Relationships among nematodes based on the analysis of 18S rRNA gene sequences: molecular evidence for monophyly of chromadorian and secernentian nematodes

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Summary. The relationships among nematodes were studied by 18S rRNA gene sequencing. On the basis of phylogenetic trees and cladistic analysis of the secondary structure of helix 49, some orders of traditional Adenophorea should be ascribed to the Secernentea. The Chromadorida and Desmodorida should be grouped with nematodes of a complex consisting of the Monhysterida, Plectida and Secernentea. This taxon may be named Chromadoria, as was proposed earlier (Drozdovsky, 1981), since chromadorids are most closely related to the common ancestor of these groups. Hence, the class Adenophorea in a traditional sense is paraphyletic and should be revised.
Key words: Nematoda, Adenophorea, Secernentea, Chromadoria, 18S rRNA, molecular phylogeny.

At present, the understanding of relationships within the phylum Nematoda is based mainly on analysis of morphological features. However, the difficulties in finding reliable systematic criteria for the gross systematics of nematodes are well known by nematologists. The majority of systematic criteria for nematodes were developed between 1918 and 1937 (Lorenzen, 1994). Until recently, no newly described features have been developed as systematic criteria for the gross phylogeny and systematics of nematodes. What is more, synapomorphic characters which are informative in deducing cladistic phylogenies comprise a small part of all phenotypic features. As a result, many systems based on morphological characters have been proposed but they are often based on conflicting criteria and do not reflect satisfactorily the real phylogenetic relationships among nematode groups of relatively high rank. These systems subdivide the nematodes either into two subclasses (Chitwood & Chitwood, 1950; Maggenti, 1981) or into three subclasses (Andrássy, 1976; Inglis, 1983; Malakhov, 1994). In trichotomic patterns, the first two subclasses [or classes according to Inglis (1983)] coincide very closely in subtaxa composition with the subclass Adenophorea in dicho-tomic patterns. Lorenzen (1994) subdivides the free-living nematodes on the basis of extensive cladistic analysis into four monophyletic groups (Chromadorida, Monhysterida, Enoplia, and Secernentea), but due to the fact that the inter-relationships between them are not resolved, and, therefore, grouping the higher taxa is possible in any way to form two, three or four subclasses, he retains Chitwood's classification into the Adenophorea and Secernentea.

It is evident that the problem of phylogenetic relationships within nematodes can not be solved by analysis of morphological characters alone and that it is necessary to use different modalities of characters. Molecular phylogenetic analysis is one such route to derivation of phylogenies. There are a number of arguments in favour of ribosomal RNA genes for this purpose (Hillis & Dixon, 1991). Ribosomal RNA genes are universally present in all living organisms and sufficiently conserved in structure to allow comparison and sequence alignment; they contain regions of differing sequence variability which allow assessment of phylogenetic relationships at multiple different taxonomic levels.

In this work 18S rRNA sequence comparisons were used to examine the phylogenetic relationships

of some groups within the Nematoda. 18S rRNA gene sequences of several species of nematodes from the orders Enoplida, Chromadorida, Desmodorida, and Monhysterida, traditionally placed to the subclass Adenophorea, were sequenced and compared with nematode 18S rRNA sequences available in the public databases.

MATERIAL AND METHODS

Biological material and DNA extraction. The animals investigated in the present study are *Pontonema vulgare* (Enoplida, Oncholaimidae), *Paracanthonchus caecus* (Chromadorida, Cyatholaimidae), *Chromadoropsis vivipara* (Desmodorida, Chromadoridae), and *Daptonema procerus* (Monhysterida, Xyalidae). They were collected by Prof. V.V. Malakhov in summer 1996 at Kandalaksha Bay of the White Sea. Nematodes were fixed in 70% ethanol.

DNA of nematodes was extracted from several intact animals essentially as described by Arrighi *et al.* (1968) with some modification (Sambrook *et al.*, 1989).

Amplification and sequencing of the 18S rRNA genes. 18S ribosomal RNA coding regions were amplified using the polymerase chain reaction with two primers complementary to the 5' and 3' termini of eukaryotic 16S-like rRNAs (Medlin *et al.*, 1988). Full-length products of amplification were purified by agarose gel electrophoresis and cloned in the plasmid pBluescript KS+. Several clones were sequenced on both strands using Sequenase Version 2.0 USB kit, a set of 18S rRNA specific internal primers and universal M13 sequencing primer.

Alignment, tree construction and analysis of secondary structure elements. Complete or nearly complete 18S rRNA gene sequences determined were submitted to GenBank under the following accession numbers: *Pontonema vulgare* AF047890, *Paracanthonchus caecus* AF047888, *Chromadoropsis vivipara* AF047891, *Daptonema procerus*, AF047889. Other previously published 18S rRNA gene sequences from nematodes and some invertebrate phyla were derived from GenBank. A list of the sequences is given in Figure legends.

In initial analyses, two different alignments were analyzed to be certain that differences of alignment have no significant effect on the tree topologies. In order to prepare these alignments the sequences were fitted either into our own alignment (alignment I), or (alignment II) into an alignment of small subunit rRNA sequences (Van De Peer *et al.*,1996). The alignments are available from the authors on request via e-mail. All analyses of alignment I were based on 1104 unambiguously aligned sites, including all conserved regions, excepting helices 1-5, and 50 for

which the complete sequence data are not available, and helices E23-1, E23-2, and apical part of hairpin E10-1 and 43, for which unambiguous alignment was not possible. Appropriate subsets of these alignment were analysed by both distance and maximum parsimony (MP) methods. Distance neighbor-joining (NJ) trees were inferred with the program TREECON (Van De Peer & De Wachter, 1994), using Kimura distances (Kimura, 1980), modified to take gaps into account (Van De Peer *et al.*, 1990) as well as distances considering the substitution rates of the different alignment positions (Van De Peer *et al.*, 1996). MP trees were constructed using Dnapars program within the PHYLIP 3.572 package (Felsenstein, 1993) with options search for best tree and randomisation of input order of sequences. Maximum likelihood (ML) trees (Felsenstein, 1981) were inferred using fastDNAml (Olsen *et al.*, 1994) with global branch exchange and randomisation of input order as well as PUZZLE with the Hasegawa *et al.*, (1985) model of nucleotide substitution (Strimmer & von Haeseler 1996). Confidence in NJ and MP trees was determined by analysing 1000 bootstrap replicates (Felsenstein, 1985). Confidence in the ML trees derived by PUZZLE and fastDNAml was determined using 1000 puzzling steps or 20 bootstrap replicates, respectively.

Elements of secondary structure of the 18S rRNA were constructed manually using the model proposed by Van De Peer *et al.* (1996).

RESULTS

The newly-determined 18S rRNA sequences from *Pontonema vulgare, Paracanthonchus caecus, Chromadoropsis vivipara,* and *Daptonema procerus* were 1753, 1750, 1742, and 1748 nucleotides in length (without primer regions), respectively. These sequences do not show significant G + C differences from the majority metazoan species. They have no large insertions or deletions and/or regions which are difficult to align.

Figure 1A shows the results of NJ analysis of the sequence set from alignment I on the basis of Kimura (1980) distances of the set of nearly complete 18S rRNA gene sequences of several representatives of various orders of the Nematoda and some representatives of various metazoan phyla. In this tree, the nematodes comprise a monophyletic group supported by 77% of bootstrap replicates. A further three monophyletic groups supported by more than 70% of bootstrap replicates can be distinguished within this group. The first of them (group I, 100% of bootstrap replicates) includes a part of the rhabditids and all Strongylida; the second one (group II, 76% of bootstrap replicates) consists of the Monhysterida,

Plectida, and Secernentea; and the third (group III, 75% of bootstrap replicates) includes species from Chromadorida (*Paracanthonchus caecus*) and Desmodorida (*Chromadoropsis vivipara*) in addition to representatives of all three groups named above. Analysis after application of the substitution rates correction of Van De Peer *et al.* (1996) yielded principally the same grouping of nematodes (not shown), except for the positions of *Strongyloides stercoralis* and *Daptonema procerus* which form a clades at the base of the Plectida and Secernentea.

A congruent topology was obtained by maximum parsimony analysis (Fig. 1). The only significant difference is that *Paracanthonchus caecus* (Chromadorida) and *Chromadoropsis vivipara* (Desmodorida) do not form a distinct clade, but branch off separately from the main stem of the Chromadorida-Secernentea. Monophyly of the Nematoda is supported in this tree by 86% of bootstrap replicates. The MP analysis displays the same three major clusters (I, II, III) as the NJ analysis which are supported by 98, 95, and 87% of bootstrap replicates, respectively. Thus, the reliability of the novel groups within the Nematoda inferred by NJ analysis is confirmed by MP analysis.

The results of maximum likelihood (ML) analysis with fastDNAml (Olsen *et al.*, 1994) are shown in Fig. 1C. The topology is essentially the same as in Fig. 1B, differing in that *Plectus* sp. (Plectida) does not form a clade with *Ascaris* sp. and *Brugia malayi* as it does in NJ analyses, but lies basal to the Secernentea. The branching order of the Chromadorida and Desmodorida is similar to that found by MP analysis. Monophyly of the Nematoda is supported by 90% of bootstrap replicates. Major clusters (I, II, III) within nematodes are the same as in NJ and MP trees, but they have somewhat higher bootstrap support (100, 95, and 95%, correspondingly). Similar results (not shown) were obtained by another maximum likelihood method implemented in PUZZLE (Strimmer & von Haeseler, 1996).

In addition, subsets of the sequences from two different alignments were analyzed by the NJ and MP methods in order to examine the dependence of the internal structure of the trees upon the alignment type, the sequence set, and outgroup taxa. Trees derived by MP analyses of sequence sets from alignment II using priapulids, kinorhynchs, and nematomorphs as outgroup taxa are shown in Fig. 2. These trees have the similar topologies independent of outgroup taxa used. A congruent topology (not shown) was also derived by MP analyses on the basis of the same sequence set from alignment I. All these trees are similar to the MP tree in Figure 1B derived from more extensive set of the 18S rRNA sequences, except that the bootstrap support of major groups (I,

II, III) is slightly higher. Similarly, the NJ analyses gave the same results. Thus, all examined factors (type of alignment, type of outgroup and set of species) have no or little effect on the tree topologies.

The data above show the congruence of the most elements of trees derived by various methods. Some differences are due to the position of several rhabditid branches. The 18S rRNA sequences of the Rhabditida, especially *Strongyloides stercoralis* and *Pellioditis typica*, evolve much more rapidly than those of all other nematodes and even all metazoans. In keeping with the tendency to place long branches at the basal position of the clade (Felsenstein 1978, 1984; Hillis *et al.*, 1994), the NJ analysis gives more basal position of *Strongyloides stercoralis* and *Pellioditis typica* than the MP and ML analyses do (see Fig. 1). Contrary to the Rhabditida, the orders Ascaridida and Spirurida, traditionally considered as specialized parasitic groups, appear to have much more slowly evolving 18S rRNA sequences. Artificial clustering of these groups with Plectida in the NJ and MP trees is due to the dividing the Secernentea into two groups: the Rhabditida + Tylenchida, having long branches, and all the others. ML analysis, which is more tolerant to differences in rates of nucleotide substitutions (Nei, 1991), places Plectida in an intermediate position in the phylogenetic tree.

The results of phylogenetic analysis of 18S rRNA gene sequences support monophyly of the Nematoda as a whole as well as monophyly of three major groups, one included in another of higher rank (Fig. 1): part of the Rhabditida + Strongylida (I); Monhysterida + Plectida + Secernentea (II); Chromadorida + Desmodorida + Monhysterida + Plectida + Secernentea (III). These clades are strongly supported by bootstrap analyses and congruence between trees derived by various methods.

A cladistic analysis of certain region of nucleotide sequence near the 3'-end of 18S rRNA supports this hypothesis. This region forms a conserved element of the 18S rRNA secondary structure, named hairpin 49 according to the model of Van De Peer *et al.* (1996). The primary structure of this region of some species of the nematodes is compared to that of several species of metazoans, plants, and protists in Fig. 3. All these eukaryotic taxa were in turn analyzed as the outgroup. While *Enoplus brevis*, *Pontonema vulgare*, and *Trichinella spiralis* have retained the presumed ancestral state of this region, other nematodes traditionally ascribed to class Secernentea have acquired 35 substituted nucleotide positions (shown boxed in Fig. 3) that may be regarded as synapomorphic characters. Since these nucleotides constitute the complementary pairs in the double helix of the hairpin, they have presumably arisen as the result of 21 separate evolutionary events (14 of them are
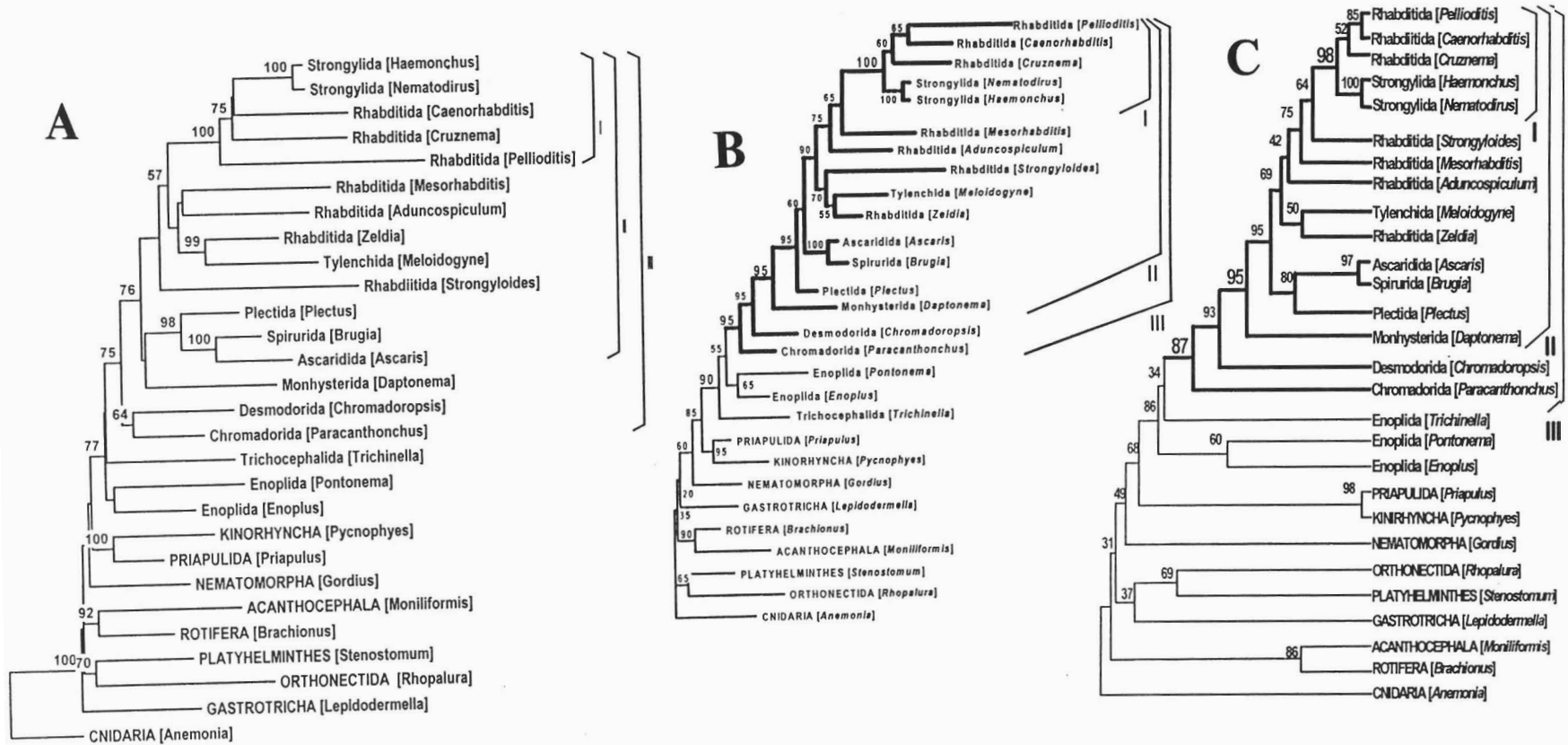
Fig. 1. A. Phylogram of nematode and other taxa derived from NJ analysis of 18S rRNA sequences. Bootstrap values above 50% of 1000 replicates are shown in percentages at the internodes. The three main nematode groups (I, II, III) well supported by bootstrap analysis are indicated with brackets. List of the phylum, binomial name, and GenBank accession number of species used is as follows: Nematoda - *Enoplus brevis*, U88336; *Trichinella spiralis*, U60231; *Caenorhabditis elegans*, X03680; *Strongyloides stercoralis*, M84229; *Ascaris* sp., (partial 18S rRNA sequence was compiled from M58348, X06225, X05836, X06713, M74584, M74585); *Haemonchus similis*, L04152; *Nematodirus battus*, U01290; *Cruznema tripartitum*, U73449; *Pellioditis typica*, U13933; *Mesorhabditis* sp., U73452; *Aduncospiculum halicti*, U61759; *Zeldia punctata*, U61760; *Meloidogyne arenaria*, U42342; *Plectus* sp., U61761; *Brugia malayi*, (nearly complete sequence was compiled from H30951, H31020, H35866, H39237-H39239, H39242, H39245, H48210, H48950, H48988, H52893, H77269, H91508, R86409, R95205); Nematomorpha - *Gordius aquaticus*, X87985; Priapulida - *Priapulus caudatus*, X87984; Kinorhyncha - *Pycnophyes kielensis*, U67997; Acanthocephala - *Moniliformis moliniformis*, Z19562; U41400; Rotatoria - *Brachionus plicatilis*, U29235; Gastrotricha - *Lepidodermella squammata*, U29198; Platyhelminthes - *Stenostomum* sp., U95947; Orthonectida - *Rhopalura ophiocomae*, X97158; Cnidaria - *Anemonia sulcata*, X53498. **B.** Phylogram of the same taxa as in A, but derived by maximum parsimony. Bootstrap values of 1000 replicates are shown in percentages at the internodes. Branches of the Chromadoria cluster are shown in bold. **C.** Cladogram of the same taxa in A, but derived by maximum likelihood. Bootstrap values of 20 replicates are shown in percentages at the internodes. Branches of the Chromadoria cluster are shown in bold. Branch lengths were derived from analysis of unbootstrapped sequence set results of which differed from consensus tree in position of *Strongyloides stercoralis* and *Lepidodermella squammata*.
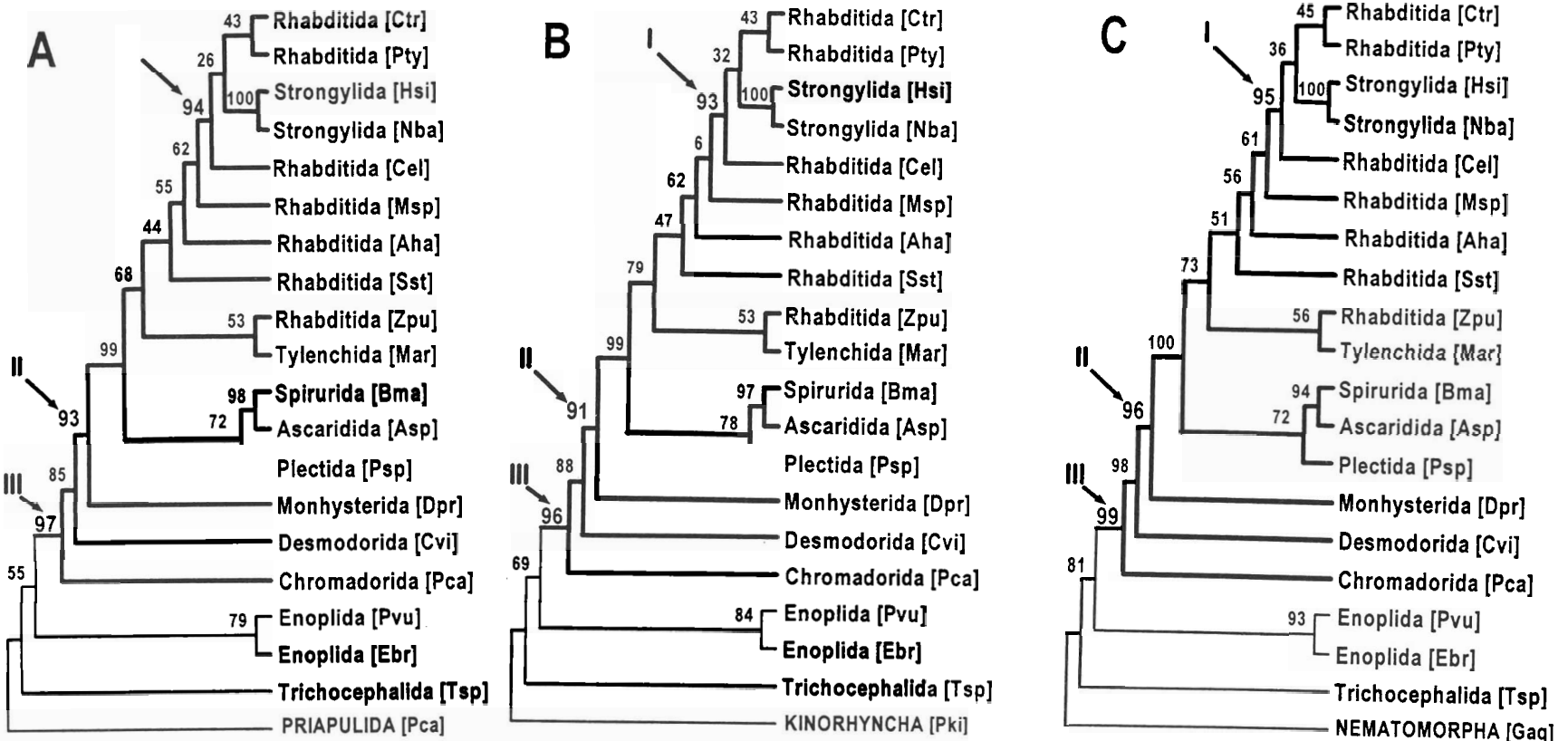
Fig. 2. Phylograms of restricted set of taxa derived by maximum parsimony using priapulids (**A**), kynorhynchs (**B**), and nematomorphs (**C**) as outgroup taxa. Bootstrap values of 100 replicates are shown in percentages at the internodes. Main three groups, the same as in figures above, are indicated by arrows to the corresponding bootstrap values. List of the phylum, binomial name, three-letter abbreviation of species used is as follows: Nematoda - *Enoplus brevis* [Ebr]; *Trichinella spiralis*, [Tsp]; *Pontonema vulgare* [Pvu]; *Paracanthonchus caecus* [Pca]; *Chromadoropsis vivipara* [Cvi]; *Daptonema procerum* [Dpr]; *Caenorhabditis elegans* [Cel]; *Strongyloides stercoralis* [Sst]; *Ascaris* sp. [Asp]; *Haemonchus similis* [Hsi]; *Nematodirus battus* [Nba]; *Cruznema tripartitum* [Ctr]; *Pellioditis typica* [Pty]; *Mesorhabditis* sp. [Msp]; *Aduncospiculum halicti* [Aha]; *Zeldia punctata* [Zpu]; *Meloidogyne arenaria* [Mar]; *Plectus sp.* [Psp]; *Brugia malayi* [Bma]; Nematomorpha - *Gordius aquaticus* [Gaq]; Priapulida - *Priapulus caudatus* [Pca]; Kinorhyncha - *Pycnophyes kielensis* [Pki].

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brown alga (Fucus)            TCGCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACCGGAAC-...-CGAAGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAA
yast (Saccharomyces)          TCGCTAGTACCGATTGAATGGCTTAGTGAGGCCTCAGGATCTGCTT-...-AGCGGAGAATTTGGACAAAACTTGGTCATTTAGAGGAACTAAAA
bean (Glycine)                TCGCTCCTACCGATTGAATGGTCCGGTGAAGTGTTCGGATTGCGGC-...-TTGTGAGAAGTCCACTGAACCTTATCATTTAGAGGAAGGAGAA
PORIFERA (Scypha)             TCGCTACTACCGATTGAATGGTTTAGTGAGATCTTCGGATTGCTGG-...-CAGCGAGAAGTTGATCAAACTTGATCATTTAGAGGAAGTAAAA
PLACOZOA (Trichoplax)         TCGCTACTACCGATTGGATGGTTTAGTGAGGTCTTCGGATTGGTGA-...-TATCGAGAAGTTGATCAAACTTGATCATCTAGAGGAAGTAAAA
PLATYHELMINTHES (Stenostomum) TCGCTACTACCGATTGAATGGTTTAGCAAGGTACTCGGATTGGTCT-...-TGCCGAGAAGAAGGCCAAACTTGATCATTTAGAGGAAGTAAAA
PLATYHELMINTHES (Gyrodactylus)TCGCTACTACCGATTGAATGGTTTAGCAAGGTCAATAGATTGGCTG-...-GGCTGAGAAGCTGGCCGAACTTGATCATTTAGAGGAAGTACAA
CHORDATA (Homo)               TCGCTACTACCGATTGGATGGTTTAGTGAGGCCCTCGGATCGGCCC-...-CGCTGAGAAGACGGTCGAACTTGACTATCTAGAGGAAGTAAAA
MOLLUSCA (Crassostrea)        TCGCTACTACCGATTGGATGGTTTAGTGAGCCCCTTGGACTGGTCC-...-TGCCGGGAAGAAGGGCAAACTGTACTTTCTAGAGGTCGTAAAA
ANNELIDA (Lanice)             TCGCTACTACCGATTGGATGGTTTAGTGAGATCCTCGGATTGGTCC-...-TGCCGAGAAGACGATCGAACTTGATCATCTAGAGGAAGTAAAA
ARTHROPODA (Artemia)          TCGCTACTACCGATTGAATGATTTAGTGAGAACTTCGGACGACTCG-...-GGTTGAAAGGTTGTTCAAACTTGATCCTTTAGAGGAAGTAAAA
TARDIGRADA (Macrobiotus)      TCGCTACTACCGATTGAATGATTTAGTGAGGTCTTCGGACTGGCCA-...-GGTTGGGAAGACGACCAAACTGGCTCATTTAGAGGAAGTAAAA
ROTIFERA (Brachionus)         TCGCTACTACCGATTGAATGGTTTAGTGAGATCTTCGGATTGGTCC-...-TGCCGAGAAGATGATCAAACTTGATCATTTAGAGGAAGTAAAA
ACANTHOCEPHALA (Moniliformis) TCGCTACTACCGATTGAATGGCTTAGTGAGGCTACGGATAGGCTA-...-TACCTAAAAGTTGGCCGAACTTTACCATTTAGAGGAAGTAAAA
KINORHYNCHA (Pycnophyes)      TCGCTACTACAGATTGAATGATTTAGTGAGGTCTTTGGACTGGCTC-...-TGCTGGGAAGTTGACCAAACTTGATTATTTAGATGAAGTAAAA
PRIAPULIDA (Priapulus)        TCGCTACTACCGATTGAATGATTTAGTGAGGTCTTCGGACCGGTCC-...-TGCTGGGAAGATGACCAAACTTGATCATTTAGAGGAAGTAAAA
NEMATOMORPHA (Gordius)        TCGCTACTACCGATTGAATGATTTAGTGAGGTCTTTGGACGGGTCT-...-CGCCTGAAAATTGATCTAGCTCAGTCATTTCGAGGAAGTAAAA
GASTROTRICHA (Lepidodermella) TCGCTACTACCGATTGAATGGTTTAGCGAGATTCTCGGATTGGGCC-...-GTCTGAGAAGAAACTCAAGCTTGATCATTTAGAGGAAGTAAAA
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ENOPLIDA (Enoplus)            TCGCTACTACCGATTGGATGATTTAGTGAGGCCCTCGGACTGGTGC-...-TTCCGGAAAGACGGCTAACTTGATCATTTAGAGGAAGTAAAA
ENOPLIDA (Pontonema)          TCGCTACTACCGATTGGATGTGTTAGTGAGGTTTTCCGACTGGGAC-...-TCTTGGAAAGATCATCGAACTAATATATCTAGAGGAAGTAAAA
TRICHOCEPHALIDA (Trichinella) TCGCTACTACCGATTGGATGATTTAGTGAGGTGTTTTGACTGGCGC-...-CGCCGGGAAATTTCCCAAACCAAATCATCTAGAGGAAGTAAAA
CHROMADORIDA (Paracanthonchus)TCGCTACTACCGACTGGCCTAATTTTTGAGGTGTCTGGACCTGCG--...-GCGGGGAAATTCCCCGGAA-ATGTTCGCGTTGGAGGAAGTAAAA
DESMODORIDA (Chromadoropsis)  TCGCTACGAGGGACGGTGCCATTTACTGAAATTACTGGACCGAGG--...-TTTTGGAAATTTACTTTAAGTTGATGGCGTGGACCACGTAAAA
MONHYSTERIDA (Daptonema)      TCGCTGCCGGAACTGAGCGGTTTCGAGAAGCCTGGGGACTGCAG--...-CAGTGGAAACCAAGTTAATCGCAGCGGCTTGAACCGGGCAAAA
PLECTIDA (Plectus)            TCGCTGCCGGAACTGAGCCGTTTCGAGAAAGCGGGGACTGCTG--...-CGGTGGAAACCGCTTTAATCGCAGTGGCTTGAACCGGGCAAAA
SPIRURIDA (Brugia)            TCGCTGCCGGAACTGAGCCGTTTCGAGAAAGCGGAGACTGCTG--...-GGGTGGAAACTGCTTTAATCGCAGTGGCTTGAACCGGGMAAAA
TYLENCHIDA (Meloidogyne)      TCGCTGCCGGAACTGAGCCATTTCGAGAAATTTGGGGACCGTTG--...-TGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAA
"RHABDITIDA" (Zeldia)         TCGCTGCCGGAACTGAGCCATTTCGAGAAGTGTGGGGACCGTAG--...-CCGTGGAAACCATATGGTTGAACGTGGCTTGAACCGGGCAAAA
"RHABDITIDA" (Caenorhabditis) TCGCTATCCGGAACTGAACTGATTCGAGAAGAGTGGGGACTGTCG--...-TTGCGGAAACCATTTTTATCGCATTGGTTTGAACCGGGTAAAA
STRONGYLIA (Haemonchus)       TCGCTGTCCGGAACTGAGCTGTCTCGAGAGGACTGCGGACTGCTG--...-TGGCGGGAAACAGTTCAATCGCAATGGCTTGAACCGGGTAAAA
"RHABDITIDA" (Pellioditis)    TCGCTGTCCGGAACTGAACTGTTTCGAGAAGGCTGAGGACTGACT--...-TGTTGGGAATCAGTTGGATCGAATTGGTTTGAACCGGGCACAA
"RHABDITIDA" (Strongyloides)  TCGCTGCCGGAACTGAGCAATATCCAGAGGCAGGAAGA--GATG--...-TATATTAAATCCTTCCAATCGCTGTTGTTTGAACCGGGCAAAA

                              49-a  ||| -f  -g  |  -j  |  -m  |  -o     -p      -m'  | -j'  | -g'  -f' ||| 49-a'
                               -b || |  -h  -k  -n              -n' -q  -k' -r  -h'  -u
                                -c         -i      -l            -l'       -s  -i'
                                -d                                              -t
                                -e                                                    -d'
                                                                                      -e'
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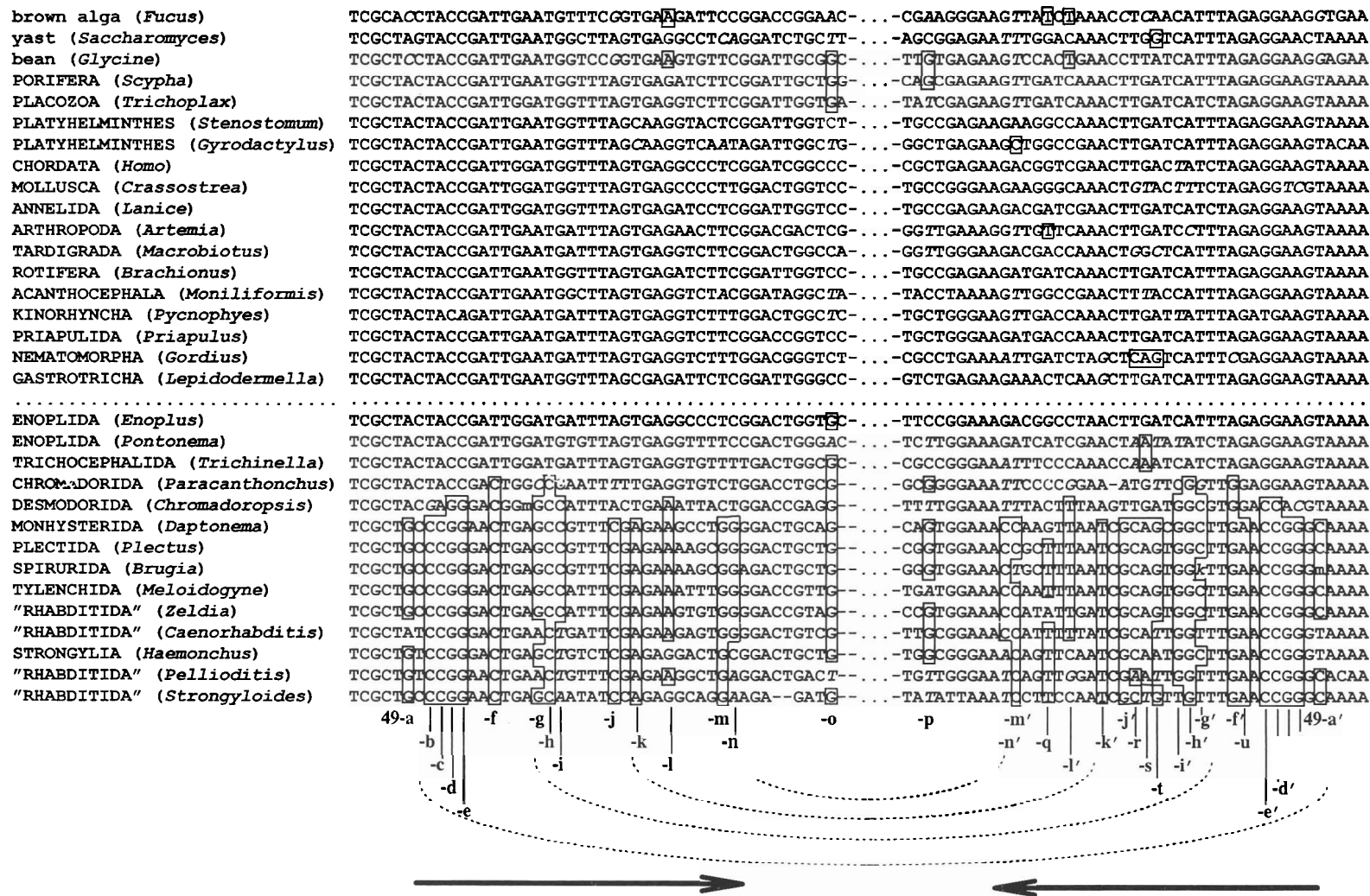
Fig. 3. Alignment of 18S rRNA sequence regions comprising helix 49 of the secondary structure of 18S rRNA. Nucleotide positions defined as synapomorphies for certain groups are boxed. Arrows show the folding of the sequence stretches to form the helix 49. Pairs which are complements in the helix are indicated by the same letters, for example a and a'; b and b' and so on. In addition to the sequences listed in figure 1 legend, the following 18S rRNA sequences were used: Chordata, *Homo sapiens*, M10098; Mollusca, *Crassostrea virginica*, X60315; Annelida, *Lanice conchilega*, X79873; Tardigrada, *Macrobiotus hufelandi*, X81442; Platyhelminthes, *Gyrodactylus salaris*, Z26942; Placozoa, *Trichoplax* sp., Z22783; Porifera, *Scypha ciliata*, L10827; Plantae - *Glycine max*, X02623; Fungi - *Saccharomyces cerevisiae*, J01353; Phaeophycea, *Fucus gardneri*, X53987.
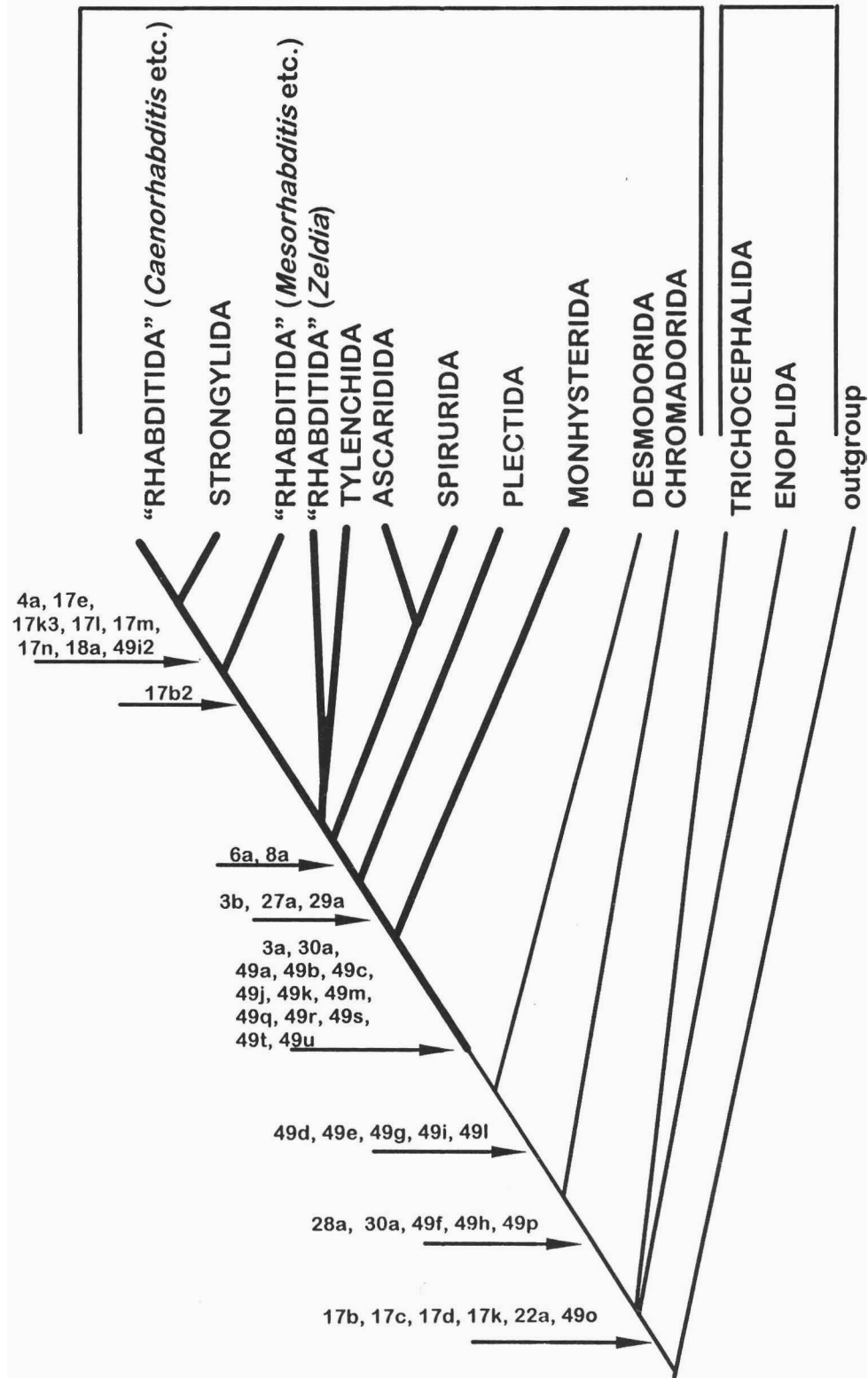
Fig. 6. Summary phylogram depicting the phylogeny of the nematode taxa studied. This tree illustrates a consensus of trees generated by NJ, MP, and ML methods. Arrows indicate the branches where synapomorphic nucleotide substitutions have arisen. Synapomorphies from hairpin regions of 18S rRNA are indicated by corresponding numbers.

related to each other in pairs, and the remaining events are a single). This region of 18S rRNA molecule in Secernentea thus forms a unique "secernentean stem". The origin of the "secernentean stem", which is a reliable phylogenetic marker for the taxa studied, is very important for understanding relationships within the Nematoda. Representatives of adenophorean orders Plectida and Monhysterida also have the state of this structure peculiar to the typical Secernentea. This could be considered a proof of their monophyletic origin with the Secernentea. Plectida have been quite often considered probable ancestors of secernentean nematodes, but the presence of the "secernentean stem" within the monhysterids is somewhat unexpected.

Some elements of the "secernentean stem" are apparent also in species of the Chromadorida and Desmodorida. In *Paracanthonchus caecus* (Chromadorida) this structure demonstrates the initial phase of its origin which is characterized by presence of the first three of the 21 synapomorphies included in the "secernentean stem": 49f, 49h, 49p (Fig. 2). In *Chromadoropsis vivipara* (Desmodorida) another 5 characters of the "secernentean stem" are present (49d, 49e, 49g, 49i, 49l). This supports the separate branching of these two orders from the main lineage of the nematodes seen in MP and ML analysis. On the whole these data, as well as the results of reconstruction of phylogenetic trees described above, suggest that the Chromadorida and Desmodorida should be ascribed a complex taxon "Monhysterida + Plectida + Secernentea". Fig. 4 shows the allocation of synapomorphies from the helix 49 region which support this affiliation. Synapomorphies supporting this branching order can also be found in other elements of the 18S rRNA secondary structure. Thus, nucleotide substitutions in helix 17 indicate synapomorphies for all the nematodes (Fig. 4, 17b, 17c, 17d, 17k) or for the cluster "a part Rhabditida + Strongylida" (Fig. 4, 17b2, 17e, 17k3, 17l, 17m, 17n). These characters, as well as the results of the NJ, MP, and ML analyses demonstrate convincingly the paraphyly of the traditional order Rhabditida.

DISCUSSION

The data described above provide fresh insight into the inter-relationships within the phylum Nematoda. Both comparative analysis of the full-length 18S rRNA gene and comparison of the primary structure of the helix 49 region show unambiguously that the traditional division of the Nematoda into classes Adenophorea and Secernentea (Chitwood & Chitwood, 1950) should be revised. The data suggest that the earliest divergence in the nematodes did not occur between the Adenophorea and Secernentea,

but between Enoplia and Chromadorida + Desmodorida + complex of "Monhysterida + Plectida + Secernentea". In a like manner, monophyly of Chromadorida + Plectida + Secernentea was also shown by Blaxter *et al.* (1998) on the basis of 18S rRNA gene sequences, but our analyses demonstrate that Monhysterida should be decidedly included into this complex.

Similar views were developed earlier on the basis of analysis of morphological characters alone (Maggenti, 1963, 1970) or morphological and embryological (Drozdovsky, 1975, 1981) characters. The main difficulty in the reconstruction of phylogeny of the Nematoda consists in the lack of reliable criteria. As a result, pragmatic considerations such as creating some order amongst nematode characteristics were preferred to their phylogenetic significance. The study of molecular characters makes it possible to derive reliable criteria, for example in the helix 49 region of 18S rRNA. The nucleotide substitutions accumulated in this region are clear synapomorphies which serve to divide the nematodes into two sharply outlined groups and to trace the branching order in one of them.

Molecular evidence suggest that neither the traditional dichotomic patterns of the Nematode system (Chitwood & Chitwood, 1950; Maggenti, 1981), nor the trichotomic ones (Andrássy, 1976; Inglis, 1983; Malakhov, 1994) reflect their true evolutionary history. The settled separation of the Nematoda into Adenophorea and Secernentea is based upon a division which is untenable in terms of phylogenetic systematics: one class includes, for the large part, aquatic free-living generalized forms, while the other includes mainly terrestrial and highly specialized parasitic ones. The deduction from this analysis and other work (Malakhov, 1994) is that these groups contain both primitive generalized and specialized forms. After having separated at an early stage, these two groups have evolved according to general principles common for all nematodes. Hence the Chromadorida and Enoplida have certain morphological and ecological traits in common being the representatives of generalized lineages in their separate groups.

On the basis of analysis of molecular characters it is proposed the commonly accepted pattern of dichotomy in nematode evolution should be abandoned. The class Adenophorea in its traditional sense is paraphyletic. It is proposed that the two branches of the nematodes are Enoplia (in a broad sense) and all other nematodes; this second branch includes the Chromadorida, Desmodorida, and the complex of the Monhysterida + Plectida + Secernentea. Synapomorphies in the helix 49 region of 18S rRNA give unambiguous resolution of the branching order in the

second lineage of the Nematoda. As the Chromadorida lie basal to the other members of this second clade, we propose that it should be named Chromadoria, as was proposed earlier first by Gadea (1973) on the basis of Maggenti's arguments (1963) and then by Drozdovsky (1975, 1981) on the basis of peculiarities of morphology and embryogenesis. According to Drozdovsky (1981), Chromadoria have two diagnostic characters. First, in Chromadoria the ducts of subventral pharyngeal glands open into the esophageal lumen at the same level, while the duct of dorsal pharyngeal gland opens always ahead of the former. In Enoplia the ducts of pharyngeal glands open into the esophageal lumen at the different levels independently of one another. Furthermore, in Chromadoria the endoderm precursors are held in the posterior blastomere of two-cell stage, while in Enoplia they are present at this stage in the anterior blastomere. Both these traits are also characteristic of the Monhysterida (Malakhov, 1981; Coomans et al., 1996). In the present work, such a proposition is based on the phylogenetic analysis of all the molecular characters provided by the 18S rRNA sequence and well defined synapomorphies provided by the hairpin 49 region.

Relationships within Enoplia remain unresolved because neither phylogenetic reconstruction based on a full-length sequences nor an analysis of some elements of the secondary structure resolve the branching order in this group. By these criteria within Enoplia there are two clades: Enoplida (sensu stricto) and Trichocephalida that form with the Chromadoria a trichotomy. Though many adenophorean orders were not represented in this analysis, the similar results were obtained by Blaxter et al. (1998) on basis of more representative 18S rRNA sequence set. In their analysis, the Enoplida (sensu stricto) on the one hand and Dorylaimida + Mononchyda + Mermithida + Trichocephalida on the other hand form only weakly supported clusters. Beyond that point the morphological and embryological characters suggested by Drozdovsky to distingush Chromadoria from Enoplia (sensu lato) are not fully uniform for Enoplida (sensu stricto) and Dorylaimida + Trichocepalida. In Enoplida (sensu stricto) all the pharyngeal glands open into stoma or nearly stoma, while in Dorylaimida and Mononchida pharyngeal gland outlets locate far from the stoma. Furthermore, in Mermithida and Trichocephalida pharyngeal glands form the stichosome in the cardial part of oesophagus. In Enoplia excluding Enoplida (sensu stricto) the endodermal precursor always derives from the anterior blastomere, while for Enoplida (sensu stricto) individual variations of its localization were described (Voronov & Panchin, 1995; 1998). Therefore, additional data are necessary to verify the monophyly of Enoplia.

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Алешин В.В., Кедрова О.С., Милютина И.А., Владыченская Н.С., Петров Н.Б. Родственные отношения нематод на основе анализа последовательностей 18S рибосомальной РНК: молекулярные доказательства монофилии Chromadoria и Secernentea.

Резюме. Изучены родственные отношения нематод путем сравнения генов 18S рибосомной РНК. Филогенетические деревья полных последовательностей 18S рРНК и кладистический анализ вторичной структуры шпильки 49 свидетельствуют, что некоторые отряды из традиционного класса Adenophorea следует относить вместе с Secernentea к одному классу. Отряды Chromadorida, Desmodorida и комплекс Monhysterida+Plectida+Secernentea образуют монофилетическую группу. Этот таксон можно назвать Chromadoria, как предлагалось ранее Дроздовским (1981), так как Chromadorida наиболее близки общему предку этих групп. Внутри Chromadoria установлен следующий порядок отделения клад: Chromadorida, Desmodorida (независимо от Chromadorida); Monhysterida; Plectida+Secernentea. Класс Adenophorea в традиционном смысле представляет собой парафилетическую группу и должен быть пересмотрен.