Assessment of the configuration of the posterior cells of the nematode embryo as a potential phylogenetic marker

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Summary. We have reconstructed the evolutionary history of an early developmental character by mapping the configuration of the posterior cells in the early embryo onto the SSU-based molecular phylogeny of Holterman (2006). We have analyzed the early embryonic development of 19 species, complemented with 20 species from the literature, covering representatives in each clade. We show that the *Caenorhabditis elegans* configuration, found in most species in clade III-X, is the ancestral state for these clades. Alternative configurations arose independently in clade IX, (*Diploscapter coronatus*), X (*Halicephalobus gingivalis*), XI (all investigated species) and XII (*Meloidogyne incognita*). A variable configuration of the posterior cells arose at least twice independently during nematode evolution, once in clade IX (*D. coronatus*) and once in an ancestor, shared by clade XI and XII and thus can be used as a phylogenetic marker to delineate these clades. Statistical tests based on our data-set show that the presence of a variable configuration is related to developmental tempo and egg shape.

Key words: embryology, evolution, Nematoda, phylogenetic marker, spatial configuration.

The potential significance of features of embryonic development as phylogenetic markers in the phylum *Nematoda* is little recognized, although researchers have made several attempts to reconstruct the evolution of embryonic diversity within nematodes. Drozdovsky (1967) assessed the arrangement of the blastomeres in the four-cell stage to infer phylogenetic relationships. Voronov *et al.* (1998) pointed out that three distinct patterns of early embryonic development reported for nematodes are in good agreement with the classification of Blaxter *et al.* (1998), which divides the phylum into three groups. Goldstein *et al.* (1998) and Goldstein (2001) mapped several early developmental characters, such as axis specification, onto the molecular phylogeny of Blaxter *et al.* (1998). However, they examined the scored characters in only a limited number of taxa. Schierenberg (2000) identified features that are typical for specific taxa: the timing of early cleavages, gastrulation and establishment of bilateral symmetry. Dolinski *et al.* (2001) were the first to map the evolution of some early developmental characters on a large scale. For 70 species they scored the spatial arrangement of the four-cell stage, whether the AB and P1 lineages proceed at a synchronous or asynchronous rate, and the time when the germ founder cell P4 is established. Recently, Schierenberg (2004) investigated two developmental events, namely, the establishment of a visible germline and the type of gastrulation, and found four taxon-specific character combinations that could be used to infer phylogenetic information.

Most studies of the early embryonic development of nematodes have focused on species belonging to clades VI-XII (12-clade phylogeny of Holterman *et al.*, 2006). These species develop with a fixed cleavage pattern (Sulston *et al.*, 1983; Schierenberg, 1987; Skiba & Schierenberg, 1992; Malakhov, 1994; Spieler & Schierenberg, 1995; Goldstein *et al.*, 1998; Wiegner & Schierenberg,

1999; Borgonie *et al.*, 2000; Dolinski *et al.* , 2001; Lahl *et al.*, 2003; Houthoofd *et al.*, 2003; Hasegawa *et al*, 2004; Laugsch & Schierenberg, 2004; Houthoofd *et al.*, 2006; Lahl *et al.*, 2006; Houthoofd & Borgonie, 2007; Houthoofd *et al.*, 2008; Vangestel *et al.*, 2008; Schulze & Schierenberg, 2008). Development starts with a series of asymmetric stem cell-like divisions: the zygote P0 divides in an anterior daughter AB, which contributes to the somatic tissues and a posterior daughter P1, the germline precursor cell. P1 divides to generate an anterior somatic cell EMS, which defines the future ventral side of the embryo, and a posterior germline cell P2, which continues to cleave in a stem cell-like pattern for two more cycles. In intact embryos of *Caenorhabditis elegans* (Rhabditidae, clade IX), beginning with the second germline division, the polarity is reversed (Schierenberg, 1987), so when P2 divides the anterior daughter cell P3 is pushed to the ventral side and C is pushed to the dorsal side. Also P3 gives rise to a germline cell P4 at the ventral side and a somatic cell D, which is located at the dorsal side of the embryo. With this one reversal of polarity the configuration of the posterior cells from ventral to dorsal is P4-D-C (Fig. 1C). Another configuration was described for *Acrobeloides nanus* (Cephalobidae, clade XI) by Skiba & Schierenberg (1992). In intact embryos of this species, no reversal of polarity occurs, which results in the following spatial configuration from ventral to dorsal: C-D-P4 (Fig. 1A). Recently Houthoofd & Borgonie (2007) found that in *Halicephalobus gingivalis* (Panagrolaimidae, clade X) the polarity in the germline divisions changes twice, once with the division of P2 and again with the division of P3, resulting in the configuration D-P4-C (Fig. 1D). Because this configuration seems to vary between species, and is well defined and easy to determine, it prompted us to study more species over the phylum to see how this putatively taxon-specific character has evolved. Schierenberg (1987) first described the characteristic developmental phenomenon of polarity reversal in the *C. elegans* embryo. In this study partial embryos were generated by puncturing the eggshell with a laser micro-beam and gently removing the anterior AB cell in order to give space for the posterior germ line cells. However, this method requires experimental interference. This is not compatible with our goal of analyzing a readily visible and easily scored developmental character, which can be determined with great ease on recordings of developing embryos for many species. Depending on whether there is a reversal of polarity in the division of P2 and/or P3, four different configurations of the posterior cells are possible (Fig. 1). After scoring this character in 39 investigated nematodes (data for 20 nematodes were obtained from literature), it will be mapped on a molecular phylogeny to evaluate its level of congruence with major clades in the phylogeny.

MATERIAL AND METHODS

Strains. The analyzed strains and the strains found in the literature can be found in Table 1. The strains whose development was recorded using 4D-microscopy are marked in bold.

Culture of nematodes. *Acrobeloides butschlii, Acrobeloides maximus, Acrobeloides thornei, Caenorhabditis elegans, Cephalobus cubaensis, Caenorhabditis remanei, Halicephalobus gingivalis, Mesorhabditis longespiculosa, Mesorhabditis miotki, Oscheius dolichuroides, Panagrolaimus detritophagus, Pristionchus pacificus, Panagrellus redivivus, Panagrolaimus rigidus, Procephalobus sp., Pelodera strongyloides, Rhabditella axei, Rhabditophanes* sp. and *Teratorhabditis palmarum* were cultured on 1% agar plates. *Pellioditis marina* was cultured on artificial sea agar plates, as described by Houthoofd *et al.* (2003). *Plectus aquatilis* was cultured on low salt agar plates made from 10g agar/500 ml distilled water. All agar plates contained 5 mg/l cholesterol. The uracil-requiring strain of *Escherichia coli* OP50 was used as a food source. *Prionchulus punctatus,* a predator on other nematodes, was cultured on humus extract based agar plates; the prey species used was *Oscheius* sp. (Rhabdititidae). Humus extracts were made by cooking rotting leaves in distilled water for 45 min in a microwave oven and filtering them through coffee filters. Humus extracts were stored at −20°C. 1% humus agar was made with nutrient agar diluted with 2/3 distilled water and 1/3 humus extract. Handling was as described by Brenner (1974). *Meloidogyne incognita* was cultured *in vitro* on *Pisum sativum* on Knop medium (Sijmons *et al.*, 1991) under sterile conditions.

Slide preparation. For *M. incognita*, galls were collected from the roots 4-6 weeks after the initial inoculation and gently cut in M9 buffer with a scalpel to release the eggs/embryos. All other embryos were collected by either cutting open gravid hermaphrodites or females in a drop of distilled water with a scalpel, or by flooding agar plates with distilled water using a drawn-out Pasteur pipette. Embryos in the one- or two-cell stage were transferred to a microscope slide

Table 1. Analyzed species and their source

Clade	Taxon	Species (nr of examined species)	Code	Source	Available literature
1	Enoplida	*Enoplus brevis*			Voronov and Panchin (1998), Voronov et al., (1998)
		Pontonema vulgare			Malakhov (1994), Malakhov (1998), Voronov *et al.* (1998); Voronov (1999)
	Triplonchida	*Tobrilus diversipapillatus*			Schierenberg, 2005
2	Mononchidae	*Prionchulus punctatus* (n=3)	GB0021	G. Borgonie, University of Ghent, Belgium	
	Mermithidae	*Gastromermis hibernalis*			Malakhov, 1994
	Mermithidae	*Romanomermis culicivorax*			Schulze & Schierenberg, 2008
3	Desmodoridae	*Desmodora serpentulus*			Malakhov, 1994
4	Chromadoridae	*Hypodontolaimus inaequalis*			Malakhov, 1994
5	Xyalidae	*Daptonema setosum*			Malakhov, 1994
	Axonolaimidae	*Axonolaimus paraspinosus*			Malakhov, 1994
6	Plectidae	*Plectus aquatilis* (n=4)	PDL0018	P. De Ley, University of California, Riverside	Lahl et al., 2003
		Plectus minimus			Lahl et al., 2003
		Tylocephalus auriculatus			Lahl et al., 2003
7	Teratocephalidae	*Teratocephalus lirellus*			E. Schierenberg, pers. comm.
8	Ascarididae	*Parascaris equorum*			zur Strassen (1906) in Schierenberg, 1987

Table 1 (continued). Analyzed species and their source

Clade	Taxon	Species (nr of examined species)	Code	Source	Available literature
9	Neodiplogastridae	*Pristionchus pacificus* (n=12)	PS 312	CGC, University of Minnesota	Vangestel et al., 2008
	Rhabditidae	*Caenorhabditis elegans* (n=8)	N2	CGC, University of Minnesota	Schierenberg, 1987
		Caenorhabditis remanei (n=3)	PB206	CGC, University of Minnesota	
		Rhabditella axei (n=3)	DF5006	CGC, University of Minnesota	
		Oscheius dolichuroides (n=3)	DF 5018	CGC, University of Minnesota	
		Pellioditis marina (n=3)	TM02	CGC, University of Minnesota	
		Teratorhabditis palmarum (n=3)	DF5019	CGC, University of Minnesota	
		Mesorhabditis miotki (n=5)	AF72	CGC, University of Minnesota	
		Mesorhabditis longespiculosa (n=4)	DF5017	CGC, University of Minnesota	
		Pelodera strongyloides (n=3)	DF5022	CGC, University of Minnesota	
	Diploscapteridae	*Diploscapter coronatus*			Lahl, 2007
10	Parasitaphelenchidae	*Bursaphelenchus xylophilus*			Hasegawa et al., 2004
	Panagrolaimidae	*Halicephalobus gingivalis* (n=3)			Houthoofd en Borgonie, 2007
		Panagrolaimus detritophagus (n=3)	BS0008	A. Burnell, National University of Ireland, Maynooth	
		Panagrolaimus rigidus (n=3)	AF36	CGC, University of Minnesota	
		Procephalobus sp. (n=3)	JU 169	P. De Ley, University of California, Riverside	
		Panagrellus redivivus (n=3)	PS1163	CGC, University of Minnesota	
	Alloionematidae	*Rhabditophanes sp.* (n=3)	PDL0036	P. De Ley, University of California, Riverside	
	Rhabdiasidae	*Rhabdias bufonis*			Spieler and Schierenberg, 1995
11	Cephalobidae	*Acrobeloides butschlii* (n=4)	DWF1107	CGC, University of Minnesota	
		Acrobeloides thornei (n=5)	DWF1109	CGC, University of Minnesota	
		Acrobeloides maximus (n=3)	DWF5048	P. De Ley, University of California, Riverside	
		Cephalobus cubaensis (n=3)	PS1197	R. Rhode, University of California, Davis	
12	Meloydoginidae	*Meloidogyne incognita* (n=5)		G. Gheysen, University of Ghent, Belgium	

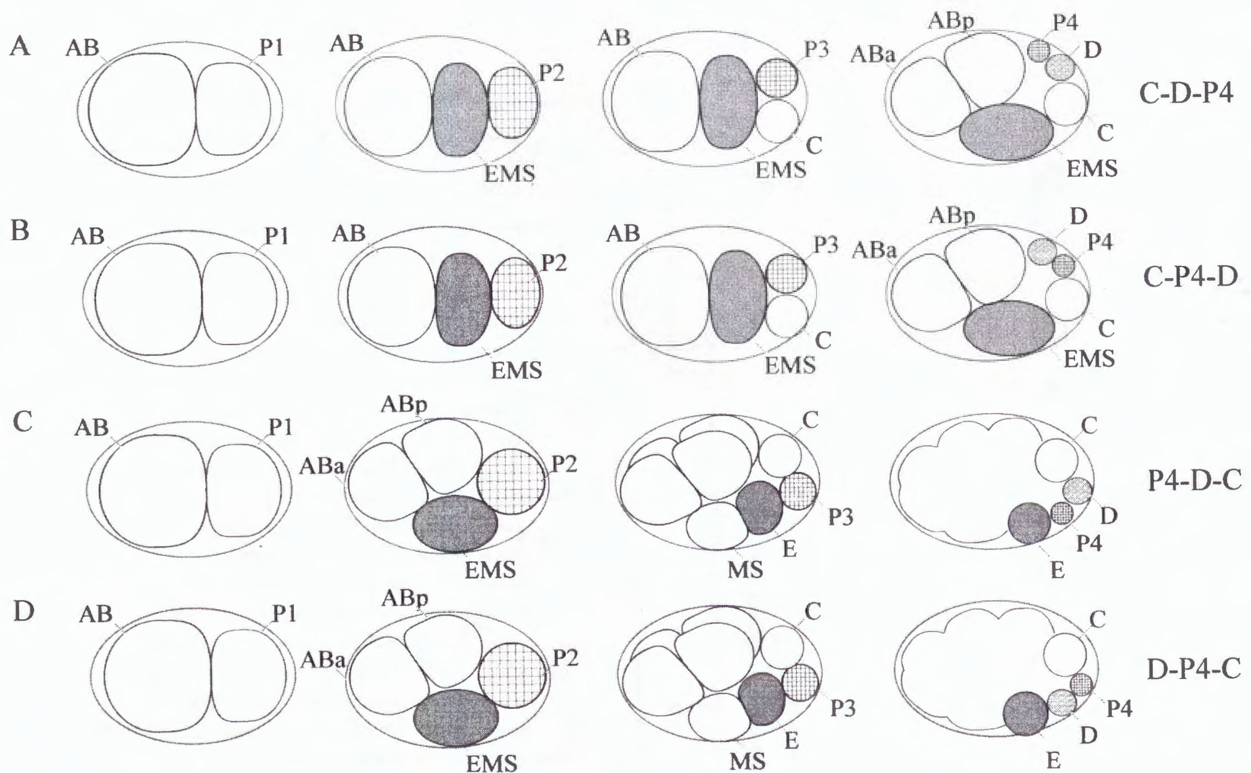

Fig. 1. Scheme representing the four possible configurations of the posterior cells, from ventral to dorsal, after P3 has divided. A: no reversal of polarity in germline divisions leading to the configuration C-D-P4; One reversal of polarity in P2, leading to B: the configuration C-P4-D or C: the configuration P4-D-C (*C. elegans* pattern); D: double reversal of polarity in germline divisions, leading to the configuration D-P4-C.

carrying a thin pad of 5% agar. Embryos were covered with a cover slip and sealed with Vaseline (Sulston & Horvitz, 1977).

4D microscopy. Recordings of embryos were obtained using 4D microscopy (Hird & White, 1993), a multi-focal plane and a time-lapse recording system. Depending on the developmental tempo, every 30-60 s (15-30 min for *M. incognita*), a Hamamatsu Newvicon camera (C2400-07) recorded 30 focal planes through the embryos (with a distance between 1 and 1.2 m between two focal planes), and the software Simple PCI 5.3/6.1.0 (Compix, Inc., USA) stored the images on disk. The lineage of each recording was constructed using the Simi Biocell software (version 4.0, Simi Gmbh, D-85705 Unterschleissheim, Germany) (Schnabel *et al.*, 1997). The recordings could be replayed as required for further analysis. The embryonic cell lineage was established by identifying all cells and cell divisions in space and time. By clicking with the mouse pointer on the nucleus of the cell in the window displaying the digitized image, the cell positions were marked and stored in a file. By establishing the positions of each cell, 3D reconstructions of the embryo were made and cellular migrations could be followed. All embryos were recorded at 20°C, except for *P. marina, H. gingivalis* and *Rhabditophanes* sp. (25°C).

Evaluation of the configuration of the posterior cells. The final configuration of the posterior cells from ventral to dorsal was scored as followed: after P3 divided, we looked at the configuration of the following cells: the endodermal precursor (EMS or E), C, D and P4. When a nematode shows no polarity reversal the configuration of the posterior cells from ventral to dorsal is C-D-P4 (Fig. 1A). When a nematode has a single polarity reversal the configuration of the posterior cells from ventral to dorsal is either C-P4-D (Fig.1B) or P4-D-C (Fig. 1C). When a double polarity reversal occurs, the spatial configuration of the posterior cells from ventral to dorsal is D-P4-C (Fig. 1D).

Determination of relative early developmental tempo. For all recorded nematodes the early developmental tempo, measured as the time between

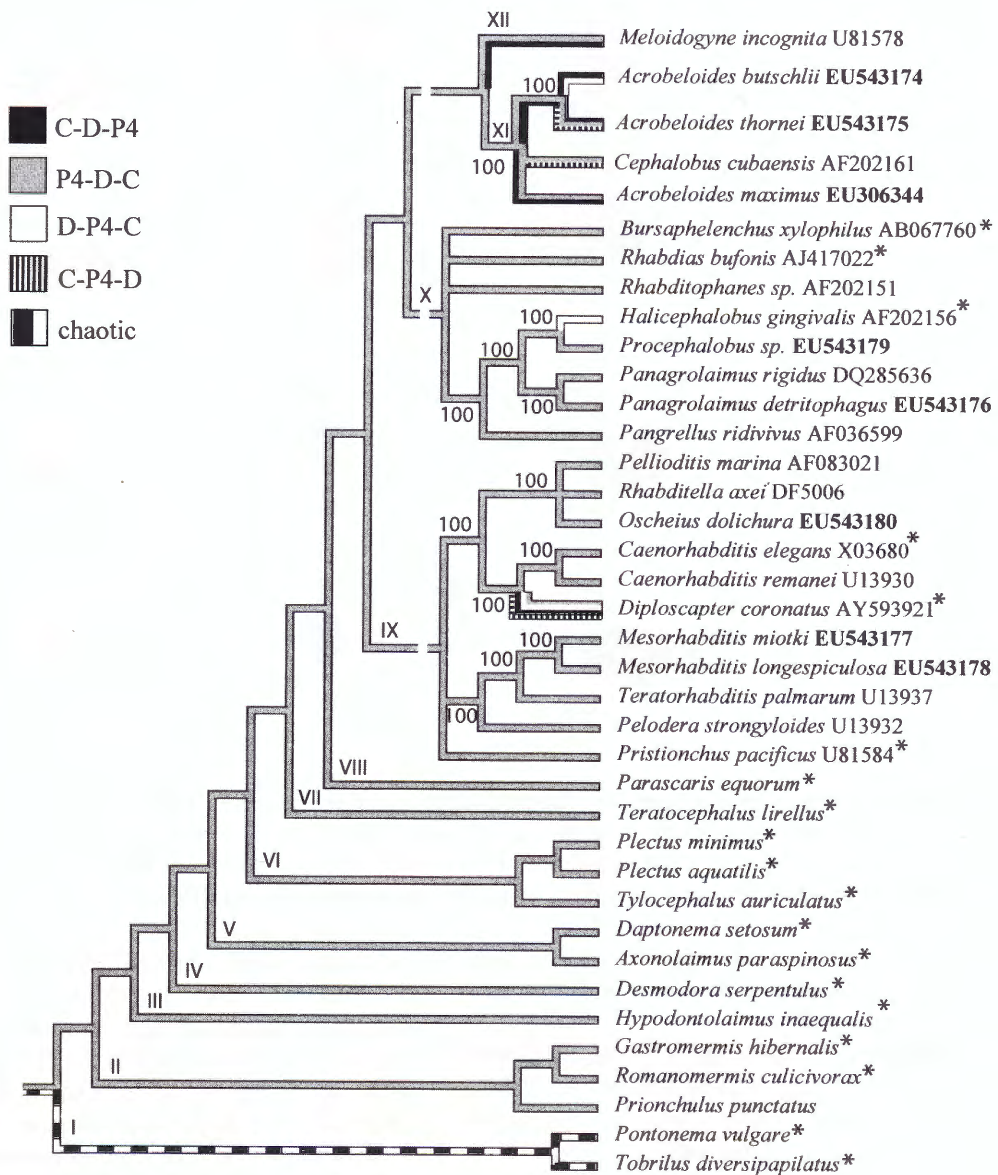

Fig. 2. Parsimonious reconstruction of the posterior cells' configuration onto the phylogenetic tree. This tree is a combination of the nematode phylogeny presented by Holterman *et al.* (2006) and newly obtained consensus phylogenies based on SSU rDNA data of clades IX, X and XI. Data obtained from literature are marked with an asterisk (see Table 1). Species, where the configuration could not be determined because of homology problems, are shown in grey. Branch support is indicated with posterior probability (PP). Newly obtained SSU rDNA sequences are marked in bold. The numbering of the clades (I-XII) refers to the phylogeny of Holterman *et al.* (2006).

the second division of the zygote (AB in case of *C. elegans*, *C. remanei*, *O. dolichuroides*, *P. redivivus*, *P. rigidus*, *P. marina*, *P. strongyloides*, *P. aquatilis*, *P. pacificus*, *Rhabditophanes* sp., *R. axei*, *T. palmarum*; and P1 in case of *A. butschlii*, *A. maximus*, *A. thornei*, *C. cubaensis*, *H. gingivalis*, *M. incognita*, *M. longespiculosa*, *M. miotki*, *P. detritophagus* and *Procephalobus* sp.), and the division of the endodermal precursor cell E, was calculated. This time was divided by the time *C.*

elegans needed to develop from the division of AB until the division of E at 20°C (or at 25°C for *P. marina*, *H. gingivalis* and *Rhabditophanes* sp.), and was called the relative early developmental tempo. For *B. xylophylus* (Hasegawa *et al.*, 2004) and *R. bufonis* (Spieler & Schierenberg, 1995), this early developmental tempo was deduced from lineages in the literature.

Determination of egg shape index. The Egg Shape Index (ESI) was measured on three recordings and calculated as follows: ESI=100 x A/B, with A= egg width and B = egg length.

Phylogenetic analysis and mapping the configuration of the posterior cells. To study character evolution based on a phylogenetic hypothesis, separate trees were assembled (a 'supertree' approach). The backbone of the presented phylogenetic tree was based on the framework presented by Holterman *et al.* (2006) and agreed with Meldal *et al.* (2007). We did this because sequences were not available for all analyzed nematodes and we preferred to use well-established phylogenetic hypotheses based on many taxa. However, *de novo* phylogenetic analyses were made for the taxon dense clades Rhabditomorpha (clade IX), Panagrolaimorpha-Aphelenchoidea (clade X) and Tylenchomorpha-Cephalobomorpha (clades XI and XII), based on seven new and 17 GenBank SSU sequences. DNA amplification and sequencing were done as described by Bert *et al.* (2008). The sequences were aligned with Clustal W (Thompson *et al.*, 1997) and manually checked and edited. Bayesian inference (BI) was performed with MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) with a general time-reversible model with rate variation across sites and a proportion of invariable sites (GTR + I + Γ), as estimated by PAUP/Mr Modeltest 1.0b (Nylander, 2004). Analyses were run for four million generations and trees were generated using the last three million generations, well beyond the burn-in value. LogDet-transformed distance analyses (LogDet; Lockhart *et al.*, 1994) were performed using PAUP* 4.0b10 (Swofford, 2002), especially to cope with the possible effects of compositional heterogeneity across taxa. Consensus trees were built from the outcome of both analyses based on a pronounced conservative approach; conflicts from both analyses and/or branches with lower than a 95 Bayesian posterior probability were presented as unresolved. Finally, our own analyses and the Holterman (2006) framework were combined into a single tree in Mesquite v1.11 (Maddison & Maddison, 2006). Character evolution of developmental character states was traced using parsimony reconstruction. The character states at the internal nodes were reconstructed with the "reconstruct Ancestral States" module implemented in Mesquite.

RESULTS

The configuration of the posterior cells. We evaluated the configuration of the posterior cells on recordings using 4D microscopy for 19 species, complemented with 20 species from the literature. The results are discussed per clade, according to the phylogeny of Holterman *et al.* (2006). For clarity, the data obtained from the literature are included in the results section.

The early embryonic development of clade I differs from the other clades. Malakhov (1994), Voronov & Panchin (1998), Voronov *et al.* (1998), Malakhov (1998) and Voronov (1999) showed that marine nematodes of the order *Enoplida* lack early asymmetric cleavages and a recognizable germline. Schierenberg (2005) studied another member of clade I, belonging to the order Triplonchida, and confirmed the symmetric cleavage and the absence of distinct cell lineages. Therefore the configuration of the posterior cells cannot be determined in this clade.

In clade II, *P. punctatus*, belonging to the family Mononchidae was studied. Drozdovsky (1969) found that in this nematode the intestine is derived from the anterior instead of the posterior blastomere. This was also observed by Malakhov & Spiridonov (1981) in *Gastromermis* and by Schulze & Schierenberg (2008) in *Romanomermis culicivorax*, both belonging to the family Mermithidae. As a result, because of homology problems (cells with a similar fate and position in the embryo have a different lineal origin), this character cannot be coded in this clade.

In descriptions of five species within clades III-V, observed by Malakhov (1994), a small primordial germ cell at the ventral side is in contact with the intestinal precursor, thus leading to the pattern P4-D-C. We assume that no prior cell rearrangements occur, since this is nowhere mentioned in the text.

Within clade VI, *P. aquatilis* showed the *C. elegans* pattern P4-D-C. This finding confirmed the results of Lahl *et al.* (2003), who studied this nematode together with two other *Plectus* species and *T. auriculatus*. All showed the *C. elegans* pattern P4-D-C.

In clades VII and VIII, predominantly containing animal parasites, one representative for each clade was found in the literature. Both *P. equorum* (formerly known as *Ascaris*

megalocephala) (zur Strassen, 1906) and *T. lirellus* (E. Schierenberg, pers. comm.) showed the *C. elegans* pattern P4-D-C.

In clade IX, containing mostly bacterial-feeding nematode families, 11 representatives of three families were studied. Within the family Rhabditidae, to which the model organism *C. elegans* belongs, all nine species showed the *C. elegans* configuration P4-D-C. Also the satellite organism *P. pacificus* had a pattern similar to that of the members of the family Rhabditidae (Vangestel *et al.*, 2008). However, one species within this clade showed a different pattern: *D. coronatus* (Diploscapteridae) is characterized by a variable configuration of the posterior cells (Lahl, 2007).

In clade X, also containing mostly bacterial-feeding nematode families, we investigated representatives of four families: Parasitaphelenchidae (one species, Hasegawa *et al.*, 2004), Panagrolaimidae (five species), Alloionematidae (one species) and Rhabdiasidae (one species, Spieler & Schierenberg, 1995). All species display the P4-D-C pattern, except one species within the family Panagrolaimidae: *H. gingivalis* has the D-P4-C configuration (Fig. 1D) (Houthoofd & Borgonie, 2007). In this species contact between the germline and the endodermal progenitor is restored when the two daughters of the primordial germ cell migrate between the two daughter cells of D, after which they migrate inwardly together.

In clades XI and XII, the configuration C-D-P4 was prevalent. Remarkably, there was considerable intraspecific variation in cellular positioning and subsequent rearrangements within these clades. Variations in spatial patterns were already described for *Cephalobus* sp. (later referred to as *Acrobeloides nanus* that develops 5 times slower than *C. elegans* (Skiba & Schierenberg, 1992). They found that alternative orientations of the cleavage spindle in AB result in two different arrangements of blastomeres in the 5-cell stage. Within clade XI, which comprises mainly bacterial feeding families, we studied four other species of the family Cephalobidae, all of which showed variable arrangements of blastomeres after the division of P3. *A. butschlii* showed two possible configurations: D-P4-C (3/4; double polarity reversal) and P4-D-C (1/4; the *C. elegans* configuration). In the three cases of double polarity reversal, subsequent migration of P4 over D resulted in the *C. elegans* configuration before gastrulation started. The other case exhibited the *C. elegans* pattern immediately and no migrations were observed. For *A. thornei* we observed three different configurations. The configuration C-D-P4 (Fig. 1A) was evident in three out of five cases. All three cases displayed intense cellular migrations, in which C migrated from its ventral position toward the dorsal side, and P4 and D switched positions. While P4 migrated over the D cell ventrally, D translocated to a more dorsal position. One specimen was found with the configuration P4-D-C, showing no cellular migrations. In one recording the configuration C-P4-D (Fig. 1B) was found. After the division of EMS, C started to migrate toward the dorsal side, leading to the *C. elegans* configuration. *A. maximus* displayed two different configurations: C-D-P4 (2/3) and P4-D-C (1/3). In the first case migrations typical for the C-D-P4 pattern were observed. Finally, in *C. cubaensis* we found two configurations: C-P4-D (2/3) and P4-D-C (1/3); in the first case, migration of the C blastomere to the dorsal side lead to the *C. elegans* configuration.

In clade XII, dominated by plant parasites, we studied the root-knot nematode *M. incognita*. The following configurations of its posterior cells were found: P4-D-C (2/5) and C-D-P4 (3/5). This finding contrasts with the observations of Goldstein *et al.* (1998), who mentioned an absence of polarity reversal for *M. incognita*. There are alternative orientations of the cleavage spindle of P2, possibly resulting in two different arrangements of blastomeres in the five-cell stage. This does not depend on the orientation of the cleavage spindle of ABs, because two AB cells are already present when P2 divides.

Mapping the configuration of the posterior cells. The most parsimonious reconstruction of the evolution of the configuration of the posterior cells along the phylogram (Fig. 2) suggests that a fixed configuration of the posterior cells and, more precisely, the configuration P4-D-C, is the ancestral state for all the investigated clades (III-XII). However, this is because Mesquite makes ancestral reconstructions for clades I and II, while in these clades the character cannot be coded. In the phylogram it seems as if there is no distinction between clade I (no cells can be identified because of equal cleavages) and clade II (cells are not homologous), but this is because the program makes no distinction between missing data and non-applicable data. From the configuration P4-D-C the pattern D-P4-C evolved once in *H. gingivalis*. According to our analysis a variable configuration of the posterior cells evolved at least twice independently, once in an ancestor of clades XI and XII and once in *D. coronatus* (clade IX).

A variable configuration of the posterior cells versus the relative early developmental tempo and the shape of the egg. We tested whether the developmental tempo is possibly linked to the organization of the posterior cells in the early embryo, because *A. nanus*, with a variable configuration of its posterior cells, has a slow developmental tempo (13.3 times slower than *C. elegans*, measured from the division of P0) (Skiba & Schierenberg, 1992). A Wilcoxon Mann-Withney test showed that there is a significant difference in developmental tempo between a variable and a fixed configuration ($P<0.0001$). Looking at early developmental tempo, Fig. 3 suggests that nematodes with a fixed configuration of their posterior cells have a fast developmental tempo. As such, rapidly developing nematodes tend to show a strict regulation of the division axes of the germline divisions. With the exception of *Diploscapter coronatus*, all nematodes with a variable configuration are slow developing nematodes. However, not all slow developing nematodes have a fixed configuration. *Romanomermis culicivorax*, which has a fixed configuration, develops at least 20 times more slowly than *Caenorhabditis elegans* (based on total embryogenesis, Ginarte & Mijares, 1994). Alternatively, we could hypothesize that the shape of the eggshell also exerts a determining influence on the cells' configuration. We may expect that in long eggs the posterior blastomeres would have a fixed configuration, because there is little space for reorganization to the P4-D-C pattern, while this restriction is not present in more round eggs. A Wilcoxon Mann-Withney test showed that there is a significant difference in developmental tempo between a variable and a fixed configuration ($P=0.0147$). However, Fig. 4 reveals that long nematode eggs, with an egg shape index around 40, have both variable (*M. incognita*) and fixed (*Procephalobus* sp.) configurations. More round eggs with an egg shape index around 60 also display both variable (*C. cubaensis*) and fixed (*O. dolichuroides*) configurations.

DISCUSSION

Is the configuration of the posterior cells a valuable phylogenetic marker? With the exception of *H. gingivalis*, the configuration P4-D-C is found in all species of clade III-X. Thus, the configuration of the posterior cells is apparently uninformative. Furthermore, in clades where variation in configuration was observed (in *D. coronatus* and in clade XI+XII), this variation proved to be intraspecific. A variable configuration of the configuration of the posterior cells apparently has arisen at least twice independently within the phylum: once in an ancestor of clades XI and XII, and once in a member of clade IX (*D. coronatus*). Thus, the configuration of the posterior cells is a good phylogenetic marker to delineate clades XI and XII; however, as a diagnostic marker it cannot be used since it shows parallel evolution in clade IX. Using experimental interference, Laugsch & Schierenberg (2004) also found variable configurations within clade IX. They analyzed three *Rhabditis* species (family Rhabditidae) in this way and found that in two of them, *R. belari* (2/4) and *R. dolichura* (9/11), a reversal of polarity in P3 was seen in some cases, resulting in the configuration C-P4-D. By looking at the configuration of the posterior cells, we have shown that *M. incognita* and Cephalobidae both have a similar variable positioning of their posterior cells. This is in agreement with the sister relation of the Tylenchomorpha and the Cephalobidae (Holterman *et al.*, 2006; Bert *et al.*, 2008). Other parameters typical for clades XI and XII were described by Goldstein *et al.* (1998), who analyzed how asymmetry is generated along the antero-posterior (A-P) axis by analyzing the presence of a cytoplasmic rearrangement in the uncleaved embryo, and whether the site of sperm entry predicts the posterior of the embryo. They found that the mechanism, typical for *Acrobeloides* sp., is an apomorphic character, which may have arisen once in an ancestor of clades X, XI and XII. In addition Dolinski *et al.* (2001) described similar early developmental characters (asynchronous cleavage and early establishment of the P4 cell) for Cephalobidae and Tylenchomorpha with ancestral morphological characters (Tylenchidae and Anguinidae). In contrast, typical for the more derived Tylenchomorpha is a synchronous cleavage and a late establishment of the P4 cell (our data of *M. incognita*).

Migrations restore contact between endodermal line and germline. All examined members of clades XI and XII and *D. coronatus* (clade IX) show a variable configuration of their posterior cells in their embryo. In all cases where the *C. elegans* configuration is not reached after the division of P3, subsequent cellular migrations restore the contact between the germline and the endodermal precursors, leading to the *C. elegans* spatial arrangement (P4-D-C) before the onset of gastrulation, which suggests that this configuration is needed for normal further development (Skiba & Schierenberg, 1992). Contact between germline and gut is a common feature in many species.

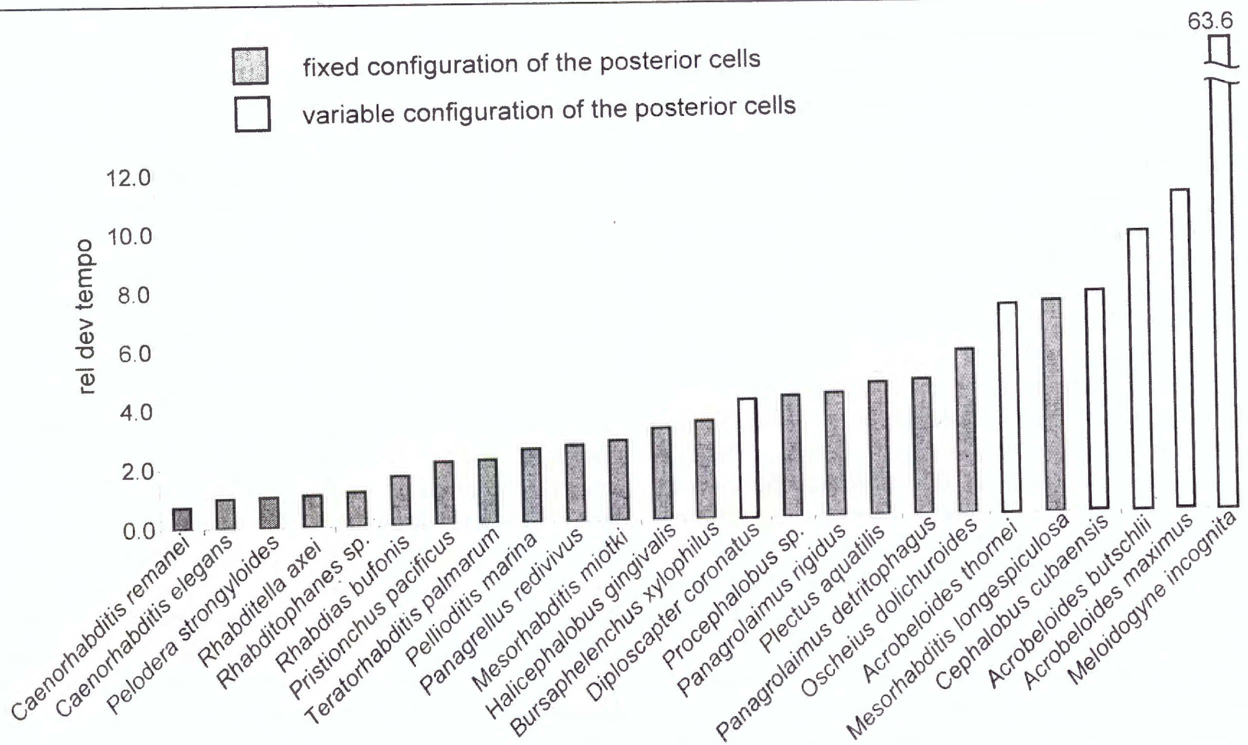

Fig. 3: Relative early developmental tempo of all analyzed species, expressed as number of times slower than *C. elegans*. (time from of the zygote's second division until the division of the endodermal precursor cell E). The configuration of the posterior cells was visualized as follows: white=variable configuration, grey=fixed configuration.

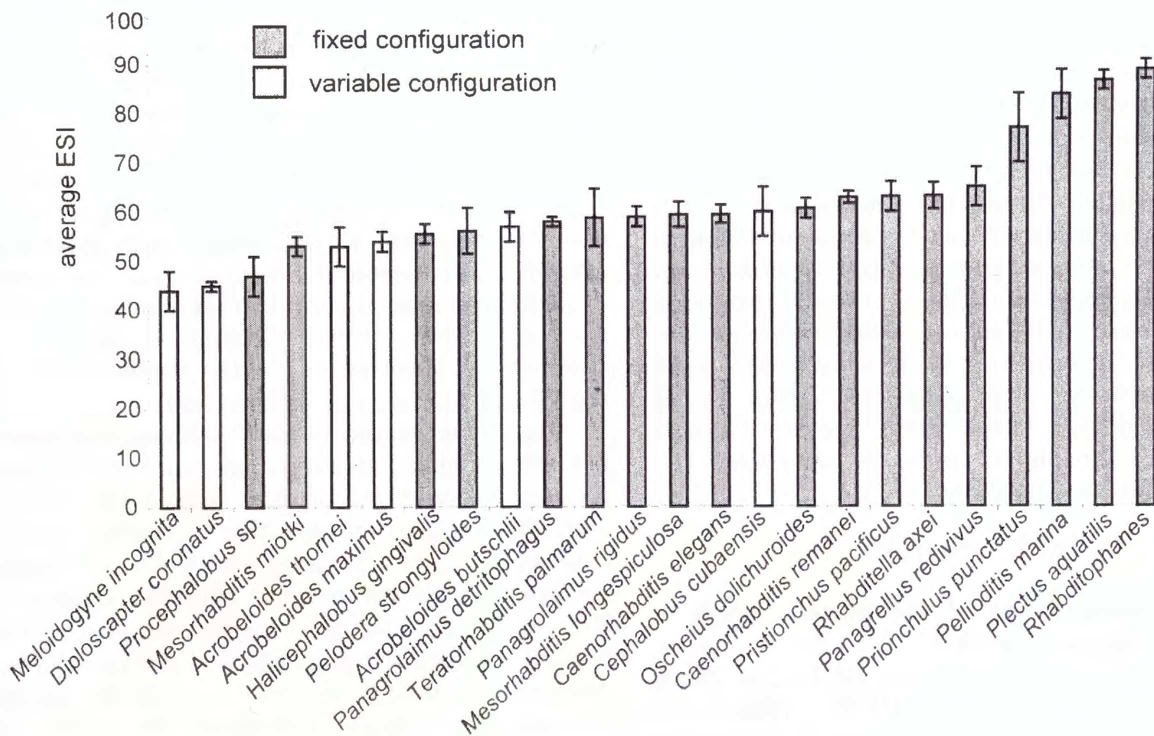

Fig. 4. Egg shape index (ESI) of all analyzed species. The configuration of the posterior cells was visualized as follows: white=variable configuration, grey=fixed configuration. Error bars represent the standard error.

Primordial germ cells have a similar pattern of migration in *Drosophila*, *Xenopus*, chick and mouse. In each case the primordial germ cells associate with the developing gut, from which they migrate to the gonads during organogenesis (Wylie, 1999).

Why is the mechanism of strict regulation lost? We conclude that a variable configuration of the posterior cells has arisen independently on at least two occasions within the phylum. Then an important question arises: why would this variable patterning evolve with no apparent evolutionary consequence, since the change is neutralized by compensatory migrations? The variable polarity of the germline divisions suggest that in these nematodes, the mechanism which tightly regulates the division axes of the germline cells is lost in these species; and time consuming migrations to restore the required *C. elegans* configuration before gastrulation are no obstacle. This agrees with the low maternal control in *A. nanus* (clade XI), in which early development proceeds very slowly and speeds up later when zygotic expression becomes active (Wiegner & Schierenberg, 1998). However, in the fast developing nematode *C. elegans*, essential decisions, including fixed division axes which assure the correct positioning of early blastomeres, have shifted to a very early phase of development in conjunction with early segregation of maternal factors. For example, in *C. elegans* the MES-1 protein, which is localized to the boundary between the germline and gut cells, is required for unequal divisions of the germline and EMS (Berkowitz & Strome, 2000; Bei *et al.*, 2002). In embryos with a mutation in this maternal-effect gene, P0 and P1 divide normally, but P2 and P3 partition P-granules to both daughters, leading to defects in cleavage asymmetry. The variable polarity in germline divisions in clades XI-XII and *D. coronatus* indicates that no tightly regulated molecular MES-1-like mechanism is present in these species, and that the ultimate position of the posterior cells must be regulated later during the compensatory migrations. Moreover, the observed variable arrangements of blastomeres within one species suggest that certain inductive cell interactions found in *C. elegans* probably do not take place in these nematodes, and cell fates are specified in another way. Wiegner & Schierenberg (1998, 1999) demonstrated that *A. nanus* shows aspects of regulative development. Whether other members of the family *Cephalobidae* also exhibit this regulative fate specification mechanism is not clear yet. Schierenberg (2000) mentioned that other *Cephalobidae* behave similarly, but until now experimental confirmation is missing.

Could it be that nematodes with a variable configuration of their posterior cells have a different strategy because their environment does not require the ability to reproduce quickly, and hence, strict regulation of the division axes of their germline is unnecessary? In many situations in the animal kingdom, environmental features change ontogeny. For example, *Daphnia* develop modified shapes in response to the presence of predators, and food supply may determine developmental pathways in other animals (Raff, 1996). When we look at the habitat of nematodes with a variable configuration of their posterior cells, we find plant-parasitic nematodes (clade XII), free-living bacteriovorous nematodes, often found in sandy soils at extreme temperatures (clade XI) (De Ley, 1992), and thermo-tolerant free-living nematodes, commonly found in compost, sewage and the rhizosphere of agricultural soils (*D. coronatus*, Gibbs *et al.*, 2005). Thus, there seems to be no correlation between the environment and this alternative developmental program without fixed division axes of germline divisions, resulting in variable configurations of the posterior cells.

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Резюме. Методом нанесения различных конфигураций расположения клеток задней части зародыша нематод на их филогенетическое древо, построенное по последовательностям малой субъединицы рибосомы (Holterman *et al.*, 2006), проведена реконструкция эволюционной истории изменений по этому признаку в пределах класса. Проанализировано эмбриональное развитие 19 видов нематод, а также добавлены литературные данные еще по 20 видам, что позволило привести данные по всем основным эволюционным линиям класса. Показано, что конфигурации свойственные *Caenorhabditis elegans* и большинству представителей эволюционных линий III-X, представляют собой предковое состояние для всех этих нематод. Альтернативные конфигурации возникают независимо друг от друга в эволюционных линиях IX, (*Diploscapter coronatus*), X (*Halicephalobus gingivalis*), XI (все изученные виды) и XII (*Meloidogyne incognita*). Изменчивые конфигурации бластомеров задней части зародыша возникают независимо как минимум дважды в эволюции нематод: в линии IX (*D. coronatus*), а также на предковой стадии для линий XI аи XII. Таким образом, данный признак может использоваться как филогенетический маркер для этих эволюционных линий. Статистические тесты, основывающиеся на исследованном материале, показывают связь между наличием в развитии изменчивых конфигураций, с одной стороны, и формой яйца и темпами развития, с другой.