens junctions reveals that other aspects of tail tip development vary in different rhabditids. For example, the tail tip cells in *C. elegans* begin to fuse at about the same time retrac-

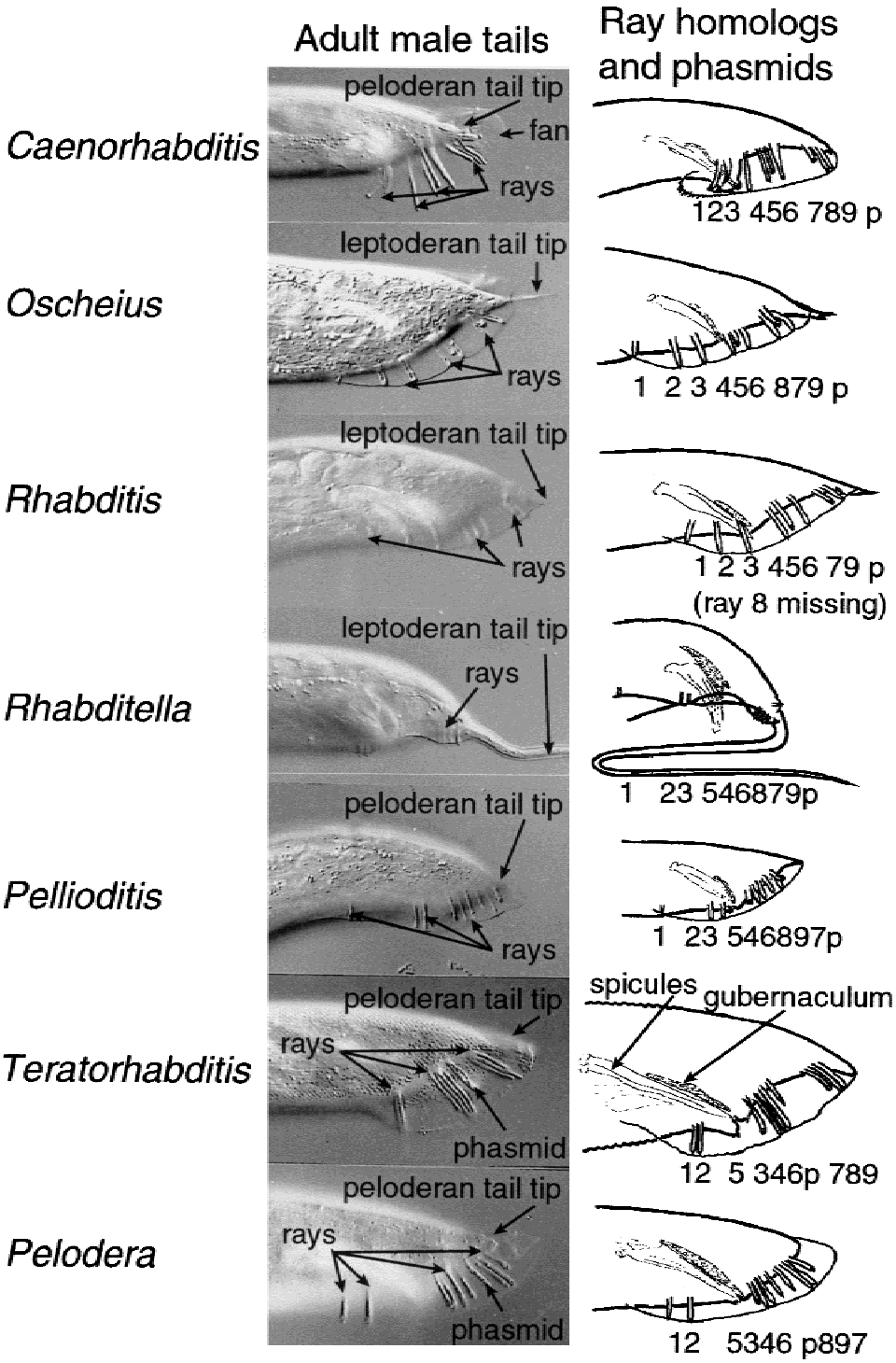

FIG. 3. Left lateral views of male tails from representatives of different species groups (Nomarski micrographs on the left, schematics on the right). Numbers beneath each schematic represent the ray homologs in their anteroposterior order; p = phasmid. There is not a direct correspondence between ray homologies and their ordinal arrangement along the anteroposterior axis; the phasmid can also be extended like a ray and lie between rays in the fan. Species shown are (top to bottom): *C. elegans, Oscheius myriophila, Rhabditis blumi, Rhabditella axei, Pellioditis typica, Teratorhabditis palmarum, Pelodera strongyloides.*

tion occurs to form the peloderan tail (Nguyen et al., 1999), but in other peloderan species such as *Pellioditis typica* or *Pelodera strongyloides,* the tail tip cells do not fuse at any time during or after their retraction (Fig. 4). Leptoderan tail tips also can vary with regard to cell fusions (Nguyen et al., 1999; Fig. 4). The number of cells used to construct the tail tip may also vary; e.g., whereas peloderan and leptoderan species of the ''*Eurhabditis*'' species group have four tail tip cells, *Teratorhabditis* and *Pelodera* species appear to only have three (Fig. 4).

Evolution of male tail characters: Plotting male tail character states on the phylogeny allows inferences about the polarities of evolutionary morphological and developmental changes (Fitch, 1997). Many of these evolutionary transformations map unequivocally to single, well-supported branches, and we can therefore reconstruct what the ancestral male tails looked like for many characters. For example, the most parsimonious reconstruction of tail tip evolution for the species represented in Figure 4 is a single change from peloderan to leptoderan. Evolution of

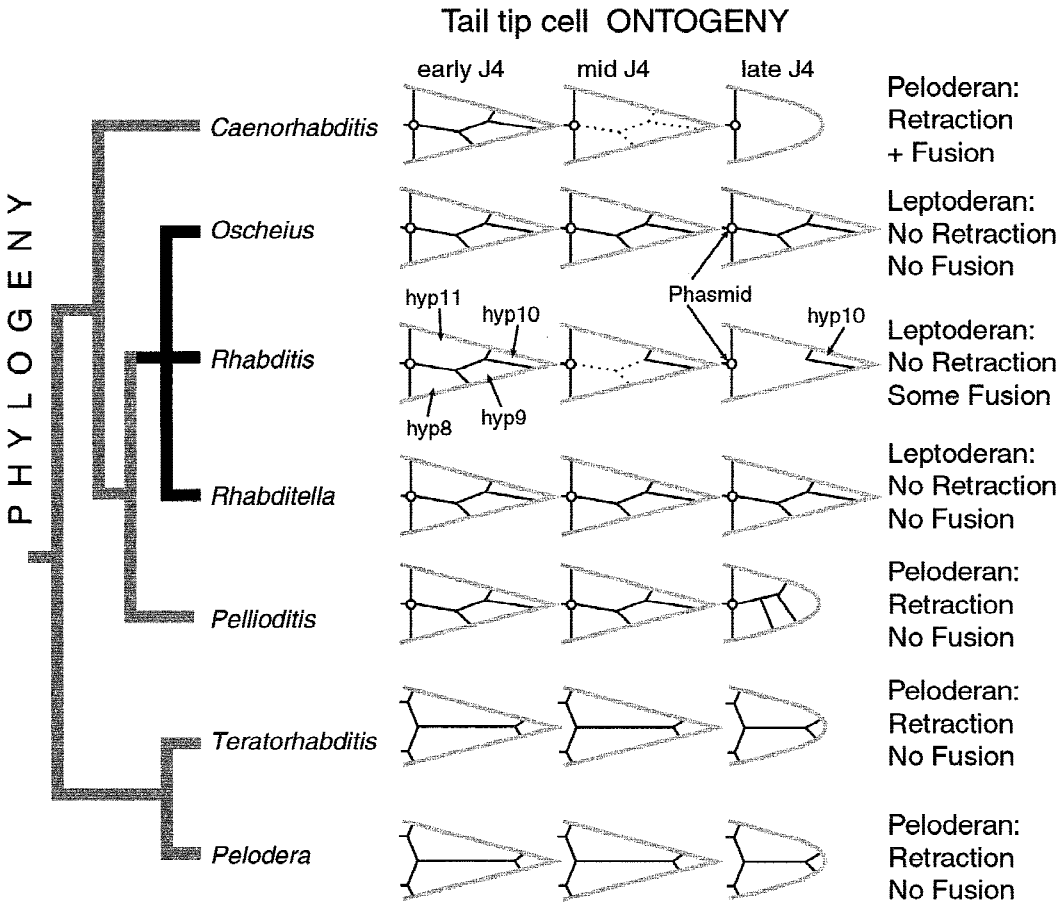

FIG. 4. Male tail tip evolution shown for a few representatives of ''Rhabditidae'' (taxa as in Fig. 3). States mapped on a portion of the molecular phylogeny are ''peloderan'' (gray lineages) and ''leptoderan'' (black lineages). For each taxon, three stages in tail tip ontogeny (during J4 development) are schematized as left lateral views. Cell boundaries (as visualized by MH27 immunofluorescent staining) are depicted as thin black lines; outlines of the tail tips are depicted as gray lines. Outlines of cells undergoing fusion during the mid-J4 stage are depicted by dashed lines. Cells homologous to the hyp8, hyp9, hyp10, and hyp11 tail tip hypodermal cells of *C. elegans* are pointed out for some taxa; homologies of individual cells in taxa with only 3-celled tail tips are unknown. Rounded tail tips represent cells changing their shape and retracting to produce peloderan tails; pointed tail tips represent unretracted cells that will result in leptoderan tails.

the changes in cell states is obviously more complex, but a single ancestral lineage can be identified in which tail tip retraction failed (Fig. 4).

Tracing the evolution of male tail characters also reveals which character states are uniquely apomorphic for particular clades. Some characters that may not have been previously recognized as such may have diagnostic importance for certain clades, whereas others previously deemed important may be homoplasious and misleading. For example, plotting phasmid position (as determined by SEM) on our molecular phylogeny has revealed that phasmids changed position in the male tail very few times during the evolution of ''Rhabditidae'' (Kiontke and Sudhaus, in press), suggesting this morphological character could be used to support major groups within ''Rhabditidae.'' On the other hand, leptoderan and peloderan tail tips have arisen multiple times throughout rhabditid evolution by apparently different mechanisms (e.g., Fig. 4; additional examples not shown). Not only are leptoderan-peloderan differences not good characters for distinguishing monophyletic groups of rhabditids, but this dichotomous classification of tail tip character states ignores possible underlying differences in cellular structure and development that may provide more robust characters for phylogenetic inference.

Tracing the morphological and developmental evolutionary changes is prerequisite for identifying underlying genetic changes. Based on similarities between developmental mutant phenotypes in *C. elegans* and inferred evolutionary changes, we identified several genes or genetic pathways that are good candidates for being involved in the evolution of ray patterning (Fitch and Emmons, 1995; Fitch, 1997). For example, the HOX gene complex is involved in anteroposterior patterning of ray identities in *C. elegans*. Some mutations in these genes closely mimic some evolutionary changes in ray positions that could also be interpreted as changes in ray identities (Fitch and Emmons, 1995; Fitch, 1997). Taking a more focused developmental genetic approach to dissect the mechanisms governing morphogenesis of just the tail tip, we have identified mutants in *C. elegans* that disrupt tail tip retraction and result in leptoderan tail tips (Nguyen et al., 1999). Mutations in one of the genes we have identified, *lep-1,* produces a leptoderan tail tip that closely mimics at the cellular level an evolutionary change from peloderan to leptoderan tail tips that occurred in at least one lineage within the ''*Eurhabditis*'' species group (Nguyen et al., 1999; Fig. 4). It is therefore possible that changes in this gene—or in the genetic pathway in which this gene is a member, or in a parallel pathway—could have resulted in the morphological change from peloderan to leptoderan in this species group.

One important question to eventually address is whether all changes between leptoderan and peloderan states involve the same pathway or different mechanisms altogether. If the former is true, ''developmental constraints'' may result in a strong bias in the evolution of form; if the latter, then selection or genetic drift would be expected to play a greater role.

Plotting the minimal male tail evolutionary changes also allows us to test correlations between form and function. According to our phylogeny, a bursa evolved early in ''Rhabditidae''; parallel mating could have appeared at the same time or afterward (Fig. 5). When a bursa was reduced in rhabditid evolution, mating position reverted to the primitive spiral type (Fig. 5). However, a reduced bursa is not required for spiral mating, which reappeared in a *Caenorhabditis* species, for example. Using the concentrated-changes test (Maddison, 1990), we find that the correlation between retaining a bursa and retaining parallel mating is significant ($P = 0.043$), suggesting that a bursa is required (and possibly preadaptive) for parallel mating.

We anticipate that the morphologically and molecularly integrated picture of ''Rhabditidae'' systematics emerging from these studies also will enhance other studies in nematode biology. Certainly ''Rhabditidae'' is rich with not only a diversity of mor-

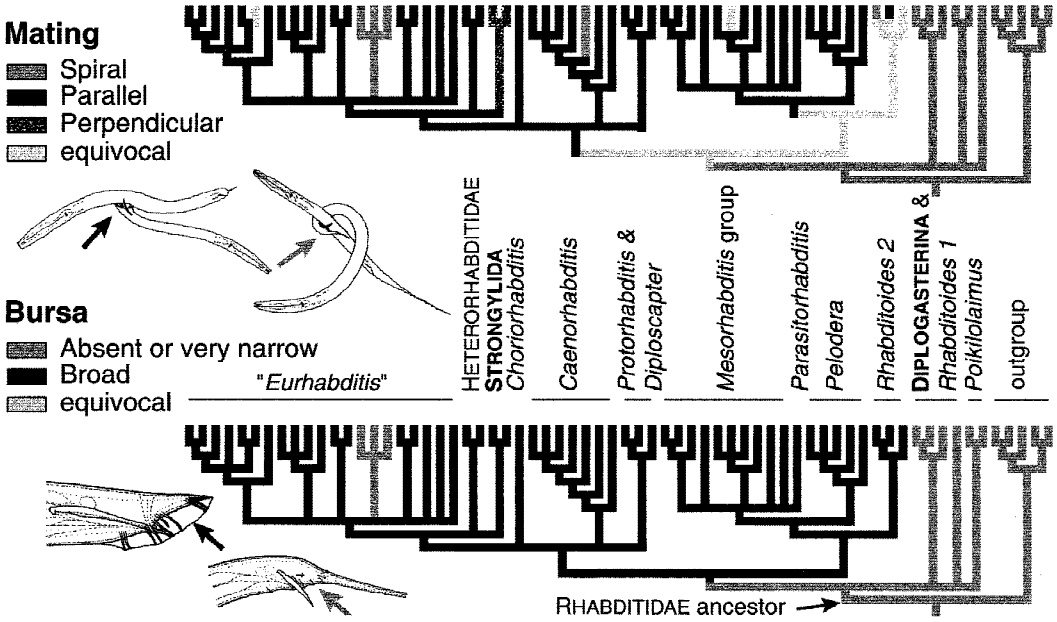

FIG. 5. Correlation between male tail morphology and male mating position. Phylogenies are the same as that in Figure 1. On the top phylogeny is traced three mating position types: spiral (dark gray), parallel (black), and perpendicular (mottled). Lineages in which there is some ambiguity about mating position type are colored light gray. On the bottom phylogeny is traced two types of male tail form: with a bursa that is broad (black), and without a bursa or with one that is very narrow (dark gray), as in the *Rhabditella* species.

phologies but also life histories and adaptations associated with commensalism, mutalism, phoresy, and parasitism, the natural history of which can be understood only in a phylogenetic context. Also, with the significant investments required for genome projects, a phylogeny of species related to *C. elegans* will be important for guiding decisions about which taxa should be targeted as best representing any particular level of phylogenetic divergence from *C. elegans* or other models.

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