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The embryonic cell lineage of the nematode *Rhabditophanes* sp.

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ABSTRACT One of the unique features of the model organism *Caenorhabditis elegans* is its invariant development, where a stereotyped cell lineage generates a fixed number of cells with a fixed cell type. It remains unclear how embryonic development evolved within the nematodes to give rise to the complex, invariant cell lineage of *C. elegans*. Therefore, we determined the embryonic cell lineage of the nematode, *Rhabditophanes* sp. (family Alloionematidae) and made detailed cell-by-cell comparison with the known cell lineages of *C. elegans, Pellioditis marina* and *Halicephalobus gingivalis.* This gave us a unique data set of four embryonic cell lineages, which allowed a detailed comparison between these cell lineages at the level of each individual cell. This lineage comparison revealed a similar complex polyclonal fate distribution in all four nematode species (85% of the cells have the same fate). It is striking that there is a conservation of a *'C. elegans'* like polyclonal cell lineage with strong left-right asymmetry. We propose that an early symmetry-breaking event in nematodes of clade IV-V is a major developmental constraint which shapes their asymmetric cell lineage.

KEY WORDS: *4D microscopy, cell lineage, embryo, evolution, nematode, Rhabditophanes sp*

The model organism *Caenorhabditis elegans* has a strict invariant cell lineage, generating a predetermined number of cells each with a fixed cell type (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). The reproducibility of this lineage has allowed scientists to study developmental mechanisms on a cellular level, greatly improving knowledge in many fields of biology and medicine (Wood, 1988; Riddle *et al.*, 1997; The *C. elegans* Research Community, 2005). Recent research on other nematode species throughout the phylum has uncovered much more diversity in developmental mechanisms than was previously thought. But most of this research focuses on specific phases or areas of development, like the early development (reviewed in Schierenberg, 2006) or development of the gonad, vulva and male tail (reviewed in Sommer, 2005). So, it remains unclear whether these different mechanisms are part of a different mode of development or fit into the invariant «*C. elegans* like» mode of development. The study of the embryogenesis of more nematodes will teach us about the different strategies to form a nematode body plan and how these strategies are evolutionarily linked to one another.

The description of the embryonic cell lineage of *Pellioditis marina* demonstrated that this cell lineage is conserved within the family Rhabditidae (clade V) according to the phylogeny of Blaxter *et al.* (1998) (Houthoofd *et al.*, 2003). To examine the extent of conservation of this mode of development in nematodes, we established the embryonic cell lineages of more distantly related nematodes from the adjacent clade IV. Recently, we determined the embryonic cell lineage of *Halicephalobus gingivalis* (Houthoofd and Borgonie, 2007). Here we present the nearly complete embryonic cell lineage of the free-living nematode *Rhabditophanes* sp. KR 3021 (fam. Alloionematidae). This gives us a unique data set of four embryonic cell lineages, which allows a detailed comparison between these cell lineages on the level of each individual cell.

The embryonic development of *Rhabditophanes* sp. is comparable to that of *C. elegans, P. marina* and *H. gingivalis*, with differences in the early division sequence and gastrulation. The division of AB and P_1 occurs simultaneously and results in a short transient T-shape that immediately converts to the rhomboid configuration (Fig. 1A,B). Gastrulation starts at the 32-cell stage, with the stepwise ingression of the four granddaughters of the intestinal precursors E (Eal, Ear, Epl, Epr), which lie in a square at the ventral side of the embryo (Fig. 1C). This is in contrast with the other species compared here, where gastrulation starts with the ingression of the two daughters of E, Ea and Ep.

At muscle contraction, the consensus lineage of

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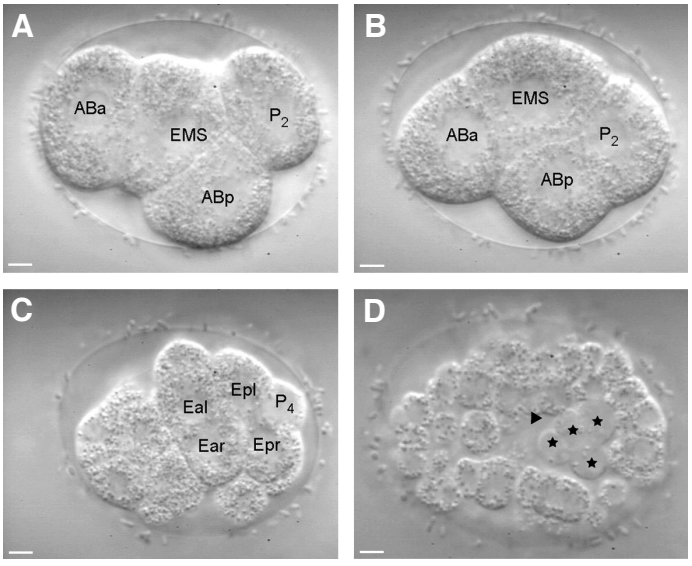

Fig. 1. DIC images of different embryonic stages in *Rhabditophanes sp.* (A) *Early T-shaped 4-cell stage, left lateral view, just after division of AB and P1.* **(B)** *Late rhomboid shaped four cell stage, left lateral view. EMS lies at the future ventral side of the embryo, ABp at the future dorsal side.* **(C)** *32-cell stage, ventral view. The four E cells lie at the ventral side just before gastrulation.* **(D)** *Ventral view of 176 cell stage (176 min), anterior to the left. The four primordial germ cells are marked with an asterisk. Arrowhead indicates the blastocoel, the space left after the inward migration of the intestinal cells. All stages anterior to the left, scalebar is 5 μm.*

Rhabditophanes sp. contains 606 terminal cells (Table 1). The position and fate of 552 of these (91 %) was determined (the position and fate of 36 cells and the division of 18 cells could not be resolved). The cellular composition of the embryonic tissues differs in some aspects with the other species. The intestinal cell lineage E forms 20 cells in a similar pattern as in *C. elegans* and *P. marina*, but it is the last but one pair of cells in the 16E stage, Ep(l/r)a, that divides to form a 7th and 8th ring, while in *P. marina* and *C. elegans,* these rings are formed by a division of the last pair of cells (Houthoofd *et al.,* 2006). The primordial gonad consists of four germ line cells, due to an extra division round of P_4 (Fig 1D). Only 21 programmed cell deaths could be identified in the *Rhabditophanes* sp. embryo (Table 1). All these cells have an equivalent cell death in *C. elegans* and 15 of those cells are early cell deaths after the ninth division round. Of the 70 cells that undergo cell death in *C. elegans*, but not in *Rhabditophanes* sp., the fate and position of 47 could be determined (neurons (35), pharynx (10), epidermis (2)). Of the remaining 23 cells, the position and fate could not be ascertained from the *Rhabditophanes* sp. recordings. It is however possible that these cells also undergo cell death, but this could not be confirmed in the recordings.

Cells are built according to similar cell lineages

A first striking conclusion after a cell-by-cell comparison between the four species is that cells are built according to a very similar cell lineage in the four species (Fig. 2). The lineage similarity between *Rhabditophanes* sp. and *C. elegans* is 93.6%, indicating that 517 of the 552 determined terminal cells have an equivalent terminal cell with the same lineage history in the *C. elegans* cell lineage. Next, we compared the fate of each equivalent cell (with an identical lineage history) between the four cell lineages (Fig. 2). For example, 454 of the 517 terminal cells in *Rhabditophanes* sp. have the same fate as in *C. elegans*. That makes a fate similarity of 88% (Fig. 2). This fate similarity is unequally distributed over the different tissues. Intestine (80-100%), body muscle (94-100%) and epidermis (88-95%) in *Rhabditophanes* sp. have a high fate similarity compared to the other species. Pharynx (70-87%) and nervous system (67-79%) have a lower similarity (similar percentages for other species, data not shown).

A comparison of the fate distribution of the AB lineage of *Rhabditophanes* sp. with the other species shows that despite the interspecific variation, there are some important similar fate distribution patterns, which might shed light on possible underlying mechanisms. The fate distribution of AB in *C. elegans* is regulated by a series of four Notch mediated inductions, which results in a specific fate distribution for each of the 8 AB great-granddaughters (reviewed in Priess, 2005) (Fig. 3). Firstly, an induction from the germline precursor P_2 to ABp results in a

TABLE 1

COMPARISON OF THE NUMBER OF CELLS PER TISSUE TYPE (ROW) AND FOUNDER CELL (COLUMN)

	AB				MS				E				C				D				P4				total			
	Rh	Hg	Pm	Ce	Rh	Hg	Pm	Ce	Rh	Hg	Pm	Ce	Rh	Hg	Pm	Ce	Rh	Hg	Pm	Ce	Rh	Hg	Pm	Ce	Rh	Hg	Pm	Ce
Pharynx	63	60	79	56	32	31	33	30																	95	91	112	86
Neuron	214	199	186	211	2	1	7	6					2		2	2									218	200	195	219
Muscle	1	1	1	1	29	33	28	28					32	32	32	32	20	20	20	20					82	86	81	81
Epidermis	72	74	114	66			3						14	16	14	13									86	90	131	79
Intestine									20	18	20	20													20	18	20	20
Other	16	18	20	20	10	7	10	11																	26	25	30	31
Gonad																					4	2	2	2	4	2	2	2
Mitosis				26				3																				29
Total survivors	366	352	400	380	73	72	81	78	20	18	20	20	48	48	48	47	20	20	20	20	4	2	2	2	531	512	571	547
Cell death	12	18	58	78	9	4	9	13		2															21	24	67	91
Unresolved mitosis	15	13			3																				18	13		
Unresolved cell	33	38			3	10																			36	48		
Total cells produced	426	421	458	458	88	86	90	91	20	20	20	20	48	48	48	47	20	20	20	20	4	2	2	2	606	597	638	638

Comparison between *C. elegans, H. gingivalis, P. marina* and *Rhabditophanes* sp. of the number of cells per tissue type (row) and founder cell (column). *Rhabditophanes* sp. (Rh), *H. gingivalis* (Hg), *C. elegans* (Ce) and *P. marina* (Pm).

reversed distribution of the epidermal precursors arising from ABa (mainly in posterior granddaughters ABa(l/r)p) and ABp (mainly in anterior granddaughters ABp(l/r)a). This pattern is adapted by a second induction in the 12-cell stage, where a signal from MS to ABalp and ABara induces pharyngeal potential in those cells (Priess, 2005). We observed a similar fate pattern in the 8AB descendants of *Rhabditophanes* sp., *H. gingivalis* and *P. marina* although in varying percentages (Fig. 3). A third and fourth Notch interaction influence subsets of the 8AB precursors in the *C. elegans* embryo (Priess, 2005). At the 24-cell stage, an interaction occurs between ABalap and ABplaa, which induces the anterior daughter ABplaaa to develop differently from its bilateral homologue ABpraaa, forming left head epidermal precursors that are bilaterally symmetrical with ABarpap instead. Also in *Rhabditophanes* sp., *P. marina* and *H. gingivalis*, ABalap contacts ABplaaa but not ABpraaa. Moreover, the ABplaaa descendants lie bilaterally symmetrical with the ABarpap descendants to form left and right head epidermal precursors. Finally the fourth induction occurs in the *C. elegans* embryo between MSap and ABplpa (Moskowitz and Rothman, 1996; Hutter and Schnabel, 1995), whereby the excretory cell is produced by a descendant of ABplpa (ABplpappaap). Again, both the initial positioning of cells and the resulting fate in the four species compared here are identical. These results might suggest that the inductions in those nematode species may be similar if not the same, strengthening the hypothesis that Notch inductions may be an underlying mechanism common to nematodes of clade IV and V. However, the presence of these inductions in the other species needs to be tested by laser ablation experiments.

Secondary bilateral symmetries in AB are identical in clade IV and V

It is clear from the fate distribution in AB in the four species compared here, that in some equivalent left-right sublineages of the ABa lineage the bilateral symmetry is lost, while the symmetry is largely retained in the ABp lineage. These equivalent sublineages give rise to non-equivalent cell types (for example, compare fate distribution in ABala and ABara in Fig. 3). This bilateral asymmetry in the AB lineage originates at the left –right division of the 2AB cell stage in the 6-cell stage embryo. The left daughters of the 2AB cells, ABal and ABpl, are skewed in the anterior direction, and their progeny lie in more anterior positions than their lineal equivalents ABar and ABpr. Still in all species studied, a bilaterally symmetrical nematode arises from these asymmetrical lineages. The bilateral symmetry in the embryo is restored by asymmetric lineage pairs. We scored the position of the descendants of these sublineage pairs in *P. marina*, *Rhabditophanes* sp. and *H. gingivalis*. Sixteen of the secondary symmetries found in *C. elegans* are also scored in the *Rhabditophanes* sp. embryo and 14 in *H. gingivalis*.

In *C. elegans*, this bilaterally asymmetric lineage is the result of a signaling pathway, which superimposes the bilateral symmetry of the nematode body plan on a 6-cell stage embryo that is asymmetrically arranged after the skewed left-right division of ABa and ABp (Sulston *et al.,* 1983; Wood, 1991). This single symmetry breakage event in the early embryo was found in each species we studied in clade IV and V. Although the underlying cell specification mechanisms remain to be ascertained, the result is

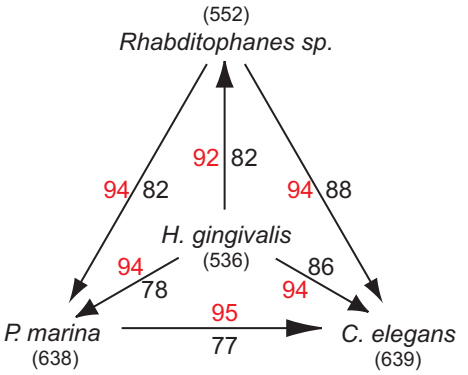

Fig. 2. Lineage and fate similarities between the embryonic cell lineages of *C. elegans, H. gingivalis, P. marina* and *Rhabditophanes sp.* *Lineage similarity, indicated in red, is expressed as the number of terminal cells in the first species that have an identical lineage history as in the second species. Fate similarity, indicated in black, is expressed as the percentage of equivalent cells (with identical lineage history) of the first species that has the same cell fate as in the second species. The direction of the arrow indicates how the comparison was performed. The number of resolved terminal cells in the cell lineage of each species is indicated between brackets.*

the same: an asymmetric cell lineage that compensates for the asymmetry in the early embryo. We hypothesize that this symmetry-breaking event in the ancestor of the nematodes of clade IV-V is a major developmental constraint that shapes the subsequent asymmetric polyclonal cell lineage in those nematodes. The studies on the early development of nematodes from clade I-III indicate that early embryos in these more basal clades are symmetric (Malakhov, 1994; Lahl *et al.,* 2003). It remains to be seen however if these symmetric cells in early embryos give rise to a symmetric cell lineage.

Materials and Methods

Cultures

The soil nematode *Rhabditophanes* sp. KR3021 is cultured on 1% agar plates with Escherichia coli OP50 as food source. Culture and handling is as described by Brenner (1974).

4D microscopy and cell lineage analysis

Early stage embryos are obtained by cutting gravid females in distilled water. One-cell embryos are selected under a dissecting microscope, mounted on a slide with a 5% agar pad, covered with a coverslip and sealed with Vaseline (Sulston and Horvitz, 1977).

The cell lineage of the embryo is established using 4D microscopy described in detail by (Schnabel *et al.,* 1997; Houthoofd *et al.,* 2003). The recordings are analyzed with the software program Simi Biocell (Simi Gmbh, D-85705 Unterschleissheim, Germany). The embryonic cell lineage is established by identifying all cells and cell divisions in space and time. By establishing the positions of all the nuclei of the cells, 3D-reconstructions of the embryo are made and cell migrations can be followed. At muscle contraction, all tissues are clearly distinguishable, so the cell fate of each terminal cell is determined. It is not always possible to accurately identify the cell type of a given tissue. When discussing the nervous system, no distinction can always be made between neurons, sockets or sheaths; similarly, for cell types of the pharynx.

The lineages presented here are based on three recordings. A

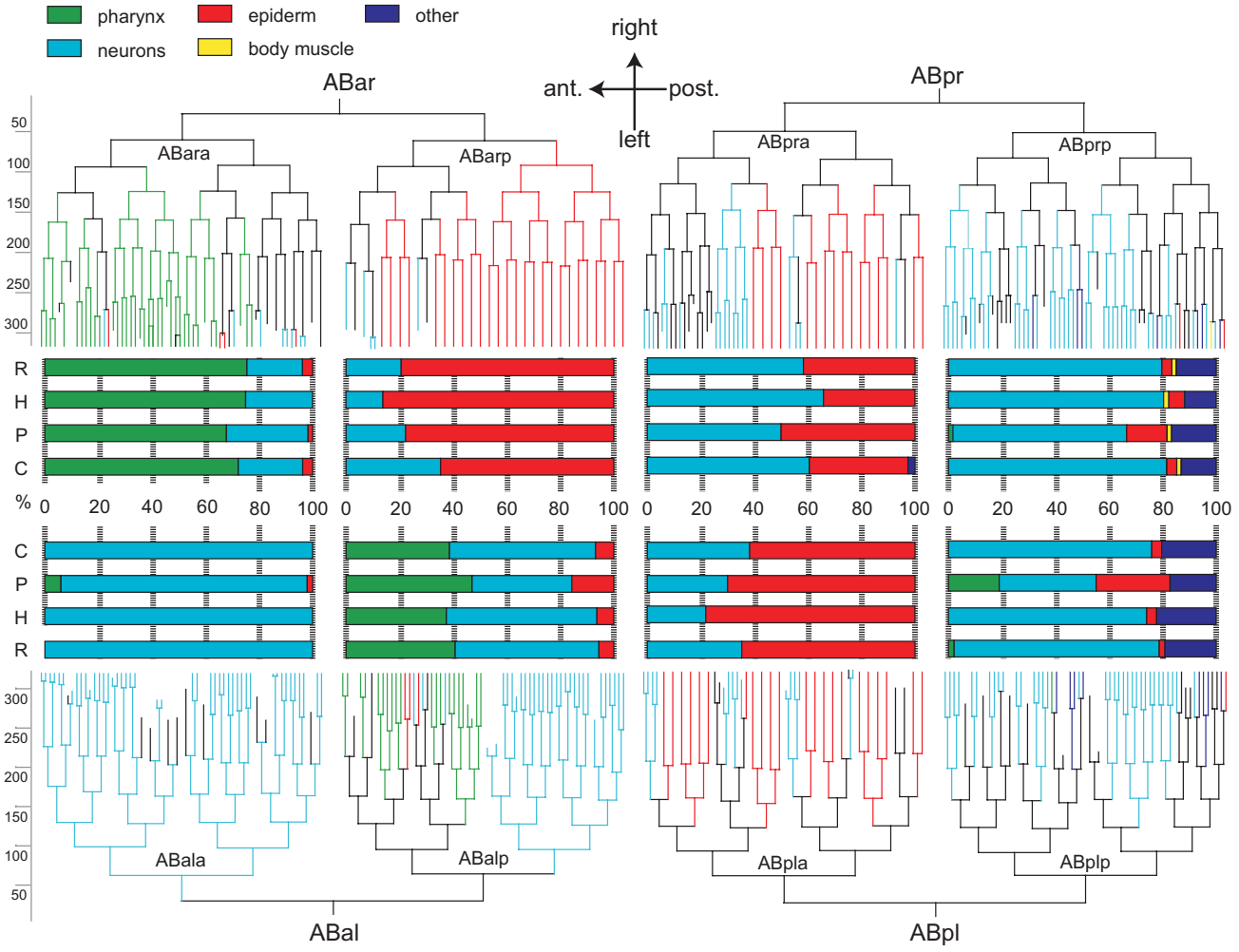

Fig. 3. AB cell lineage of *Rhabditophanes sp.*: comparison with *C. elegans, H. gingivalis* and *P. marina* of the fate distribution in the 8AB granddaughters. *The lineages of the 8 granddaughters are oriented according to their spatial position in the embryo (dorsal view, anterior to the left). Terminal cells and precursor cells that give rise to one tissue type are colored according to cell fate: pharynx: green; neuron: blue; epidermis: red; body muscle: yellow; other: dark blue. Cell deaths or cells with unresolved cell fate are colored black. The fate distribution of each AB granddaughter is compared between* Rhabditophanes sp. (R), H. gingivalis (H), P. marina (P) *and* C. elegans *(C) in percentage of cells of each cell fate to the total number of terminal cells in each granddaughter. Timeline is given to the left of the figure in minutes after first division.*

consensus lineage is formed, by which uncertainties in two complete recordings were resolved by comparison with the third recording. In *Rhabditophanes* sp. the mitoses of some cells could not be resolved in all three recordings and are marked as unresolved mitoses. Also the terminal position and/or fate of some terminal cells could not be resolved and are marked as unresolved cells.

The cells are named according to (Sulston and Horvitz, 1977, Deppe *et al.,* 1978; Sulston *et al.,* 1983). Here we repeat shortly the nomenclature for better readability of the study. Founder cells formed in the first division rounds are given arbitrary names in capital letters (Deppe *et al.,* 1981). When a founder cell divides, each daughter is named by adding to the name of the mother cell a single low-case letter representing its position immediately after division relative to its sister cell. For divisions in anterior-posterior direction the anterior and posterior daughter are indicated respectively with an 'a' and 'p', dorso-ventral divisions are indicated with 'd' and 'v', left-right divisions are indicated with 'l' and 'r'. For example when founder cell AB divides in anterior-posterior direction, the daughters are named ABa and ABp and when ABp divides in left-right direction its daughters are named ABpl and ABpr. A pair of cells may be designated by the use of internal parentheses, e.g., ABa(l/r)aa means ABalaa and ABaraa. In the cell lineage tree the 'a', 'd' and 'l' daughters are represented by the left branches, and the 'p', 'v' and 'r' daughters by right branches.

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