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The 18S ribosomal RNA gene of *Soboliphyme baturini* Petrow, 1930 (Nematoda: Dioctophymida) and its implications for phylogenetic relationships within Dorylaimia

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Summary – Phylogenetic estimations from 18S rDNA sequence data reveal close relationships of dioctophymids with Trichinellida (Trichocephalida), the latter represented by the families Trichinellidae and Trichocephalidae (Trichuridae). This phylogeny is congruent with a scenario of molecular evolution deduced from conservative motifs within the V4 and V9 regions of 18S rRNA. A phylogenetic approach to analyse data containing highly unequal rates of sequence evolution is proposed. The entire gene possesses only a few conservative molecular synapomorphies of a clade consisting of Dorylaimida, Mononchida and Mermithida. Dioctophymida, together with Trichinellida, are inferred as a sister taxon to this clade which jointly constitute the Dorylaimia. Molecular data juxtaposed with morphology were used to reconstruct some of the putative features of the common ancestor of Dorylaimia which is speculated to have possessed a spear and, as found in extant Enoplia and Dioctophymida, pharyngeal gland outlets located in the stoma. Mononchids are postulated to have secondarily lost the spear contrary to all previously published phylogenies. Reduction of caudal glands and transformation of pharyngeal glands into the stichosome are not parsimonious across the tree of Dorylaimia. There are no unequivocal adult morphological synapomorphies for Dorylaimia; the only non-molecular diagnostic feature is the unique specification of the endodermal precursor in early embryogenesis.

Keywords – Bayesian inference, homoplasy, long branch attraction artefact, maximum likelihood, maximum parsimony, molecular phylogeny, secondary structure, SSU rRNA.

Phylogenetic systematics of Nematoda remains a difficult task (see Lorenzen, 1981; Malakhov, 1986; De Ley & Blaxter, 2002, for reviews). Molecular evidence has been decisive in adducing many issues in nematode phylogeny (Blaxter *et al.*, 2000; Coomans, 2000; De Ley, 2000). The 18S rRNA gene was successfully employed for inferring internal phylogeny of Rhabditida, the large-scale relationships of Chromadoria and delimitation between Enoplia and Dorylaimia (Aleshin *et al.*, 1998a; Blaxter *et al.*, 1998; Sudhaus & Fitch, 2001; De Ley & Blaxter, 2002).

The Dioctophymidais a small group of nematodes with about 30 species in three families (Karmanova, 1968), some of them having strong veterinarian and medical impact (Karmanova, 1968; Beaver & Theis, 1979; Measures, 1985; Eberhard *et al.*, 1989; Anderson, 2000). All dioctophymids are animal parasites with two or three hosts in the life cycle. The larvae persist in oligochaetes and then transfer to a vertebrate definitive host. Dioctophymida is undoubtedly a monophyletic group and is currently assigned ordinal status (De Ley & Blaxter, 2002). All representatives exhibit features unique to this taxon (autapomorphies), amongst which are *i*) muscular caudal alae (bursa) in males, and *ii*) muscle cells ('mesenteries') stretching between the body wall and the intestine. The head region in many dioctophymids is transformed into a mouth sucker (Petrow, 1930) or a spinose non-retractile platform (Schmidt-Rhaesa, 2000). Presence

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of a spear[1] in the JI is typical for all dioctophymids (Karmanova, 1968; Malakhov & Spiridonov, 1983) and links them to Dorylaimida *sensu* Lorenzen (1981), while the location of pharyngeal gland openings inside the stoma suggests a link to Enoplia. Different systems have placed the group inside the Enoplida as a suborder along with Enoplina and Dorylaimina (Chitwood, 1933), as a suborder inside the Trichinellida (Spassky, 1956), as an independent order (Filipjev, 1934) or an order related to Dorylaimida (Ryzhikov & Sonin, 1981; Malakhov, 1986; De Ley & Blaxter, 2002). No molecular evidence on the Dioctophymida has been previously examined for phylogenetic implication.

In this study we report analysis of the sequence of the 18S rRNA gene of *Soboliphyme baturini*, a representative of the family Soboliphymidae (Petrow, 1930). We discuss the phylogeny of Dorylaimia with respect to patterns of evolutionary change in the molecular structure of the gene and discuss possible ancestral states for some important morphological characters for this clade.

Materials and methods

Specimens of *Soboliphyme baturini* Petrow, 1930 were obtained from the stomach of sable (*Mustela zibellina* L.), its definitive host, caught in Kolyma Province, Siberia. Bodies of shot animals were stored frozen. Semi-thawed stomachs were dissected and nematodes were rinsed with ice cold physiological saline and fixed immediately with 96% ethanol. Three out of nearly 200 sables dissected contained specimens of *S. baturini.*

DNA was isolated with phenol extraction and precipitated with ethanol. The entire small subunit ribosomal RNA (18S rRNA) gene was amplified using universal eukaryotic primers for nuclear 18S rRNA coding regions (Medlin *et al.*, 1988). PCR products were purified on an agarose gel, cloned in the pGEM T-Vector System (Promega), and sequenced using the *fmol* DNA sequencing system (Promega) on both strands. The sequence was deposited with GenBank under accession number AY277895.

[1] Historically, protractile mouth structures are referred to under different terms in different nematode taxa (*e.g.*, onchiostyle in Trichodoridae and Dioctophymidae, odontostyle in Dorylaimida, (stomato)stylet in Tylenchida). In order to avoid misleading interpretations of the homology of this character across nematode taxa discussed, the neutral term spear is used throughout the text.

For phylogenetic analyses the 18S rRNA sequences were manually aligned according to the eukaryotic SSU rRNA secondary structure model of Van de Peer *et al.* (2000). Taxon names with corresponding GenBank accession numbers are given in Fig. 1. All alignment positions were utilised in phylogeny reconstruction, except for some positions in the occasionally incomplete flanking regions. In some analyses variable positions were assigned weights inversely proportional to the rates of substitution calculated for corresponding sites (for eight categories with invariants) either with the aid of TREE-PUZZLE 5.0 (Strimmer & von Haeseler, 1996) under the HKY model of molecular substitution (Hasegawa *et al.*, 1985), or with RevDNArates version 1.0.3 (Korber *et al.*, 2000) under the REV model (Yang, 1994).

Maximum parsimony (MP) and neighbour-joining(NJ) phylogenies were inferred with Dnapars.exe and Neighbor.exe programs from the PHYLIP 3.6a2.1 package (Felsenstein, 1993). MP search was conducted with the 'Print out steps in each site' option in effect. We traced the states of predicted apomorphic characters on a large alignment of metazoan 18S rRNA sequences compiled from the universal 18S rRNA Database (The University of Antwerp, http://oberon.rug.ac.be:8080/rRNA/; Van de Peer *et al.*, 2000). Maximum likelihood (ML) phylogenies were estimated with fastDNAml version 1.2.2 (Olsen *et al.*, 1994) and fastDNAml modified to incorporate the REV model of molecular substitution (Korber *et al.*, 2000). MP and NJ analyses were conducted with 1000 bootstrap replicates (Felsenstein, 1985). ML analyses had 20 bootstrap replicates due to time limitations. All suboptimal ML trees found were taken into account. Suboptimal trees obtained in ML searches were compared by Kishino and Hasegawa (1989) test. Bayesian inference (BI) was conducted with four simultaneous runs of Markov chain Monte Carlo (MCMC) algorithm implemented in MrBayes version 2.01 (Huelsenbeck & Ronquist, 2001). The Markov chains were run for 1 000 000 generations with sampling every ten generations for a total of 100 000 samples per run. The states of the chain before reaching stationarity were discarded as burn-in. Likelihood parameters for BI corresponded to the General Nonreversible Model (settings nst = 12, ncat = 8, rates = invgamma, shape = estimate, basefreq = estimate).

The closest living relative of Nematoda remains unknown, and several taxa were taken as outgroups including representatives of Ecdysozoa (Aguinaldo *et al.*, 1997) and of some 'pseudocoelomate' phyla. Such a multicompound outgroup is recommended in cases when the

ancestral character states are unknown (Pavlinov, 1990; Philippe, 2000).

Elements of the 18S rRNA molecule's secondary structure were modelled with *mfold* (Zuker *et al.*, 1999) and visualised with RnaVis (De Rijk *et al.*, 2003).

Results

The sequence of the 18S rRNA gene of *S. baturini* (disregarding primer sites) is 1743 bp long, which is within the typical range for Nematoda. It does not contain extensive indels that would hamper the alignment procedure. The final alignment includes 18S rRNA data for most nematode orders and all non-chromadorian genera published so far. This dataset was processed with MP, ML, BI, and NJ algorithms. Most of the topological elements of inferred trees are identical and supported by high values of bootstrap proportions (BP) or posterior probability (PP). Some nodes, relating to the deeper radiations within nematodes, are dependent on the inferring algorithm and/or have BP $< 50\%$. These few ambiguous nodes are collapsed in the consensus tree (Fig. 1). 18S rRNA sequence of *S. baturini* is included in Dorylaimia on MP, ML, NJ and major-consensus trees after resampling (Clade I *sensu* Blaxter *et al.*, 1998). The resulting clade represents the conventional orders Dorylaimida, Mononchida, Mermithida, Trichinellida and Dioctophymida. The estimate of the posterior probability for the clade to be correct equals 1.000 when the sequence of *S. baturini* is included, and bootstrap support is about 90% with slight variations depending on the particular inferring algorithm (Fig. 1). When the same dataset is processed without *S. baturini*, BP support for the Dorylaimia node falls drastically ranging from 60 to 70% (Table 1). The latter value is similar to BP for Clade I as estimated in previous studies (Blaxter *et al.*, 1998, 2000; Rusin *et al.*, 2001). Thus, adding *S. baturini* to the dataset consolidates Dorylaimia. The lower of BP for Dorylaimia without Dioctophymida is related to the placement of Trichinellida closer to the root of the nematode tree in many subsamples. For instance, in MP analyses this pattern occurs in 25.1% of bootstrap replicates, which is not surprising in view of the longer branch lengths leading to trichinellids in trees (Fig. 1) and considering the computational artefacts likely to occur in such cases (Felsenstein, 1978). Despite the branch leading to *S. baturini* being no shorter from those of trichinellids, this sequence is placed to the root of the tree only in 8.8% of the replicates.

Due to the presence of the highly divergent dioctophymid and trichinellid lineages inside the Dorylaimia clade, the number of molecular synapomorphies shared by all members of this clade is very few: there is only one such substitution – an A→C transversion within the 5′ branch of helix 23. In our dataset of more than 200 nematode 18S rRNA sequences, only *Desmodora ovigera* Ott, 1976 (accession number Y16913) also shares this transversion.

As noted, the *S. baturini* branch always joins up with Trichinellida (Fig. 1). This node is reconstructed in most parsimonious trees (BP $> 90\%$), is present in the MCMC consensus topology (PP $= 1.000$) and is also robust against modifying the dataset (data not shown). About 100 synapomorphies are inferred for this clade with *Dnapars*, 20 of them falling on sites that are inferred with no more than five substitutions per the most parsimonious tree. We traced the states of these characters on a larger alignment of metazoan 18S rRNA sequences which were obtained from the rRNA WWW Server maintained by the University of Gent (http://oberon.rug.ac.be:8080/rRNA/). Some apomorphies of the node appeared to have little homoplastic occurrence among nematodes and other Metazoa (1766 sequences, including 172 nematode taxa available). When these characters do occur outside the (Dioctophymida, Trichinellida) clade, they usually represent autapomorphies of entire monophyletic groups of different rank. Thus, a specific A→G transition at position 3838 of the larger alignment is characteristic of 15 acoelan turbellarians and five myzostomids, while a G→A transition at position 9698 occurs in 12 flatworms, mostly monopistocotylean monogeneans, 36 insect taxa, mostly culicoidean mosquitoes and reduviid bugs, and in nine representatives of Acanthocephala, which constitute a monophyletic clade according to recent findings (Herlyn *at al.*, 2003). On this dataset, the molecular synapomorphies of Dioctophymida and Trichinellida mentioned above have frequencies of homoplastic occurrence less than 10^{-2}.

Three synapomorphies of the (Dioctophymida, Trichinellida) node are situated within the predicted single-stranded region of the loop of helix 18 (Fig. 2). This 17 bases long loop is hypothesised to be one of the longest single-stranded regions of the 18S rRNA molecule secondary structure (Neefs *et al.*, 1993; Wuyts *et al.*, 2000). However, some residues in this region are potentially able to form Watson-Crick pairs, which are maintained in Dioctophymida and Trichinellida. Comparative analysis of a few substitutions known at these positions shows that single mutations were fixed less frequently than were

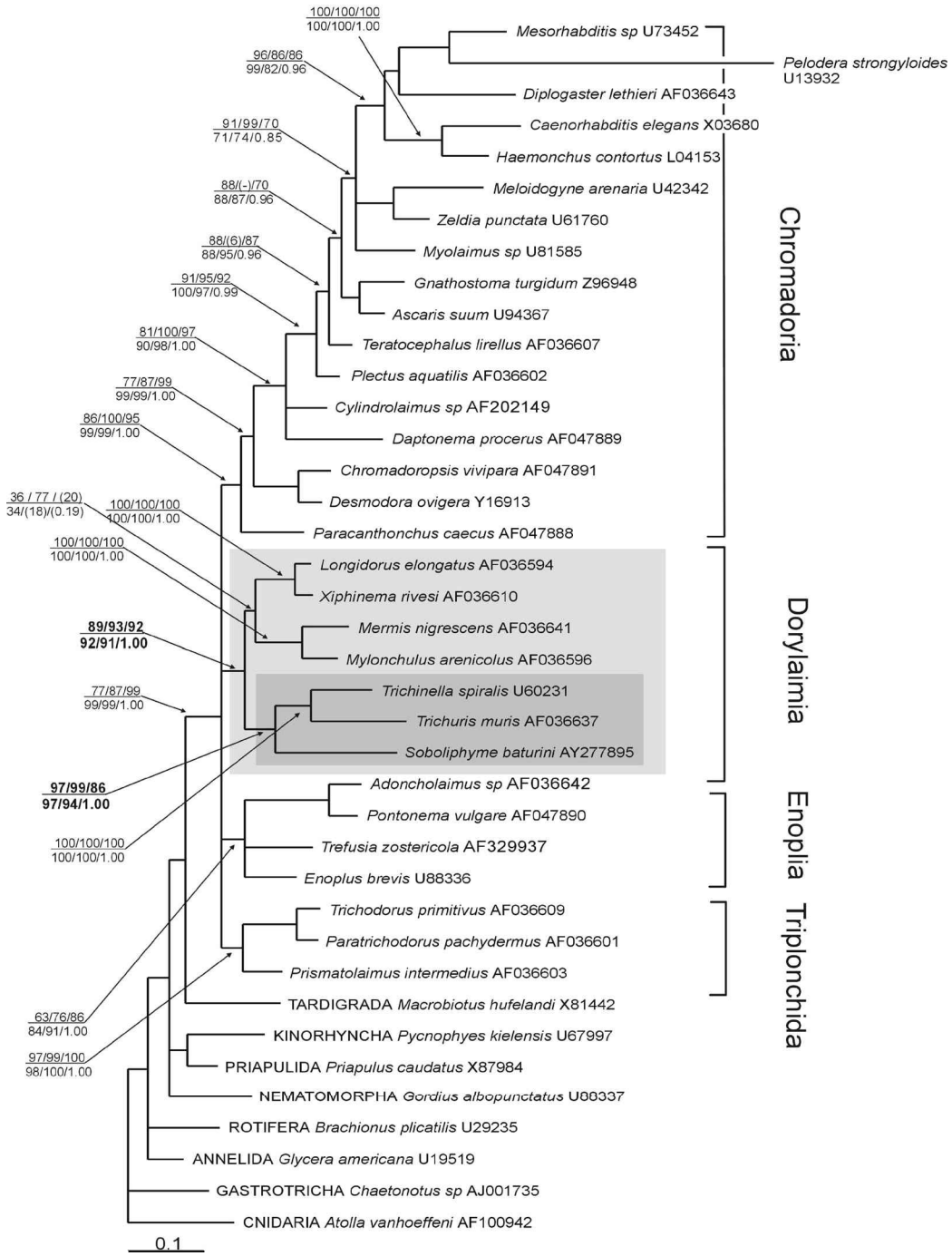

Fig. 1. *Phylogenetic position of Dioctophymida on the nematode tree inferred with 18S rDNA sequence data. Most nodes included in this general consensus trees are reconstructed with all algorithms. Ambiguous nodes are collapsed. Branch lengths are estimated with TREE PUZZLE 5.0. Values of support are given for selected nodes only. Values above the bar are MP, NJ and ML bootstrap percentages without gamma correction for rate heterogeneity, while values below the bar are bootstrap percentage for MP and ML with gamma correction and posterior probabilities for BI. If a node was not present in the 50%-majority rule consensus topology of an analysis, the corresponding statistics are given in brackets. Numbers of pseudoreplicates, evolutionary models employed, number of rate categories and BI parameters are described in text.*

Table 1. *Effect of taxon sampling on values of statistic support for alternative clustering of dorylaimian lineages.*

	All taxa					Dioctophymida excluded					Trichinellida excluded			
	MP (−Γ)	MP (+Γ)	ML (−Γ)[a]	ML (+Γ)[b]	BI	MP (−Γ)	MP (+Γ)	ML (−Γ)[c]	ML (+Γ)[d]	BI	MP (−Γ)	MP (+Γ)	ML (−Γ)[e]	ML (+Γ)[f]
a) Dorylaimia	89	92	92	91	1.000	66	72	75	78	1.000	89	94	95	96
b) Dioctophymida/Trichinellida	97	97	97	94	1.000	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
c) Mononchida/Mermithida	100	100	100	100	1.000	100	100	100	100	1.000	100	100	100	100
d) Mononchida/Mermithida/ Dorylaimida	**36**	**34**	**56**	**50**	0.189	39	32	20	18	0.003	25	17	1	11
e) Dioctophymida/Trichinellida/ Mononchida/Mermithida	35	29	8	18	**0.627**	**57**	**62**	**60**	**69**	**0.984**	**54**	**45**	1	8
f) Dioctophymida/Trichinellida/ Dorylaimida	26	34	34	27	0.183	3	5	12	11	0.012	19	38	**97**	**80**

Values of MP bootstrapping are obtained for 1000 pseudoreplicates, values of ML bootstrapping are obtained for 20 pseudoreplicates (unless otherwise indicated). Values of BI support indicate posterior probabilities of a cluster to be correct. The number of suboptimal trees (estimated with KH test) saved in ML bootstrap analyses was as follows: [a]6826 trees; [b]6313 trees; [c]4541 trees in ten pseudoreplicates; [d]4162 trees; [e]3170 trees in ten pseudoreplicates; [f]5232 trees. Underlined values indicate clusters observed in the best topology, values in bold indicate clusters kept in the major-rule consensus tree. For each dataset analyses were conducted with and without Γ correction (+Γ and −Γ, respectively).

double compensatory mutations (data not shown). Such a pattern of co-evolution is typical for residues involved in molecular interaction (Woese *et al*., 1983). Thus, hairpin 18 actually possesses a long imperfectly paired stem and a nine bases long single-stranded loop, rather than a 17 bases long loop (Fig. 2).

The presence of three monophyletic clades; *i*) Dioctophymida and Trichinellida; *ii*) Mononchida and Mermithida, and *iii*) Dorylaimida, allows for three possible basal topologies for the Dorylaimia (Fig. 3). Ironically, all the three combinations were estimated as equally parsimonious. *Dnapars* reconstructed approximately equal amounts of putative synapomorphies for each; 43, 46 and 46 synapomorphies being inferred for topologies A, B and C, respectively (see Fig. 3).

Two taxa can be erroneously clustered together due to high levels of homoplasy in hypervariable regions of the sequences compared. A conventional way to reduce this artefact is to assign more variable positions a lower weight in phylogeny reconstruction, *e.g*., by introducing a gamma distribution approximation to evolutionary models (Yang, 1996; Whelan *et al*., 2001). However, in the case of Dorylaimia, the resulting ML topology and the consensus of suboptimal trees after bootstrap resampling were influenced little by the gamma correction, depending more upon taxonomic composition of the data set (Table 1). This may be accounted for by high disparities in nucleotide composition of 18S rRNA genes of representatives of Dorylaimia. Of 39 taxa in our dataset, two were rejected with 95% confidence by χ^2 test to fit the HKY model (TREE-PUZZLE 5.0 analysis) and both these taxa (*Mylonchulus arenicolus* Clark, 1961, AF036596 and *Trichuris muris* Schrank, 1788, AF036637) belonged to Dorylaimia. The corresponding *P* values for the taxa in the dataset are plotted in Fig. 4. Representatives of Dorylaimia, except for Dorylaimida, are characterised by low *P* values. The figure illustrates disparities in *P* values among the sampled Dorylaimia. It suggests that the 18S rRNA gene in two dorylaimian lineages, (Dioctophymida, Trichinellida) and (Mononchida, Mermithida), may have followed modes of molecular evolution different from those of the majority of taxa, and perhaps from Dorylaimida as well. It is known that poor choices of substitution model render ML phylogenetic reconstructions unreliable (Yang, 1996). The same applies to the BI algorithm, which is susceptible to incorrect estimates of likelihoods of trees and actually produces similar results (Table 1).

To resolve the internal structure of Dorylaimia, selected molecular signatures were assayed for variability using data from the metazoan 18S rRNA database. It appeared that only two topologies, namely A and C in Fig. 3, are supported by substitutions showing relatively low levels of homoplasy. The most plausible characters that support the sister group status of (Dioctophymida, Trichinellida) clade with respect to the rest of Dorylaimia are two

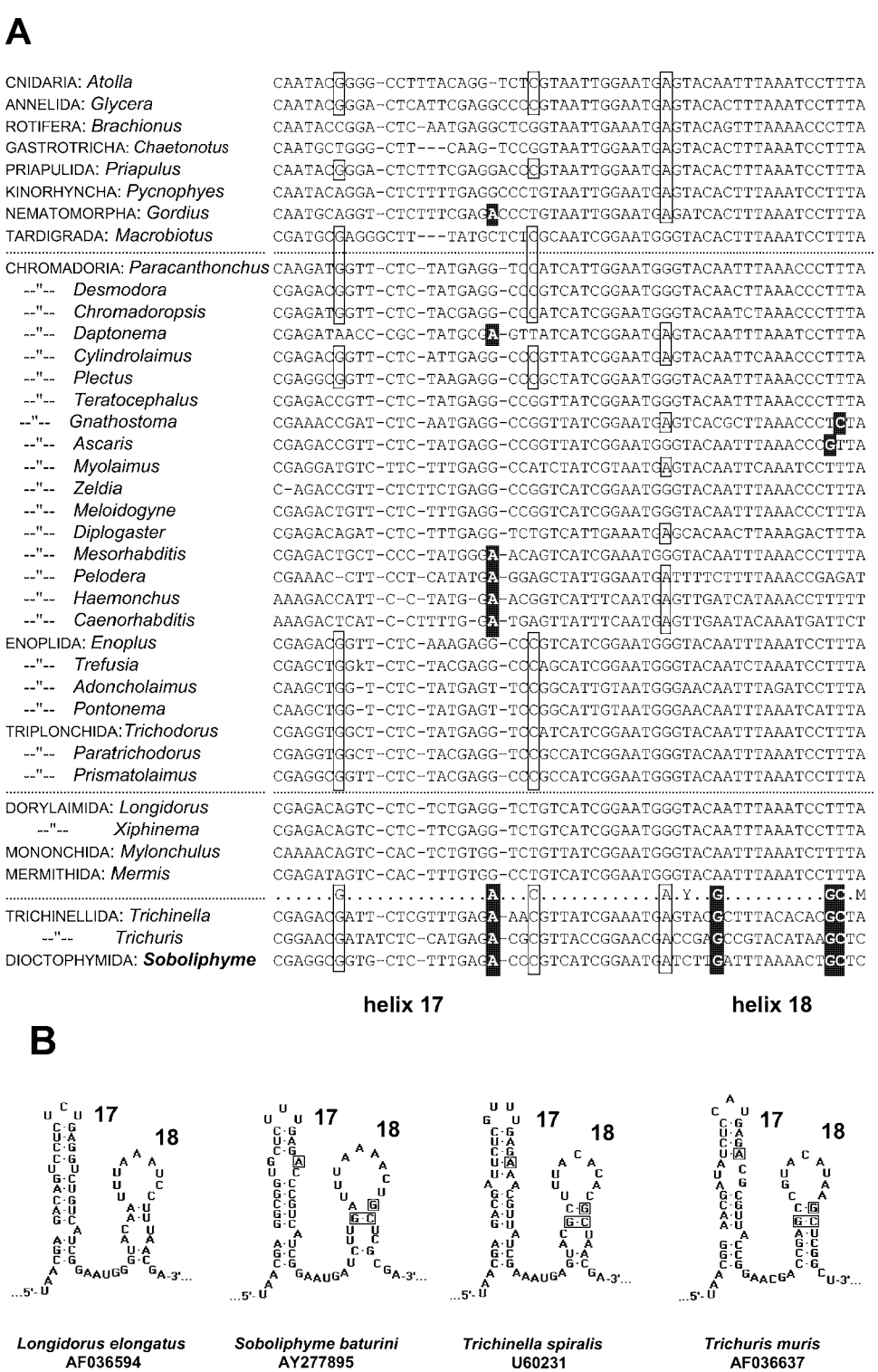

Fig. 2. *A: Synapomorphies of Dioctophymida and Trichinellida; B: Predicted secondary structure in the V3 region of helix 18 of the 18S rRNA molecule. Synapomorphic substitutions inferred with* Dnapars *are shown on the lower dotted line. Possible synapomorphies are in black boxes, those representing possible symplesiomorphies for Nematoda are enclosed in empty boxes.*

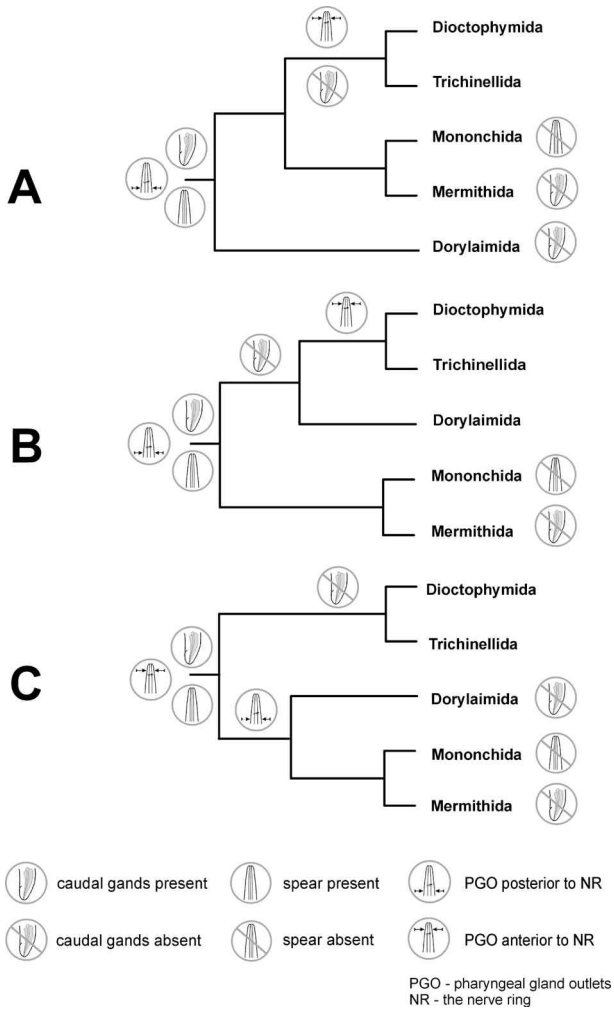

Fig. 3. *Three possible internal topologies (A, B and C) for Dorylaimia mapped with primary morphological characters.*

synapomorphies of the Mononchida/Mermithida/Dorylaimida grouping: a specific deletion of one residue in the V4 region and a relatively rare R→Y transversion within region V9 of helix 49. The alternative topology with (Dioctophymida, Trichinellida) and (Mononchida, Mermithida) as closest relatives is supported by a conservative T→C transition also situated within helix 49. The fact that molecular characters generating conflicting phylogenetic signals are located close to each other within the molecule prompted us to model the secondary structure of the corresponding region. We found that the folding pattern of helix 49 differs slightly among the groups of Dorylaimia (Fig. 5). A plausible interpretation of this is presented in below.

Discussion

We will focus here on four points: *i*) reliability of Dioctophymida as a sister taxon to Trichinellida; *ii*) feasibility of phylogeny reconstruction for the entire Dorylaimia with 18S rDNA sequence data; *iii*) morphological implications of molecular phylogenies of Dorylaimia, and *iv*) possibility of molecular diagnosis of Dorylaimia with 18S rDNA sequence data.

As may be deduced from the branch lengths on inferred trees (see Fig. 1), both the dioctophymid and trichinellid lineages contain multiple modifications in the 18S rRNA gene structure with respect to other nematodes and each other. Thus, their coalescence may be due to 'long branch attraction' artefacts (Felsenstein, 1978). However, some evidence suggests that this grouping is non-accidental. There is no sign of clustering of the *S. baturini* sequence with any of the highly divergent lineages other than trichinellids present in the dataset (*viz.*, trichodorids and rhabditids, including in particular *Pelodera strongyloides* (Schneider, 1860), accession number U13932). Furthermore, the clustering with trichinellids is very robust using all methods (Fig. 1) and receives high bootstrap support after correction for among-sites rate variation (Table 1). The number of molecular synapomorphies found in the 18S rRNA gene of *S. baturini* and representatives of Trichinellida also strongly suggests that these lineages are most closely related. The possibility of Dioctophymida and Trichinellida representing sister groups does not contradict morphological evidence. Both lineages share peculiar features such as the nerve ring positioned far anterior in the pharynx region, terminal (male) or subterminal (female) position of the anus, an obtuse tail not tapering toward the end of body, absence of lips and presence of operculated eggs. The most striking distinction of Dioctophymida, the muscular bursa in males, may be related to circular carinae on the body wall in some males of capillariids (*e.g.*, *Capillaria anceris* Madsen, 1945; *C. bursata* Freitas & Almeida, 1934). In addition, some capillariids, like dioctophymids, require an oligochaete intermediate host in the life cycle (Spassky, 1956).

Identifying the closest relative of the (Dioctophymida, Trichinellida) clade is complicated by the fact that the number of reliable synapomorphies, which would support their grouping with other dorylaimian clades, is extremely scarce. To handle this situation, cladistic analysis of individual informative characters may help to distinguish between competing topologies. This approach has already been successfully applied to phylogenies of

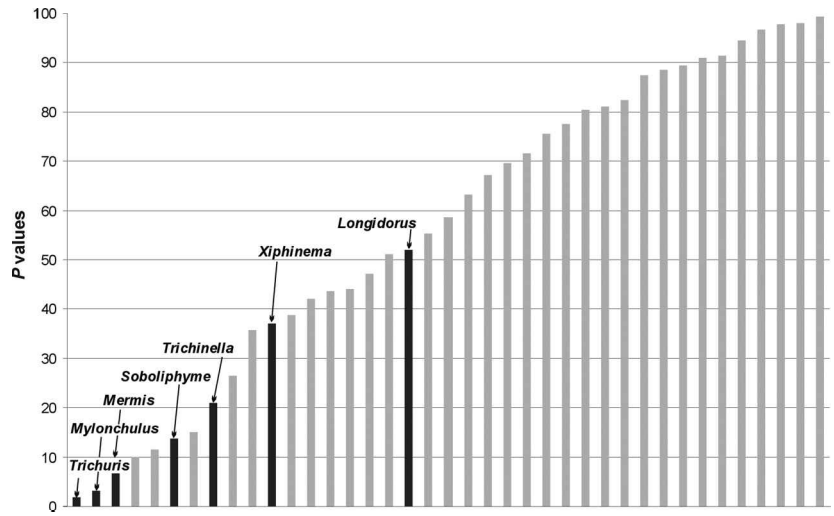

Fig. 4. *Distribution of* χ^2 P *values for 39 taxa in the dataset.* P *values indicate the probability that a sequence deviation from the HKY model is explained by chance.*

Chromadorida, Strongylida and Trefusiidae (Aleshin *et al.*, 1998a, b; Rusin *et al.*, 2001), and allows us to obtain reliable molecular evidence for monophyly of all Dorylaimia, as well as to surmise its possible internal topology (Fig. 3). The informative substitutions detected in conservative regions of the gene have very rare homoplastic occurrence among nematodes and other Bilateria.

Within the limits of this analysis, the problem of defining the internal topology of Dorylaimia narrows down to discriminating between the two conservative sites within the 18S rRNA which contribute conflicting phylogenetic signal to the data: the deletion of one base within the V4 region, which supports sister group status of (Dioctophymida, Trichinellida) with the rest of Dorylaimia, versus the T→C transition in the V9 region, which suggests Dorylaimida diverged first within Dorylaimia.

The V4 deletion is situated within a single-stranded internal loop (Van de Peer *et al.*, 2000) or a multistem loop (Wuyts *et al.*, 2000) of the predicted 18S rRNA molecule secondary structure. The apomorphic condition in all cases can be reconstructed as shortening of this segment and is conserved among the taxa. The T→C transition in region V9 is located within an imperfect stem of the native RNA. Many non-dorylaimid nematodes and non-nematode taxa exhibit uniformity in the structure of this predicted region. This hypothetical ancestral pattern is marked by a non-conventionalpyrimidine-pyrimidine pair at the 19th position from the base of hairpin 49 (Fig. 5, lower part). However, all Dorylaimia are characterised by an unusual pattern of dislocation of the unpaired pyrimidine residues and/or their unusual flanking motifs (Fig. 5, upper part). The observed structure of hairpin 49 in extant taxa cannot simply be explained by stepwise derivations of one extant condition from another. Thus, it may be speculated that the putative ancestral pattern was already altered at the level of the common ancestor of Dorylaimia. The ancestral condition may have actually been the destabilisation of the entire stem region between the 18th and the 20th positions of hairpin 49 caused by a T→C transition and a G→T transversion (see Fig. 5, in empty circles). These were then presumably followed by complete or partially compensatory changes in different subclades of Dorylaimia, either by means of back mutations or further substitutions within the 5′-branch of the helix (Fig. 5, in grey circles). Without such speculation, it would be difficult to minimise the change in conservative secondary structure of helix 49 in sampled Dorylaimia without having to postulate multiple local substitutions. According to this hypothetical scenario, the crucial plesiomorphy of Dorylaimida appears to represent a reversal from the condition apomorphic for all other recent Dorylaimia.

This reversal in recent Dorylaimida affects only the primary structure of the molecule and does not restore its presumed ancestral secondary structure. Analogously, the reversal at the secondary structure level observed in *S. baturini* presumably occurred by means of numerous nucleotide substitutions. This example is a good illustration of Dollo's law of irreversible evolution in application to

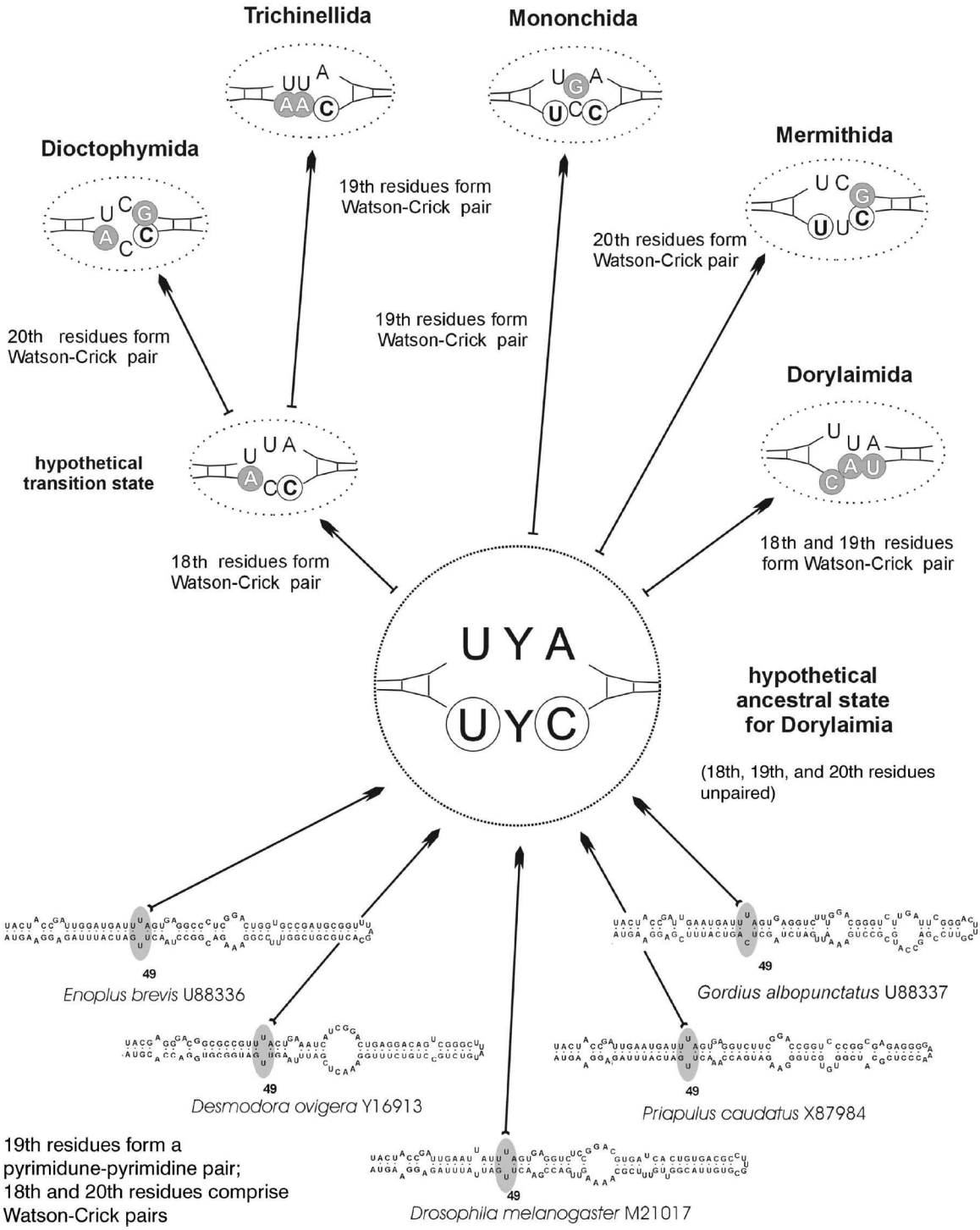

Fig. 5. *Hypothetical scenario of molecular evolution of helix 49 of the 18S rRNA in Dorylaimia. Folding patterns for this region observed in outgroups are given in the lower part. Substitutions leading to destabilisation of the hypothetical ancestral state of the loop are given in empty circles. Further substitutions leading to stabilisation of the structure are given in grey circles. YY – a non-conventional pyrimidine – pyrimidine pair at the 19th position of the hypothetical ancestral structure of the helix.*

macromolecules. The irreversibility in this case is brought about by the diversity of possibilities of shortening the internal loop of helix 49. Stabilising one or two pairs out of the three can be implemented in 18 different ways, and five of them are realised in the five subtaxa of Dorylaimia. Only one will lead to restitution of the ancestral state and it is not observed in any of the sampled Dorylaimia. If the radiation of Dorylaimia occurred relatively rapidly and gave rise to a larger number of lineages, the record of superimposed mutations in the V9 region would be difficult to decipher (Cunningham *et al.*, 1998).

Thus, the only conservative character unlikely to have experienced superimposed mutations in evolution of the 18S rRNA gene in Dorylaimia is the 1-bp deletion within the V4 region, a synapomorphy for the clade ((Mononchida, Mermithida), Dorylaimida). Blaxter *et al.* (2000) relying on statistical evidence, also conclude that the occurrence of this clade in 18S rRNA phylogenies reflects true evolutionary affinities rather than the consequences of long branch attraction of Trichinellida to the basal node of the tree.

Combining sequence data and morphology has interesting implications for understanding the general patterns of nematode evolution. Thus, in both topologies, Mononchida are placed as sister group to Mermithida at a distal node of the tree. Mononchids do not possess a spear but do have caudal glands, whilst other Dorylaimia have a spear but lack caudal glands. The distribution of the states of these characters over the entire clade makes phylogenetic assignments difficult. Two scenarios are possible; mononchids lost an ancestral spear and evolved caudal glands, or all other taxa evolved the spear and lost caudal glands. Speculating about the ancestral life strategies of particular dorylaimian lineages, one could postulate that Mononchida retained the ancestral attachment organs due to their initial radiation in fresh water environments (and more recent ingress to the soil), whilst Dorylaimida, one of the dominant nematode taxa in soils, could have originated from a soil-dwelling group. Trichinellida, Dioctophymida and Mermithida are all highly specialised parasites of unknown ancestral habitat. Alternatively, dorylaims may have evolved as a group with diversifying feeding strategies utilising a protrusible spear to pierce the food organism and ingest its liquid contents, whilst the mononchid ancestor secondarily developed a capturing apparatus allowing it to seize and swallow all or parts of its prey, thus resulting in a complete reduction of the spear. Other lineages, among them mermithids, which are inevitably placed as sister taxon to mononchids in molecular phylogenies (Blaxter *et al.*, 1998), lost the primary spear function in adults upon shifting to parasitism, yet still retain its rudiment. Some dorylaims (*e.g.*, *Actinolaimus*) are known to possess both spear and teeth in the stoma (Jairajpuri & Ahmad, 1992). Although the teeth are not likely to be homologous to those in mononchids, this case illustrates the possibility of spear-bearing forms acquiring teeth.

Another important morphological character, position of all pharyngeal gland outlets posterior to the nerve ring, does not fit the fact that dioctophymids are known to have the gland openings situated within the buccal cavity (*viz.*, at the very bottom of the mouth sucker). This peculiar arrangement of the pharynx glandular structure may be interpreted as an indication of their closer relationships with Enoplida. Homology of these systems throughout Nematoda is not questioned and thus, following topology B (Fig. 3), will require at least two evolutionary events of independent shifting of the gland orifices from the stoma down into pharynx to fit the data, unless one assumes alternative *C*, in which case the character would have changed its status only once in the evolution of Dorylaimia at the level of the common ancestor of (Mononchida, Mermithida) and Dorylaimida. On the basis of molecular evidence (Blaxter *et al.*, 2000), we assume that the pharynx in Trichinellida and Mermithida evolved into the stichosome independently.

In all Dorylaimia with a conventional, non-modified pharynx (*i.e.*, except for Trichinellida, Mermithida and Muspiceida, the latter not being studied with molecular methods to date), the pharyngeal gland orifices open close to one other into the lumen, either anteriorly in Dioctophymida, or posterior to the nerve ring in the middle part of the pharynx in Dorylaimida and Mononchida. The anterior position of the orifices also occurs in outgroups (Enoplia), which may suggest the ancestral condition of this character in Dioctophymida. Dioctophymids are also known to possess a spear-like protractile tooth (onchiostyle), which is formed ventrally in the anterior part of the pharynx at earlier stages of postembryonic development (J1) and disintegrates later. Interestingly, in common Dorylaimina (at J2-J4 stages) the replacement tooth is formed posterior to the functional odontostyle close to the level of gland outlets, while in its putative sister taxon, Nygolaimina, it is formed almost immediately posterior in the head region (Coomans & van der Heiden, 1978). This may suggest that the position of the spear and pharyngeal gland outlets actually marks the border of the ancestral stoma in Dorylaimia. So far, allocation

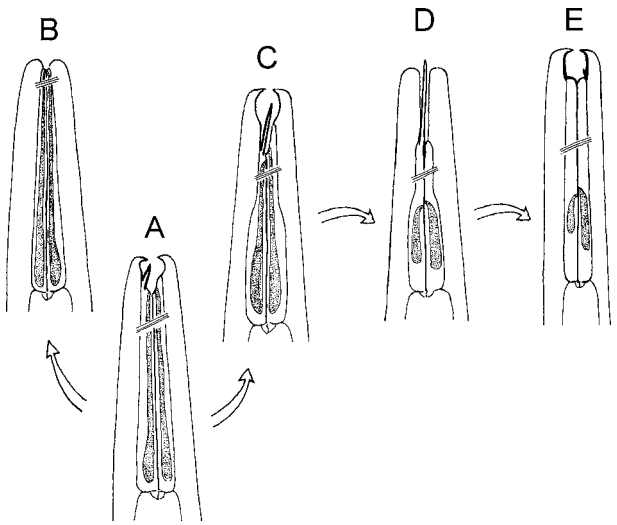

Fig. 6. *Tentative scheme of the evolution of the head region in Dorylaimia. A: Hypothetical ancestor of the Dorylaimia; B: Recent Dioctophymida; C: Hypothetical transition stage between the ancestral form and the common lineage of Dorylaimida, Mononchida and Mermithida; D: Recent Dorylaimida; E: Recent Mononchida.*

of all pharyngeal gland orifices posterior to the nerve ring is reported exclusively for Dorylaimida and Mononchida. It cannot be excluded that the stoma in the common ancestor of these groups has differentiated to form a functional stoma and its posterior part (oesophastoma) has merged with the pharynx and adopted a new function. As a result, the gland openings became displaced posteriorly in the pharynx lumen (Fig. 6). An interesting morphological evidence from pre-parasitic stages (J1) in mermithids further supports this idea. The juvenile mermithid pharynx, which is not yet modified into a stichosome, is clearly subdivided into two zones, the dorsal gland outlet being located exactly at the junction of the zones (Poinar & Hess, 1974; Rubtsov, 1977). In many recent Dorylaimina, the definitive pharynx is bottle-shaped, its anterior and posterior portions have different musculature arrangement and the pharyngeal glands also open at the junction level. This hypothesis explains the peculiar postneural position of the gland openings in Mononchida by postulating the secondary loss of the spear at later stages of the posterior elongation of the oesophastoma.

Thus, adaptive radiation of Dorylaimia gave rise to a variety of forms, which differ from each other in nerve ring position, arrangement of glandular ducts and structure and armature of the mouth cavity. Transformation of the pharynx into the stichosome and reduction of the caudal glands and spear were, probably recurring events in nematode evolution. The common ancestor of Dorylaimia is likely to have possessed a spear with the pharyngeal gland outlets inside the stoma, a feature generally lost in recent Dorylaimia with the single exception of a rare group of Dioctophymida. Thus, the only non-molecular diagnostic character of Dorylaimia available remains the unique pattern of early embryogenesis, when the endodermal precursor is strictly associated with the anterior blastomere of the egg. This is characteristic of Mononchida, Dorylaimida (Drozdovskii, 1969, 1975) and Mermithida, Dioctophymida and Trichinellida (Malakhov & Spiridonov, 1981, 1983; Malakhov *et al.*, 1984). Variability in cell fate determination in species of marine Enoplida distinguishes this group from both Chromadoria and Dorylaimia (Malakhov, 1986; Voronov *et al.*, 1998). Fresh water Triplonchida is a group most probably linked to Enoplida (Blaxter *et al.*, 1998). According to molecular evidence, *Tobrilus gracilis* (Bastian, 1865) Andrássy, 1959 belongs in the Triplonchida (De Ley & Blaxter, 2002) and is the only representative of this group for which embryogenesis has been studied so far (Drozdovskii, 1977). The highly variable early cell division in *T. gracilis* resembles that of marine Enoplida, the endodermal precursor sometimes arising from the anterior blastomere. Further studies of triplonchids employing techniques of fluorescent cell labelling may allow polarisation of the state of this character in nematode evolution. Embryological data can distinguish between major nematode lineages (Drozdovskii, 1975; Voronov *et al.*, 1998; Lahl *et al.*, 2003), though their interrelationships remain unknown. Using 18S rDNA sequence data we have defined the contents of Dorylaimia. There are few unequivocal individual molecular signatures that can be used to define its internal structure. A single reliable molecular synapomorphy for the entire Dorylaimia was detected at the 5′ end of predicted helix 23 of the18S rRNA secondary structure model. The frequency of its occurrence beyond Dorylaimia is an estimate of 10^{-2} on the dataset of all nematode sequences currently available. The scantiness of reliable molecular signatures for major lineages of Dorylaimia resembles the situation with marine Enoplida, where their representatives can only be distinguished by the presence of two low homoplastic substitutions in elements of the 18S rRNA molecule primary structure (Rusin *et al.*, 2001).

To conclude, analysis of sequence data combined with a cladistic approach for analysing molecular characters provides support for monophyly of Dorylaimia and defines its major internal structure. Comparative analysis of alterna-

tive phylogenies shows that some of the classic characters previously assigned significant value in phylogenetic reconstructions may not be parsimonious, something which forces us to reconsider conventional schemes of morphological and ecological change in nematode evolution.

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