

The demise of the Platonic worm

Ricardo B.R. AZEVEDO^{1,*}, Ana CUNHA¹, Scott W. EMMONS² and Armand M. LEROI¹

¹Department of Biology, Imperial College, Silwood Park, Ascot, Berkshire SL5 7PY, UK

²Department of Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY 10461, USA

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Summary – Nematodes are generally considered to have an adult cell number that does not vary among wildtype individuals as a consequence of invariant cell lineages (eutely). However, there is extensive evidence that at least some cell lineages can be variable in nematodes. In a comparative study of 13 free-living nematode species, we have shown that the adult epidermis of most species contained variable numbers of nuclei and that this variance was positively correlated with mean epidermal nuclear number. Here we present simulations of the lateral seam cell lineages of four species and show that variance in cell number is influenced by lineage topology, as well as by the frequency of lineage variants. We show that the epidermal variability of *Panagrellus redivivus* cannot be accounted for by the complexity of its lineage, but requires higher levels of lineage variability than are found in *Caenorhabditis elegans*, *Oscheius myriophila* and *Rhabditella octopleura*. Our findings suggest that many nematodes may have tissues composed of indeterminate numbers of cells formed from variable lineages and, as such, resemble other metazoans.

Résumé – Le décès du ver platonique – Les nématodes sont généralement considérés comme ayant un nombre de cellules invariable chez les individus de type sauvage, conséquence d'un lignage cellulaire fixe (eutélie). Cependant, il est d'évidence qu'au moins certains des lignages cellulaires peuvent varier chez les nématodes. Dans une étude comparative portant sur 13 espèces de nématodes libres, nous avons montré que l'épiderme de la plupart de ces espèces comportait un nombre variable de noyaux et que cette variabilité était corrélée positivement avec le nombre de noyaux épidermiques. Nous présentons ici des simulations des lignages cellulaires de la suture latérale de quatre espèces et démontrons que le nombre de cellules est influencé tant par la topologie du lignage que par la fréquence des variants de ce lignage. Nous montrons que la variabilité de l'épiderme de *Panagrellus redivivus* ne peut être mise au compte de la complexité de son lignage, mais demande des niveaux élevés de variabilité de ce lignage, tels ceux trouvés chez *Caenorhabditis elegans*, *Oscheius myriophila* et *Rhabditella octopleura*. Nos observations suggèrent que nombre de nématodes possèdent des tissus composés d'un nombre indéterminé de cellules dérivant de lignages variables et, de ce fait, ressemblent aux autres metazoaires.

Keywords – body size, branching process, *Caenorhabditis elegans*, cell number, Cephalobidae, eutely, lateral epidermis, Nematoda, Panagrolaimidae, Rhabditidae, simulations, variance, V cell lineage.

Nematodes are widely considered to be eutelic, *i.e.*, to have an adult cell number that does not vary among wild-type individuals as a consequence of invariant cell lineages (Malakov, 1994; Van Cleave, 1932). This Platonic view of nematode development probably results from two factors. First, the extrapolation of the remarkable constancy of *Caenorhabditis elegans* development to the rest of the phylum. Second, developmental biology tends to concentrate on robust, orderly aspects of development, rather than on variability (Sachs, 1994).

Although the majority of *C. elegans* somatic cell lineages are invariant, there are a few cases of variability in the postembryonic lineages which can lead to variation in cell or nuclear number (Sulston & Horvitz, 1977; Kim-

ble & Hirsh, 1979; Sulston *et al.*, 1983; Schnabel *et al.*, 1997). At hatching, the larval intestine usually comprises 20 cells, but occasionally an extra cell has been observed (Sulston *et al.*, 1983); four of the 14 intestinal nuclei that usually divide to generate binucleate cells may not do so (Sulston & Horvitz, 1977). In the ventral epidermis, P3.p in hermaphrodites and P9.p in males may or may not divide before fusing with the syncytium hyp7; in the male, U.1a and U.ra sometimes fail to divide; in hermaphrodites Q2.pp normally dies but may survive into the adult (Sulston & Horvitz, 1977). In hermaphrodites, P5.ppp and P7.paa sometimes divide at 25°C (Sternberg & Horvitz, 1986). It is possible that other lineage variations occurring at low frequency (*e.g.*, under 5%) have been missed,

* Corresponding author, e-mail: r.azevedo@ic.ac.uk

because it would be difficult to rule out observational error in such cases.

Furthermore, deviations from eutely have been described in other species of nematodes. *Panagrellus redivivus*, the only other nematode for which the post-embryonic lineage has been completely resolved, also shows variability (Sternberg & Horvitz, 1982). In the lateral epidermis, H2.ap, H2.pa and H2.ppa may or may not divide, and the V lineages show extensive variability; six of the 16 intestinal nuclei that normally divide to generate tetranucleate cells may fail to do so; in males U.la may or may not divide. In *Pelodera strongyloides*, P3.p and P9.p divide in nearly 50% of females and this percentage changes with temperature (Sommer *et al.*, 1994). In *Pellioiditis* sp. (VT684), the V1-V4 lineages are different at 10 and 20°C (Ambros & Fixsen, 1987). In the embryo of the marine nematode *Enoplus brevis*, at the eight-cell stage, one blastomere gives rise to only endoderm, whereas the fate of other blastomeres is not determined: they can produce neurones, pharynx, muscle and epidermal cells, of different body regions, in various proportions (Voronov & Panchin, 1998).

Cell lineage variability can also be deduced from the presence of among-individual variance (V_i) in nuclear number (bearing in mind that not all cell lineage variation will necessarily affect nuclear number). Larvae of the parasitic nematode *Oxyuris curvula* have variable nuclear numbers in the intestine, the epidermis and the vulva, but not in other tissues (Martini, 1923). *Rhabditis anomala* adults have variable nuclear numbers in the intestine, epidermis, ventral nerve chord and vulva, but not other tissues (Wessing, 1953). Several species of marine nematodes exhibit variable numbers of epidermal cells (Rusin & Malakhov, 1998; Voronov & Panchin, 1998).

The above examples suggest that deviations from eutely are quite common in nematodes, especially in the intestine and epidermis; yet the view that eutely is the rule in nematodes persists (Malakov, 1994). A definitive test of the generality of eutely in a given tissue requires a comparative study of cell lineages or, more realistically, variances in nuclear number. We have measured V_i in nuclear number for the adult lateral epidermis, in 13 species of free-living nematodes from three families (Cephalobidae, Panagrolaimidae and Rhabditidae) that spanned the range of adult body size and adult epidermal cell number typical of free-living terrestrial nematodes (Cunha *et al.*, 1999). As in *C. elegans*, all these species have epidermal syncytia containing nuclei produced by a series of lateral seam cells which cease to proliferate prior to adulthood. Here

we review the results of that study and introduce a novel approach which allows us to infer the probability of lineage variation in different species, based on their lineages. We modelled the V cell lineages of different species and determined the resulting distribution of epidermal nuclear counts by simulation.

Materials and methods

NEMATODE SPECIES

We used 13 species of free-living nematodes from three families (Fig. 1): Cephalobidae (*Acrobeloides maximus* and *A. nanus*), Panagrolaimidae (*Panagrolaimus redivivus* and *P. rigidus*) and Rhabditidae (*Caenorhabditis elegans*, *C. sp.* PS1010, *Oscheius dolichuroides*, *O. myriophila*, *Oscheius sp.* DF5000, *Pellioiditis sp.*, *Pellioiditis typica*, *Rhabditella octopleura* and *Rhabditoides regina*). The two *Acrobeloides* species are parthenogenetic. The remaining species are gonochoristic, except *C. elegans*, *O. myriophila* and *Oscheius sp.* DF5000 which are hermaphroditic.

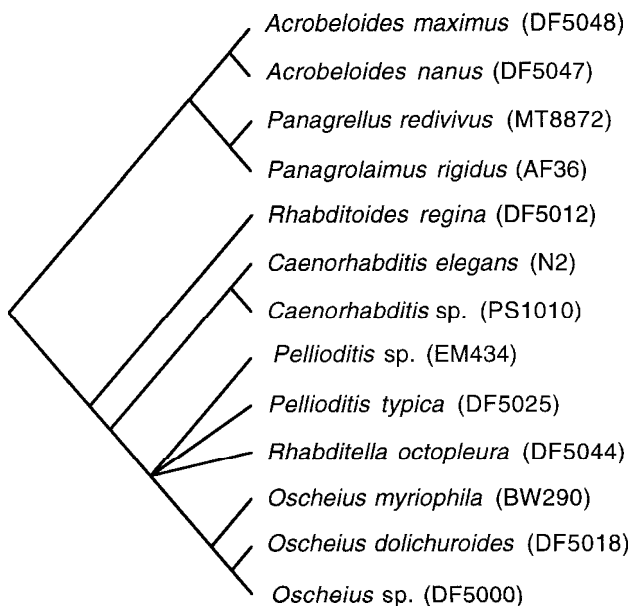


Fig. 1. Phylogenetic relationships between the 13 species of free-living nematodes studied (strain names in parentheses), based on SSU and 18S ribosomal DNA sequences (Fitch *et al.*, 1995; Blaxter *et al.*, 1998).

MEASUREMENTS

All strains used were maintained in the laboratory in standard culture conditions for *C. elegans* (Sulston & Hodgkin, 1988) at constant 20°C. Low density cultures were set up for each species and ten to 14 early adult females or hermaphrodites (*i.e.*, containing alae, open vulva but without eggs), were mounted in agar pads containing 1-3% 1 M sodium azide solution. Each individual was observed under a compound microscope using Nomarski optics at $\times 1000$ magnification. All visible lateral epidermal nuclei on one side of the body, between the posterior end of the pharynx and the anus, were photographed with a video camera connected to an Apple Macintosh computer running NIH Image 1.6. Afterwards, the worm was dismantled and allowed to recover from the anaesthetic, and then was remounted. When possible, the process was repeated again so that each worm was completely photographed two or three times on the same side. From each set of pictures for a given worm, we generated an independent reconstruction of the lateral epidermis, using Adobe Photoshop 2.5 for Macintosh, and counted the epidermal nuclei. We measured body length (L) of each individual at $\times 40$ -200 magnification using Object-Image 1.6 (Vischer *et al.*, 1994).

STATISTICAL ANALYSES

Nuclear number is the best linear unbiased estimate of nuclear number for each species from a one-way random effects linear model. V_i is the maximum-likelihood estimate of the among-individual variance component from the same model (Searle *et al.*, 1992). The model also includes the variance component for measurement error V_e . There was significant among species heterogeneity of V_e . Furthermore, V_e significantly increased with nuclear number among species (Spearman rank-correlation, $\rho = 0.64$, $P = 0.02$) but was not significantly correlated with V_i ($\rho = 0.39$, $P = 0.2$). Therefore, we can conclude that the use of repeated cell counts per individual was important in this study; if single counts had been done the variance would be overestimated in species with more cells, resulting in a spurious correlation.

Phylogenetic contrasts of log-transformed variables were obtained from CAIC 2.0, assuming equal branch lengths (Felsenstein, 1985; Purvis & Rambaut, 1995; assuming more realistic branch lengths (Blaxter *et al.*, 1998) did not affect the results). Basic statistics were calculated using JMP 3.2.2. (SAS Institute Inc., Cary, NC, USA).

CELL LINEAGES

The V cell lineages of *O. myriophila* and *R. octopleura* were determined using standard *C. elegans* methods (Sulston & Horvitz, 1977; Sternberg & Horvitz, 1982). Every cellular event in the canonical lineages was confirmed at least three times.

VIRTUAL CELL LINEAGES

The V cell lineages were modelled as a multitype, discrete-time branching process. For example, consider the *C. elegans* V1 lineage (Fig. 3). This consists of three cell types: seam cells, epidermal cells that have not fused with the syncytium and syncytial cells. We start with one seam cell (at $t = 0$). Rounds of cell division occur at successive discrete times ($t = 1, 2, 3, \dots$). This is a fair simplification given the regularity of the cell lineages, but more complex models that include variability in the times of cell division can also be constructed. At each time point, cells are 'programmed' to divide in a particular way: asymmetrically at $t = 1$, symmetrically at $t = 2$, and asymmetrically thereafter. Variant outcomes can be incorporated with specified probabilities: for example, at each time, the seam cells may divide once with a probability of 90%, not divide with a probability of 5% or divide twice with a probability of 5% (Fig. 4). To simulate an individual, each of the V1-V6 lineages is run independently and the final cell number is added up.

The lineage simulations were written in Visual Basic using Excel 98. For each combination of values of t and d (forming a log-scale 7×7 grid), 1000 replicate samples of ten individuals were simulated, and the variance in nuclear number V_i was calculated for each sample. Empirical 95% confidence intervals of predicted V_i were calculated as the 2.5 and 97.5% quantiles of the distribution of 1000 sample V_i .

Results

VARIANCE IN NUCLEAR NUMBER

C. elegans shows non-significant V_i for epidermal nuclear number (likelihood ratio test, $P = 0.08$). This is to be expected since variation in lateral seam cell lineages has never, to our knowledge, been observed (Sulston & Horvitz, 1977). Similarly, four other species (*Caenorhabditis* sp. PS1010, *O. myriophila*, *P. rigidus* and *R. octopleura*) do not display a significant deviation from eutely.

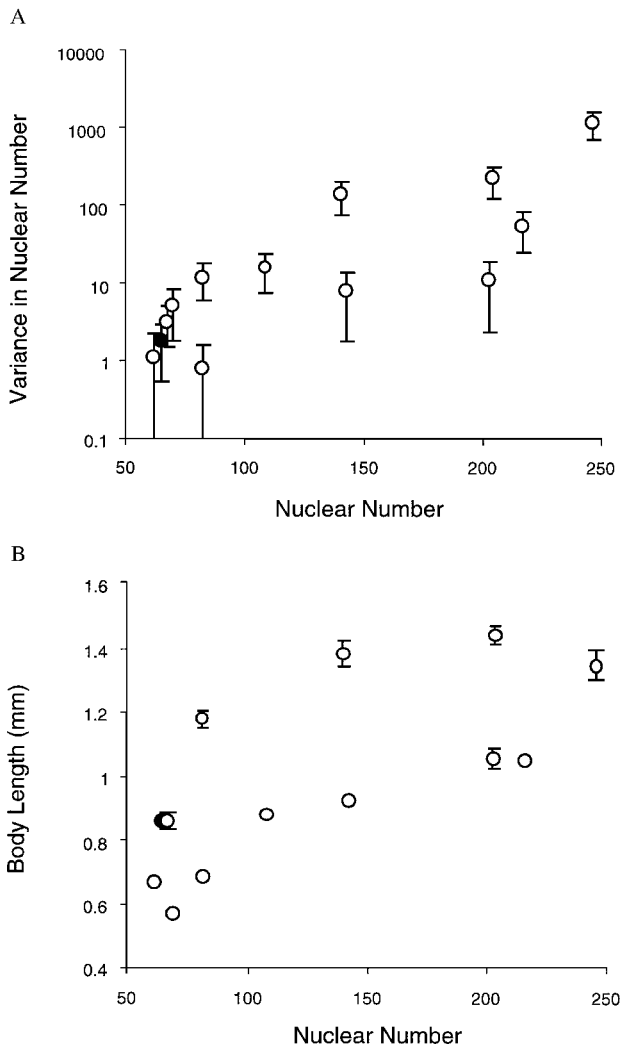


Fig. 2. Comparative study of the relationship between body length (L), number of epidermal nuclei (N) and its among-individual variance (V_i) (data from Cunha et al., 1999). *Caenorhabditis elegans* is represented by a filled circle. A: Interspecific relationship between N and V_i (\pm s.e.); B: Interspecific relationship between N and L (\pm s.e., hidden by the points in most cases).

However, the remaining eight species show significant V_i for nuclei number; notably, three species (*P. redivivus*, *Pellioditis* sp. EM434 and *R. regina*) show $V_i > 100$ (Fig. 2a).

Among species, V_i increases with nuclear number (Spearman rank-correlation, $\rho = 0.84$, $P = 0.0004$; Fig. 2A) and with body length ($\rho = 0.59$, $P = 0.03$). Body length also increases with nuclear number ($\rho = 0.73$, $P = 0.004$; Fig. 2B). We tested whether these

relationships were phylogenetic artefacts by regression through the origin of independent contrasts (Purvis & Rambaut, 1995). We find that V_i increases with nuclear number ($P = 0.006$) but not with body length ($P > 0.1$). We can conclude that the relationship between V_i and nuclear number has evolved repeatedly. Reproductive mode (parthenogenetic, hermaphroditic and gonochoristic) did not have a significant effect on any of the traits studied ($P > 0.1$).

VIRTUAL CELL LINEAGES

The evolution of V_i must be caused by evolutionary changes in lateral seam cell lineages. To understand this better we modelled the V cell lineages of four species (Fig. 3) and determined the resulting distribution of epidermal nuclear counts by simulation (the T and H lineages were ignored since our nuclear counts cover only the area between the posterior bulb of the pharynx and the anus).

The models assumed that: *i*) the canonical lineage was the modal one, *ii*) only particular kinds of variation could occur in these lineages, *iii*) variation in one part of the lineage was independent of variation elsewhere in that lineage, or other lineages within the same worm, and *iv*) the probability of variation was constant. Two kinds of variants were incorporated in the models (Fig. 4): *i*) cell fate transformations (seam cell to epidermal cell, and vice-versa), with probability t , and *ii*) gains or losses of seam cell divisions, with equal probability $d/2$. We allowed these types of variation in cellular behaviour since they, and only they, have actually been observed among wildtype individuals of any species (Sternberg & Horvitz, 1982; Ambros & Fixsen, 1987). Furthermore, these kinds of variation are analogous to lineage differences among species (Sternberg & Horvitz, 1982; Ambros & Fixsen, 1987; Sommer et al., 1994; Fig. 3). Relative to *C. elegans*, the canonical V1-4 lineages of *O. myriophila* show seam cell division gains, while those of *R. octopleura* show seam cell division gains and epidermal to seam cell transformations.

The simulations suggest two general reasons why species with many nuclei might be more variable than those with fewer. First, for any set of values of t and d , more complex lineages yield a higher V_i relative to simpler lineages. This effect can be seen by the increasing elevation of the theoretical V_i surface as cell number increases among the four species (Fig. 5). For example, for $t = 0.1\%$ and $d = 1\%$ the simulations predict the following $V_i = 2$ (95% confidence interval: 0.1-8.6) for *C. ele-*

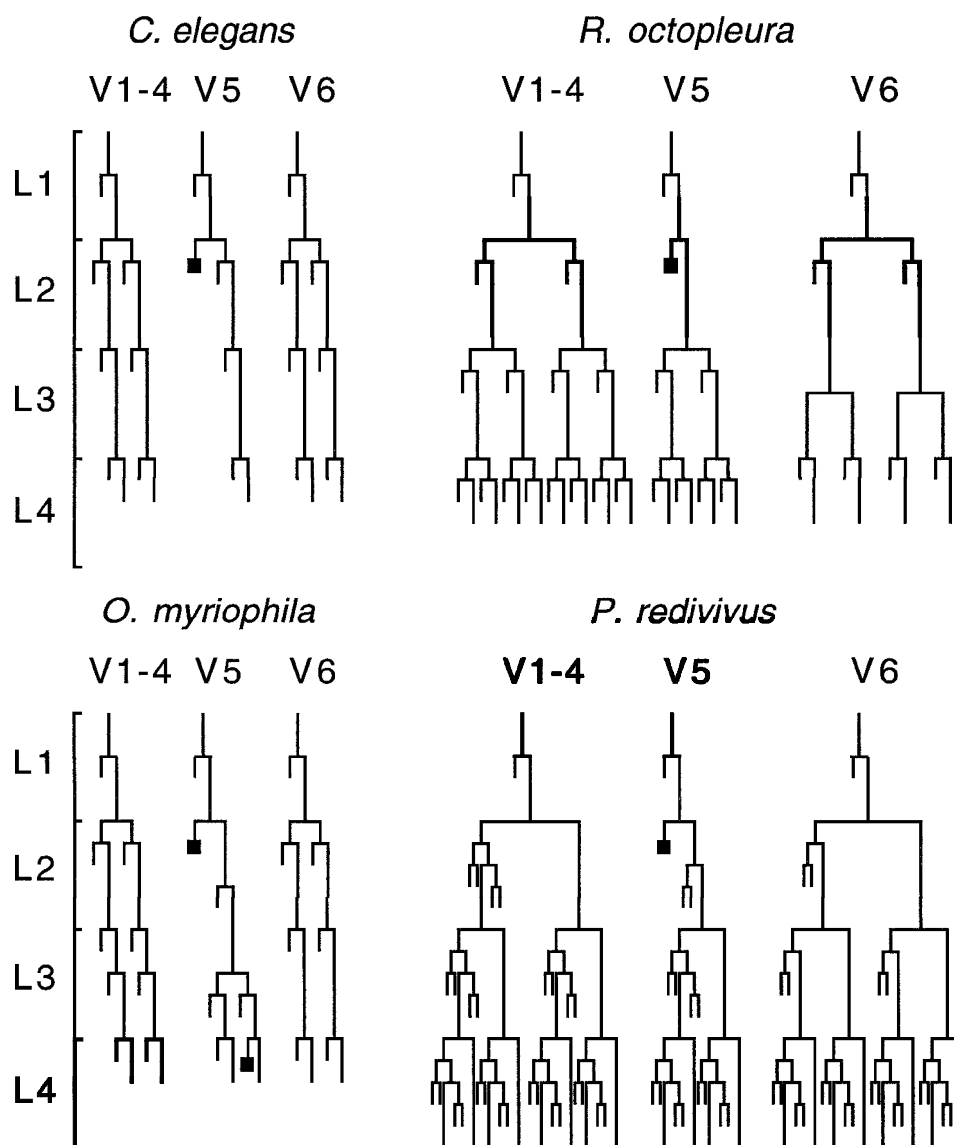


Fig. 3. Canonical V cell lineages of four species. At hatching, a row of seam cells extends from head to tail along each side of the first stage larva (L1) of every species. The V1-V6 seam cells go through a characteristic series of divisions during larval development. A seam cell either divides symmetrically producing two seam cell daughters, or asymmetrically producing a daughter that fuses with the multinucleate epidermal syncytium *hyp7*, and a daughter that will become the new seam cell. V5 lineages produce neuroblasts (■) which divide to form postdeirids (not shown). The canonical lineages of *Caenorhabditis elegans* and *Panagrellus redivivus*, were already known (Sulston & Horvitz, 1977; Sternberg & Horvitz, 1982), but those of *Rhabditella octopleura* and *Oscheius myriophila* are new.

gans, 3 (0.2-12) for *O. myriophila*, 15 (0.9-56) for *R. octopleura*, and 55 (6-201) for *P. redivivus*. Second, V_i increases with the frequency of variant epidermal lineages relative to the species' canonical lineage (Fig. 4). The results of these simulations can also be derived mathematically (Azevedo *et al.*, unpubl.).

EVOLUTION OF CELL LINEAGE VARIABILITY

Is the observed evolution of V_i in these four species merely a consequence of differences in lineage complexity, or do species with more complex lineages also deviate more often from their canonical lineage than those with simpler lineages? To distinguish between these hypothe-

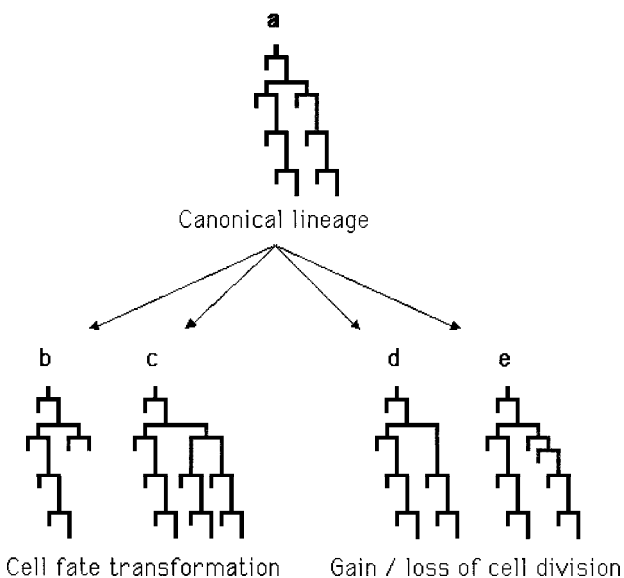


Fig. 4. Types of cell lineage variation incorporated in the simulation models, illustrated for the case of the *Caenorhabditis elegans* V6.pp asymmetric seam cell division. *a*: The canonical V6 lineage produces nine cells; *b*: If the seam cell V6.ppp becomes an epidermal cell and instead of proliferating fuses with the syncytium, an entire sublineage is lost and only seven cells are produced; *c*: Alternatively, if V6.ppa is transformed into a seam cell, an entire sub-lineage is gained and V6 gives rise to 11 cells; *d*: If the division of V6.pp is suppressed, its anterior epidermal daughter is lost and only eight cells are generated; *e*: But if there is an extra division of V6.pp, an anterior epidermal daughter is gained and ten cells are produced.

ses we looked for the range of values of t and d which predicted values of V_i matching the observed values of V_i for each species. The ranges of values of t and d compatible with observed V_i , overlap for *C. elegans*, *O. myriophila* and *R. octopleura*; however, that of *P. redivivus* does not overlap with the other species (Fig. 5). Therefore, the high observed V_i of *P. redivivus* results not only from the complexity of its lineage, but also from a greater propensity to deviate from the canonical lineage.

The highest simultaneous values of t and d (i.e., those maximising the product of the two parameters) which predict the observed V_i for each species are: $t = 0.05\%$ (upper limit, UL = 0.34%) and $d = 0.21\%$ (UL = 3.3%) for *C. elegans*; $t = 0.01\%$ (UL = 0.09%) and $d = 0.03\%$ (UL = 0.64%) for *O. myriophila*; $t = 0.02\%$ (UL = 0.20%) and $d = 0.16\%$ (UL = 1.3%) for *R. octopleura*; $t = 3.55\%$ (lower limit, LL = 0.95%) and $d = 33\%$ (LL = 8.8%) for *P. redivivus*. These values imply that anyone lineaging all the V cells of *C. elegans*

from start to finish (both sides), would expect to observe (on average) one non-canonical cell fate or gain/loss of a division in 25% of worms lineaged (5 and 35% for *O. myriophila* and *R. octopleura*, respectively). In contrast, someone lineaging the V cells of *P. redivivus* would not expect to observe any fully 'canonical' worms.

Can we predict the relationship between nuclear number (N) and V_i , for a given probability of lineage variation? We have seen that as t or d increase the expected V_i increases. However, for a large range of values of t and d , among the four species $\log V_i$ increases linearly with $\log N$ with a slope of *ca* 2.5 (Fig. 6). This means that, if the evolution of V_i is entirely a result of changes in lineage topology, without changes in probability of lineage variation, then we would expect a linear relationship between $\log V_i$ and $\log N$ with a slope of *ca* 2.5. As we have seen, the observed relationship, corrected for phylogeny, has a slope of 3.2 ± 2.0 (based on the raw data it is 3.5 ± 1.6 , 95% CL) which is steeper than 2.5, but not significantly so. In conclusion, although it is likely that, at least, *P. redivivus* has evolved a higher probability of epidermal lineage variation in the epidermis, the overall pattern in V_i seems to have been generated mostly by changes in lineage topology. Note also that there are some outliers to the general trend we are describing: *P. rigidus*, a species with a relatively high number of epidermal cells, shows very low V_i .

Discussion

Our results show that, in free-living nematodes closely related to *C. elegans*, epidermal nuclear number variability is positively associated with epidermal nuclear number (Cunha *et al.*, 1999). Furthermore, our lineage simulations show that this pattern results from combined changes in lineage topology and the probability of lineage variation.

Our model assumes that worms do not regulate cell number during development of the V lineages, but this may not be realistic. In *C. elegans*, experimental production of a large gap between Vn cells, may result in extra proliferation of Vn.a cells (Sulston & White, 1980). Evolutionary differences in V_i could, then, be explained by differences in regulation, rather than differences in t and d . However, such an explanation would imply very high levels of lineage variability (and great regulatory ability throughout the V cell lineages) in *C. elegans*, neither of which have ever been observed (Sulston & White, 1980). On the other hand, considerable lineage variability has been directly observed in *P. redivivus* (Sternberg &

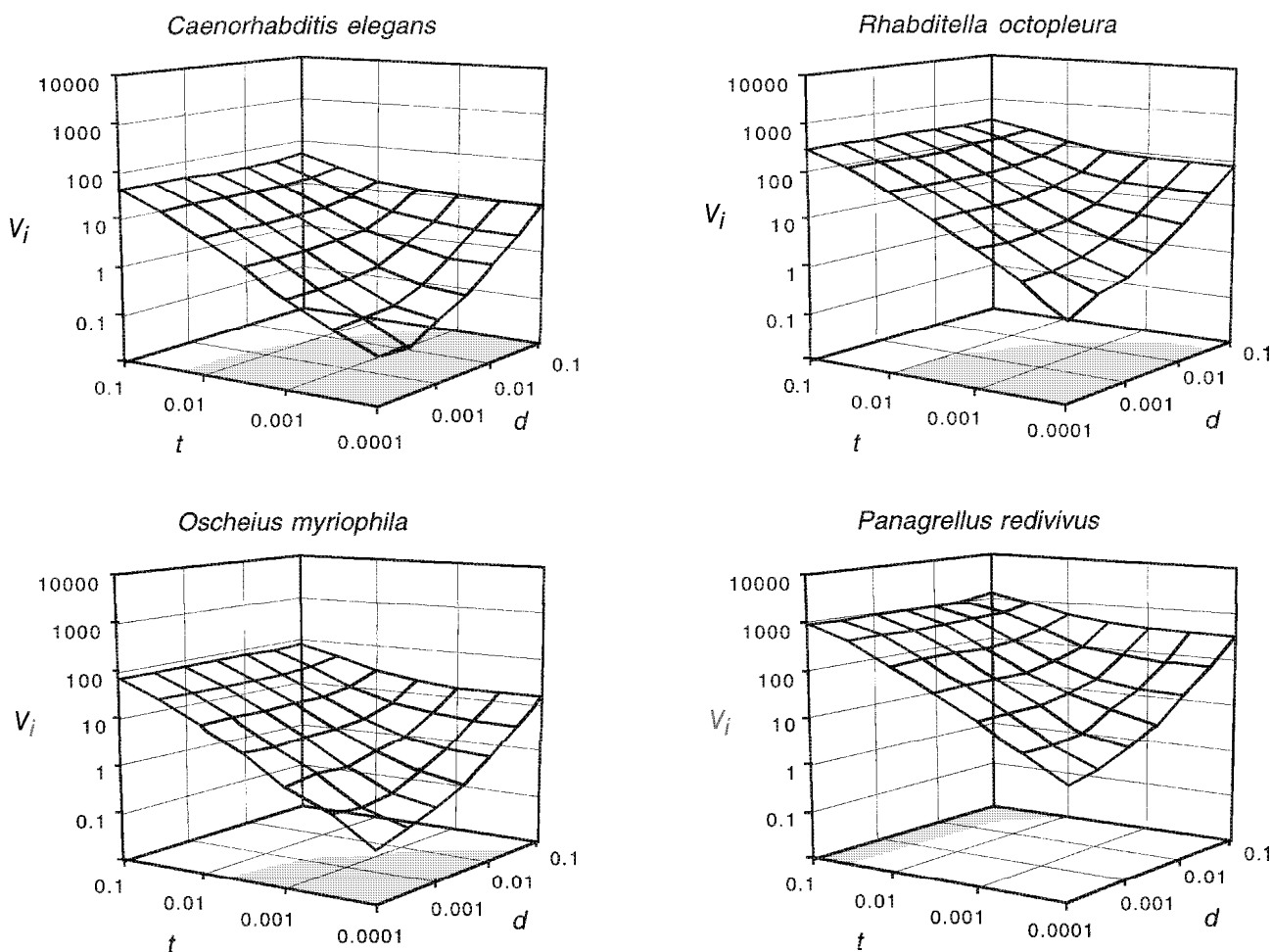


Fig. 5. Results of the V cell lineages simulations for each species (Fig. 3). The surfaces connect the means of V_i for each set of parameters (see Methods). The shaded areas indicate the parameter spaces for which observed V_i was contained within the 95% confidence intervals of predicted V_i .

Horvitz, 1982) and in another high V_i species, *R. regina*. The capacity for cell number regulation in these species deserves further investigation and would permit more realistic cell lineage models which include regulation (e.g., by specifying covariances between the numbers of cells descending from each V cell).

Does the existence of lineage variation in some species imply that they specify their lateral seam cell lineages in ways fundamentally different from that of more eutelic species such as *C. elegans*? Although V cells use cell autonomous specification mechanisms they also require particular cell-cell contacts and long range signals to specify canonical lineages (Waring *et al.*, 1992; Austin & Kenyon, 1994; Antebi *et al.*, 1998; Hunter *et al.*, 1999). It may be

that as cell lineages increase in complexity in the course of evolution, the transmission and reception of these signals becomes less accurate, thus giving rise to an explosion of lineage variants. Although this explosion is associated with evolutionary increases in body size, it may be highly tissue specific. In *P. redivivus*, extensive variability was observed in the V cell descendants (Sternberg & Horvitz, 1982), and these are the only postembryonic blast cells with very much more complex lineages than their *C. elegans* homologues (Sulston & Horvitz, 1977; Sternberg & Horvitz, 1982; Fig. 2). Even so, given that the terrestrial free living nematodes studied here are smaller (Kirchner *et al.*, 1980) and have fewer epidermal nuclei (Malakov, 1994) than most marine and parasitic nema-

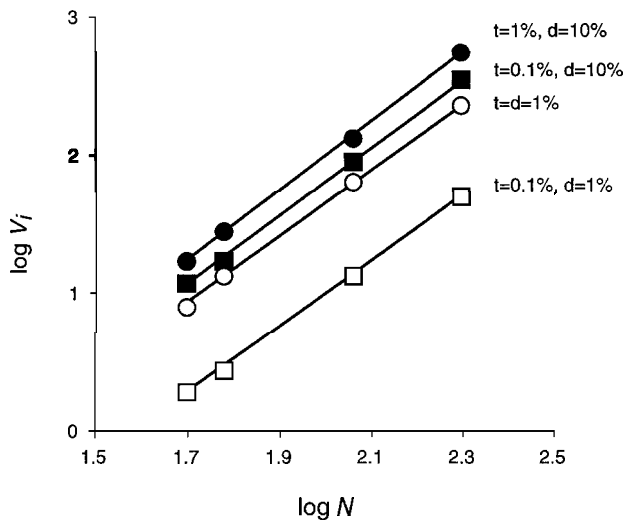


Fig. 6. Predicted values of V_i for each species from simulations using different probabilities of lineage variation plotted against the species' cell number (N). The slopes of the lines vary between 2.39 and 2.51.

todes, it is likely that the majority of nematode species are not eutelic.

Acknowledgements

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