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REVIEW AND EXPERIMANTAL ARTICLES

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Phylogeny of Molting Protostomes (Ecdysozoa) as Inferred from 18S and 28S rRNA Gene Sequences

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Abstract—Phylogenetic relationships within the group of molting protostomes were reconstructed by comparing the sets of 18S and 28S rRNA gene sequences considered either separately or in combination. The reliability of reconstructions was estimated from the bootstrap indices for major phylogenetic tree nodes and from the degree of congruence of phylogenetic trees obtained by different methods. By either criterion, the phylogenetic trees reconstructed on the basis of both 18 and 28S rRNA gene sequences were better than those based on the 18S or 28S sequences alone. The results of reconstruction are consistent with the phylogenetic hypothesis classifying protostomes into two major clades: molting Ecdysozoa (Priapulida + Kinorhyncha, Nematoda + Nematomorpha, Onychophora + Tardigrada, Myriapoda + Chelicerata, and Crustacea + Hexapoda) and nonmolting Lophotrochozoa (Plathelminthes, Nemertini, Annelida, Mollusca, Echiura, and Sipuncula). Nematomorphs (Nematomorpha) do not belong to the clade Cephalorhyncha (Priapulida + Kinorhyncha). It is concluded that combined data on the 18S and 28S rRNA gene sequences provide a more reliable basis for phylogenetic inferences.

Key words: 18S rRNA, 28S rRNA, molecular phylogeny, Protostomia, Ecdysozoa, Arthropoda, Cephalorhyncha, Nematoda, Nematomorpha

INTRODUCTION

Since its origin at the turn of the 20th century, molecular phylogeny has been developing improved methods of analysis and broadening the set of genes used for analysis. The most significant progress in molecular phylogeny was achieved by comparing the small-subunit (18S) ribosomal gene sequences. This is accounted for by the fact that, first, the 18S gene has some advantages as a phylogenetic marker at the level of large taxa [1] and, second, the use of polymerase chain reaction (PCR) has considerably simplified the procedures of gene isolation and sequencing [2, 3]. Today, the total sample of the 18S rRNA gene sequences encompasses nearly all types of metazoans, except for Loricifera and Lobatocerebrida. By July 2003, the European database (http://www.psb.ugent.be/rRNA/index.html) contained 6590 full-length aligned sequences of eukaryotic 18S rRNA genes (including 1766 genes of metazoans) and 186 sequences of 28S rRNA genes (including 29 genes of metazoans) [4].

Studies on 18S rRNA genes radically changed the views on the evolution of eukaryotes and gave rise to new phylogenetic ideas [5–11]. By the mid-20th century, phylogenetic hypotheses based on the idea of progressive evolution of the complexity of organization, which gradually increases from lower (acoelomic) to higher (coelomic) animals, were commonly accepted in zoology and became basic in textbooks [12, 13]. These hypotheses are based on the segregation of all Bilateria into coelomic and acoelomic animals, depending on whether or not they possess the coelom, a secondary body cavity, which was believed to be of key phylogenetic importance. Coelomic animals were segregated into protostomes, deuterostomes, and lophophorates; the latter were regarded intermediate between the first two groups. The ideas about sister relationships between Annelida and Arthropoda within the common group Articulata underlay the phylogeny of protostomes. A large group of animal phyla (Nematoda, Rotatoria, Nematomorpha, Priapulida, Kinorhyncha, and Gastrotricha), which, instead of the coelom, have an internal body cavity interpreted as remains of the blastocoele, did not find a place in such classification. For this reason, these groups were combined into the superphylum of Pseudocoelomates, or Aschelminthes, and placed between the acoelomic and true coelomic animals.

Phylogenetic hypotheses based on comparison of the 18S rRNA gene sequences were incompatible with these classic concepts. Ideas concerning the boundaries of the multicellular animal kingdom and the nearest relatives of bilaterally symmetrical animals have changed [14], and the phylogeny of these animals also appears in a new light. In the case of early

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segregation of protostomes and deuterostomes, the phylogeny of the former is now based on Annelida and Mollusca, the phyla whose ontogeny includes the trochophore, a ciliated planktonic larva, rather than on the previous group Articulata (Annelida and Arthropoda). On the phylogenetic trees of 18S genes, many "intermediate taxa" have found their place among trochophore animals. For example, lophophorates previously regarded as a connecting link between the protostomes and deuterostomes [15, 16]; nemerteans, regarded as the most highly organized acoelomic animals [17, 18]; or pogonophores, regarded as representatives of a new type of deuterostomes [19], together with the trochophore animals, rotifers, acanthocephalans, gnathostomulides, and flatworms (except for Acoela), formed a new large group of animals, Lophotrochozoa [9]. Other types of pseudocoelomic animals (e.g., nematodes) have found their relatives on the phylogenetic trees of 18S rRNA among the "higher coelomic" animals (specifically, arthropods) and, together with Onychophora, Tardigrada, Priapulida, Kinorhyncha, and Nematomorpha, formed another new large group of moulting animals, Ecdysozoa [20].

At the same time, some groups of invertebrates with unclear phylogenetic relationships (e.g., Acoela, Chaetognatha, Mesozoa, Orthonectida and Rhombozoa), and Myxozoa did not find their place on the phylogenetic trees of the 18S rRNA genes. The unreliable position of these groups on the 18S phylogenetic trees is explained by an insufficient resolution of relationships between animal phyla in the groups and a low level of bootstrap support. These features reflect two major problems in molecular phylogeny: the nonuniformity of the rate of evolution of nucleotide sequences in different phyletic lineages and within the same lineage, as well as the deficiency of phylogenetically informative traits.

No simple solutions to the first problem have been found thus far. The second problem can be solved by using the greatest possible number of genes in phylogenetic analysis. The goal of this study was (1) to assess the dependence between the number of nucleotide sequences analyzed and the reliability of reconstruction of phylogenetic trees and (2) to determine more precisely the phylogenetic relationships within the group Ecdysozoa (in particular, between its major groups: Nematoda, Nematomorpha, Priapulida, and Kinorhyncha). We performed analysis of the greatest set of 18S and 28S rRNA gene sequences known to date [21] and supplemented it with the previously determined sequences of 18S rRNA [22] and 28S rRNA of the kinorhynch *Pycnophyes kielensis,* and the hairworm *Gordius* sp. (determined in this work).

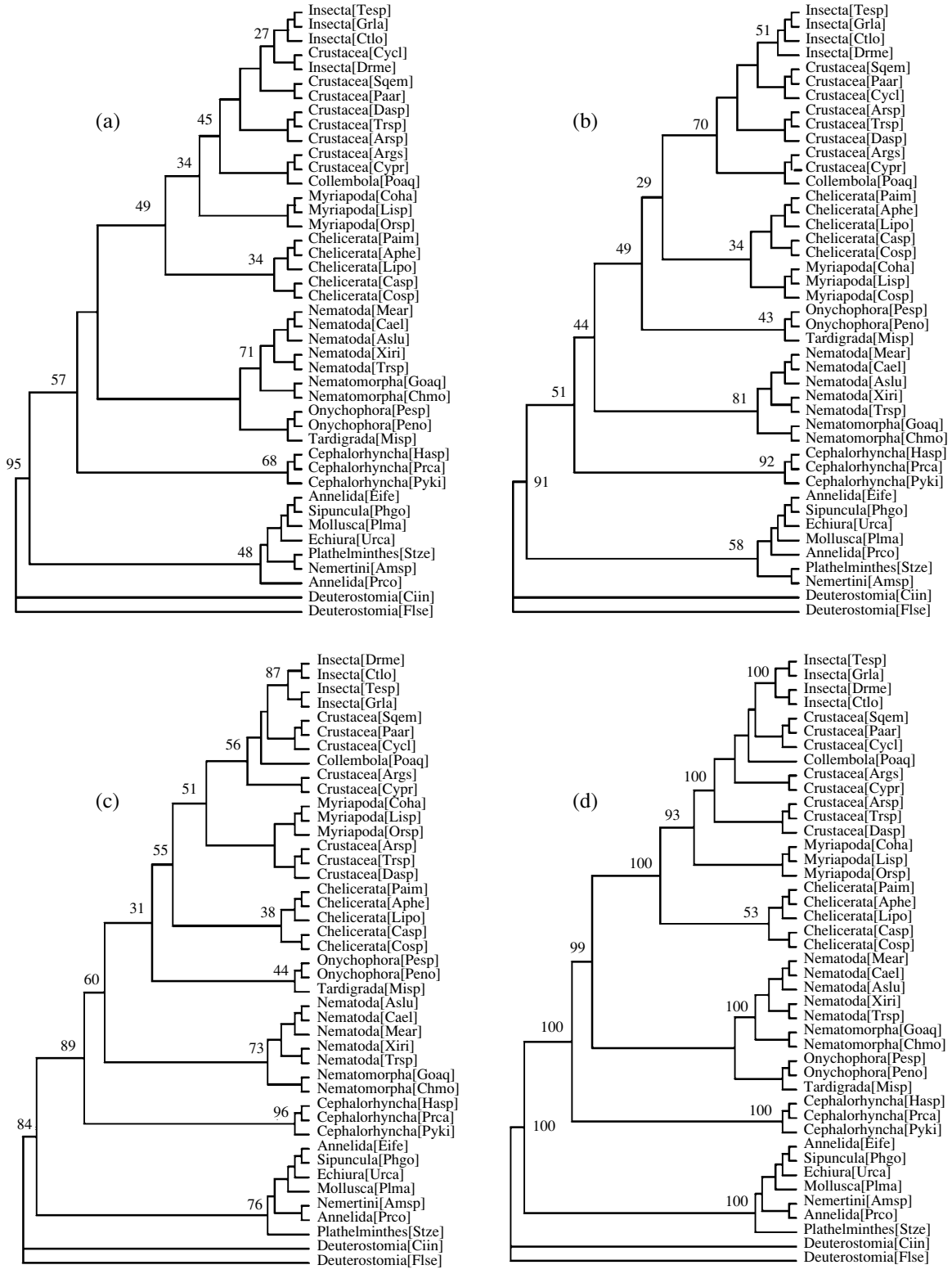
EXPERIMENTAL

Specimens of *Pycnophyes kielensis* (Kinorhyncha) were collected in the vicinity of the Kartesh Biological Station, Zoological Institute, Russian Academy of Sciences (Chupinskaya Bight of Kandalaksha Bay, White Sea) and delivered live to the laboratory. The hairworm *Gordius* sp. (Nematomorpha), found not far from the village Anisimovka (Shkotovskii rayon, Primorskii krai), was fixed in 95% ethanol.

Tissues of several kinorhynchs or several pieces of tissues of the hairworm were homogenized in 100 μl of a buffer containing 0.01 M Tris, 0.01 M EDTA, and 0.15 M NaCl (pH 8.0) and then lysed with 0.5% sodium dodecyl sulfate (SDS) and pronase (500 μg/ml) overnight at 37°C. DNA was isolated using the DI*Atom*™ DNA *Prep* 100 kit (Laboratoriya Izogen, Russia) according to the protocol provided by the manufacturer.

The rRNA genes were amplified by polymerase chain reaction (PCR). The universal eukaryotic primers for the 5' and 3' ends of genes were used to amplify the 18S rRNA genes [3], and a set of pairs of primers [23] overlapping the full length of genes was used to amplify the 28S rRNA genes. PCR products were purified by electrophoresis in agarose gel and used for determining nucleotide sequences using an automatic sequencer at the Genome Collective Sequencing Center (Institute of Molecular Biology, Russian Academy of Sciences). The sequences were deposited at the GenBank database (accession numbers AY863409–AY863411 for the 18S and 28S rRNA gene sequences of *Gordius* sp. and the 18S gene sequence of *Pycnophyes kielensis*, respectively).

Fig. 1. Phylogenetic trees of 18S rRNA gene sequences of metazoans constructed using four different methods: (a) MP, (b) NJ, (c) ML, and (d) BA. Numerals on the left of the major nodes of the trees designate the bootstrap support of these nodes, expressed in bootstrap indices (bi, %) for trees *a*, *b*, and *c* and posterior probability (pp, %) for tree *d*. The first two letters in abbreviated names in brackets designate the generic name; the last two letters designate the specific name. Insecta: *Tenebrio* sp. [Tesp], *Gromphadorhina laevigata* [Grla], *Ctenolepisma longicaudata* [Ctlo], and *Drosophila melanogaster* [Drme]; Collembola: *Podura aquatica* [Poaq]; Crustacea: Cyclopidae *Gen.* sp. [Cycl], *Squilla empusa* [Sqem], *Panulirus argus* [Paar], *Daphnia* sp. [Dasp], *Triops* sp. [Trsp], *Artemia* sp. [Arsp], *Argulus* sp. [Args], and Cyprididae *Gen.* sp. [Cypr]; Myriapoda: *Cormocephalus hartmeyeri* [Coha], *Lithobius* sp. [Lisp], and *Orthoporus* sp. [Orsp]; Chelicerata: *Pandinus imperator* [Paim], *Aphonopelma hentzi* [Aphe], *Limulus polyphemus* [Lipo], *Callipalene* sp. [Casp], and *Colossendreis* sp. [Cosp]; Nematoda: *Meloidogyne arenaria* [Mear], *Caenorhabditis elegans* [Cael], *Ascaris lumbricoides* [Aslu], *Xiphinema rivesi* [Xiri], and *Trichinella spiralis* [Trsp]; Nematomorpha: *Gordius aquaticus* [Goaq] and *Chordodes morgani* [Chmo]; Cephalorhyncha: *Halicriptus spinulosus* [Hasp], *Priapulus caudatus* [Prca], and *Pycnophyes kielensis* [Pyki]; Annelida: *Eisenia fetida* [Eife] and *Proceraea cornuta* [Prco]; Sipuncula: *Phascolopsis gouldii* [Phgo]; Mollusca: *Placopecten magellanicus* [Plma]; Echiura: *Urechis caupo* [Urca]; Plathelminthes: *Stylochus zebra* [Stze]; Nemertini: *Amphiporus* sp. [Amsp]; Deuterostomia: *Ciona intestinalis* [Ciin] and *Florometra serratissima* [Flse].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The above full-length 18S and 28S rRNA gene sequences were added to the set of full-length sequences of 18S and 28S rRNA genes of insects, crustaceans, myriapods, tardigrades, onychophores, nematodes, nematomorphs, priapulids, mollusks, nemerteans, flatworms, annelids, echiurids, sipunculids, and hemichordates [21]. Before starting phylogenetic analysis, we removed the nucleotide positions of variable V4 and V7 sites of 18S rRNA molecules and variable domains of 28S rRNA that defy unambiguous alignment. The resulting alignment pattern contained 1630 and 2795 positions of 18S and 28S genes, respectively.

Phylogenetic trees were constructed using four different methods on the basis of the sets of 18S and 28S rRNA gene sequences taken either separately or in combination (4425 positions). We used three methods from the PAUP* version 4.b10 package of phylogenetic programs [24]: the maximal parsimony (MP), neighbor-joining (NJ), and maximal likelihood (ML) methods, as well as Bayesian analysis (BA) realized in the MrBayes 3.01 program [25].

The parameters of the model of evolution of nucleotide sequences were determined using the Modeltest program [26], performing several calculation cycles.

To construct the MP trees, we performed a heuristic search with random addition of sequences in 20 replications, with subsequent optimization by the TBR algorithm [24]. Distant trees were constructed by the NJ method using genetic distances calculated by the ML method. For this purpose, we used the general model of reversible evolution, taking into account the proportion of invariant positions and corrections for heterogeneity of positions with respect to the evolution rate (GTR + Γ + I), whose parameters were determined using the Modeltest program.

The ML trees were constructed on the basis of results of a heuristic search with random addition of sequences using the same model; the trees were optimized by the SPR algorithm [24].

Bayesian analysis was performed on the basis of the general model of reversible evolution, taking into account the proportion of invariant positions and corrections for heterogeneity of positions with respect to the evolution rate (GTR + Γ + I), whose parameters were calculated using the MrBayes program. In the course of analysis, four Markov chains were run for 500000 generations, and 50000 trees were selected; 20000 trees were discarded as they did not reach the stationary state of chains, and the remaining 30000 trees were used to construct the consensus tree and estimate the posterior probability of its nodes.

The reliability of nodes of constructed trees was estimated using 500, 3000, and 100 bootstrap iterations in the case of MP, NJ, and ML methods, respectively.

The significance of differences between the trees was estimated using a set of statistical tests, including an improved AU test of Shimodaira [27], realized in the programs TREE-PUZZLE 5.2 [28] and CONSEL [29].

RESULTS

The trees constructed on the basis of the set of 18S rRNA gene sequences by the four different methods had a similar topology but were slightly different in some elements (Fig. 1). All trees included the main large monophyletic groups and the clades comprising lower-rank taxa.

Topological differences concern the position of onychophores and tardigrades and the relationships between myriapods and chelicerates. In MP (Fig. 1a) and BA (Fig. 1d) trees, onychophores and tardigrades form a clade with nematodes and nematomorphs, whereas in NJ and ML trees (Figs. 1b, 1c) they are at the base of the clade of arthropods (Myriapoda + Chelicerata + Crustacea + Collembola + Insecta), forming a monophyletic group together with them. On the NJ tree (Fig. 1b), myriapods form a clade with chelicerates, whereas on the MP and BA trees they form an individual clade at the base of the monophyletic group comprising crustaceans (Crustacea) and hexapods (Collembola + Insecta); on the ML tree, they join with a group of crustaceans within the same monophyletic group.

The topologies of the trees constructed using the same four methods on the basis of the set of the 28S rRNA gene sequences (Fig. 2), with some exceptions, exhibit mostly the same similarities and differences as the 18S rRNA trees. They are as follows. Onychophores and tardigrades in all trees enter the group of panarthropods (Onychophora + Tardigrada, Myriapoda + Chelicerata, Crustacea + Collembola + Insecta) but do not form a single group on the NJ tree (Fig. 2b). Nematodes and nematomorphs do not form a single clade on the BA tree (Fig. 2d) but comprise a paraphyletic group between panarthropods and cephalorhynchs. Myriapods form a monophyletic group only on the BA tree, whereas chelicerates do not form a monophyletic group on this tree, in contrast to other trees (Fig. 2d). In general, the monophyletic groups on the trees of 28S rRNA gene sequences are largely the same as on the 18S rRNA trees, except for Chelicerata and Myriapoda, which do not form such groups in all trees.

The trees constructed on the basis of combined 18S and 28S rRNA gene sequences (Fig. 3) do not principally differ in topology from the trees constructed on the basis of 18S and 28S rRNA genes taken separately; however, they differ from one another much less significantly. For example, onychophores and tardigrades within the same clade occupy the position at the base of the group of panarthropods on all trees; nematodes and nematomorphs always form a single

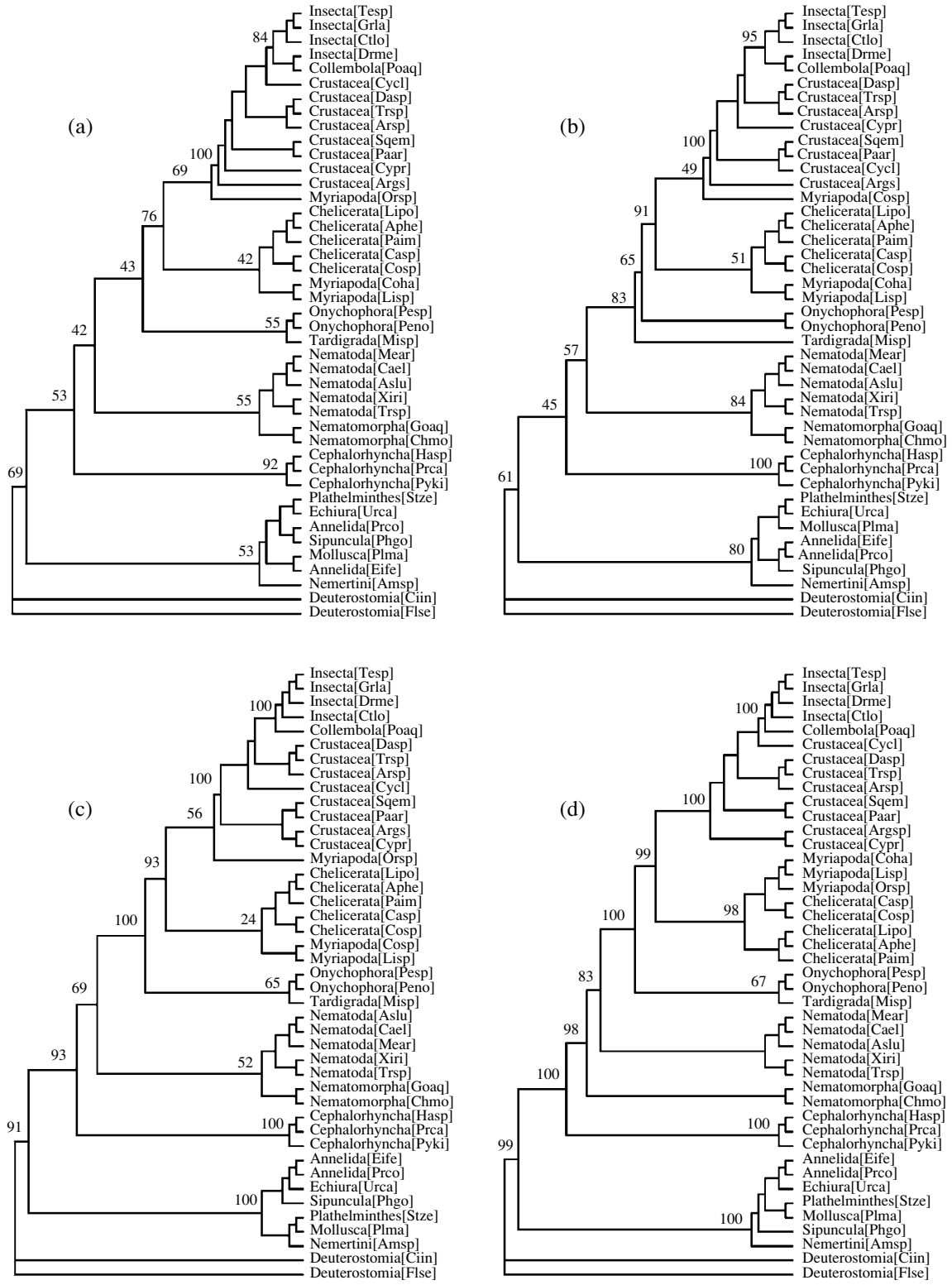

Fig. 2. Phylogenetic trees of 28S rRNA gene sequences of metazoans constructed using the four different methods. For designations, see Fig. 1. Numerals on the left of main nodes of trees show bootstrap indices of these nodes.

clade; myriapods and chelicerates (except for MP trees; Fig. 3a), join into a monophyletic group. Note that, on the trees based on all three sets of sequences, the clade Cephalorhyncha is always formed comprising kinorhynchs and priapulids; it never includes nematomorphs.

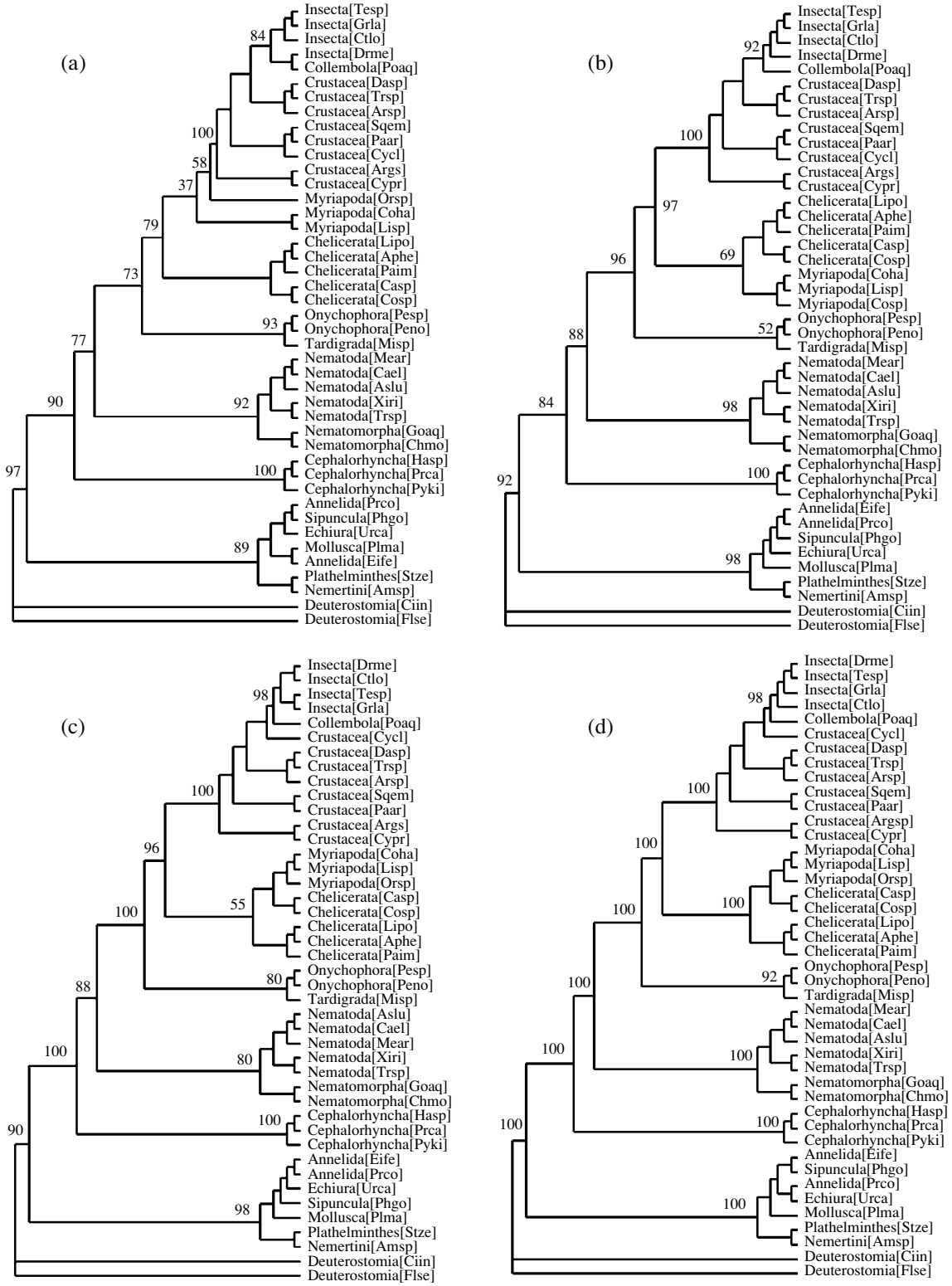

Fig. 3. Phylogenetic trees of combined 18 + 28S rRNA gene sequences of metazoans constructed using the four different methods. For designations, see Fig. 1. Numerals on the left of main nodes of trees show bootstrap indices of these nodes.

The reliability of phylogenetic constructs can be estimated from the consistency of tree topologies and from the bootstrap indices for tree nodes. These indices are shown near the corresponding nodes and summarized in the table. The nodes of trees are designated with capital letters in the direction from the roots to

Bootstrap support indices of the main nodes of phylogenetic trees of ribosomal RNA genes

Node	18S				28S				18 + 28S			
	MP	NJ	ML	BA	MP	NJ	ML	BA	MP	NJ	ML	BA
A	95	91	84	100	69	61	91	99	97	92	90	100
B	57	51	89	99	53	45	93	100	90	84	100	100
C	29	44	60	99	42	57	69	98	77	88	88	100
C_1	–	–	–	–	–	–	–	83	–	–	–	–
D	–	49	31	–	43	83	100	100	73	96	100	100
D_1	–	–	–	–	–	65	–	–	–	–	–	–
E	49	29	55	100	76	91	93	99	79	97	96	100
E_1	34	–	51	93	69	49	56	–	37	–	–	–
E_2	–	–	–	–	–	–	–	–	58	–	–	–
F	45	70	56	100	100	100	100	100	100	100	100	100
G	48	58	76	100	53	80	100	100	89	98	98	100
H	68	92	96	100	92	100	100	100	100	100	100	100
I	71	81	73	100	55	84	52	–	92	98	80	100
J	–	43	44	–	55	–	65	67	93	52	80	92
K	34	34	38	53	42	51	24	98	33	69	55	100
L	27	51	87	100	84	95	100	100	84	92	98	98

Designation of the reconstruction methods used: MP, maximal parsimony; NJ, neighbor-joining; ML, maximum likelihood; and BA, Bayesian analysis.

treetops. They join the following groups of animals (Fig. 4): A, all protostomes; B, all molting animals (Ecdysozoa); C, nematodes and nematomorphs with all panarthropods; D, panarthropods; E, all arthropods; F, group of crustaceans and insects (Pancrustacea); G, Lophotrochozoa in a broad sense (including nemerteans and flatworms except Acoela); *H*, Cephalorhyncha (priapulids and kinorhynchs); *I*, nematodes and nematomorphs; *J*, onychophores and tardigrades; *K*, myriapods and chelicerates; and *L*, insects. The appearance of additional nodes designated by letters with subscript indices (see table), correlating with the disappearance of some major nodes, reflects the instability of position of certain groups on the trees. For example, the appearance of the additional node B_1 on the BA tree of 28S rRNA genes and the disappearance of the node *I* on the same tree is related to the splitting of the generally stable group of nematodes and nematomorphs. The appearance of the node D_1 and the corresponding disappearance of the node *J* reflects the splitting of the clade of onychophores and tardigrades, which is stable in all other cases (the absence of the node *J* on the MP and BA trees of the 18S rRNA genes is accounted for by the relocation of the entire clade of onychophores and tardigrades in the clade of nematodes and nematomorphs). The appearance of additional nodes E_1 and E_2 is explained by unstable relationships within the group of myriapods and chelicerates.

In general, the distribution pattern of nodes on the trees constructed on the basis of different gene sets is more diverse in the case of the 18S and 28S trees rather than on the 18S + 28S trees. For example, out of the 18S + 28S trees constructed using different methods, only the MP tree (Fig. 3a) has two additional nodes D_1 and D_2, which reflects segregation of the group of myriapods and chelicerates, as well as segregatioin of the clade of myriapods into two branches. All the other 18S + 28S trees have the same set of nodes (table); i.e., they are identical with respect to the set of the main monophyletic groups. Topological differences between these trees boil down to the differences in relative positions of certain groups within the Pancrustacea (Crustacea + Collembola + Insecta) and Lophotrochozoa (Annelida + Sipuncula + Echiura + Mollusca + Plathelminthes + Nemertini).

The mean values of the bootstrap index for the main nodes of the 18S + 28S trees are greater than those for the nodes of the 18S and 28S trees; i.e., the reliability of the trees constructed on the basis of combined sequences of the 18S + 28S rRNA genes is higher than that of trees constructed on the basis of the 18S and 28S rRNA gene sequences taken separately.

The high degree of topological similarity between the 18S + 28S trees obtained by different methods allowed us to construct a consensus tree (Fig. 4) on the basis of 5600 trees obtained as a result of bootstrap analysis using the MP, NJ, and ML methods. The consensus tree contained all monophyletic groups that were present on the trees constructed by different methods using all three sets of 18S and 28S rRNA gene sequences. The only exception were chelicerates, which did not form a single clade on the consensus tree. On the whole, the values of the bootstrap index for the main nodes of this tree were significantly higher than those for the nodes of the trees constructed for the 18S and 28S rRNA genes taken separately.

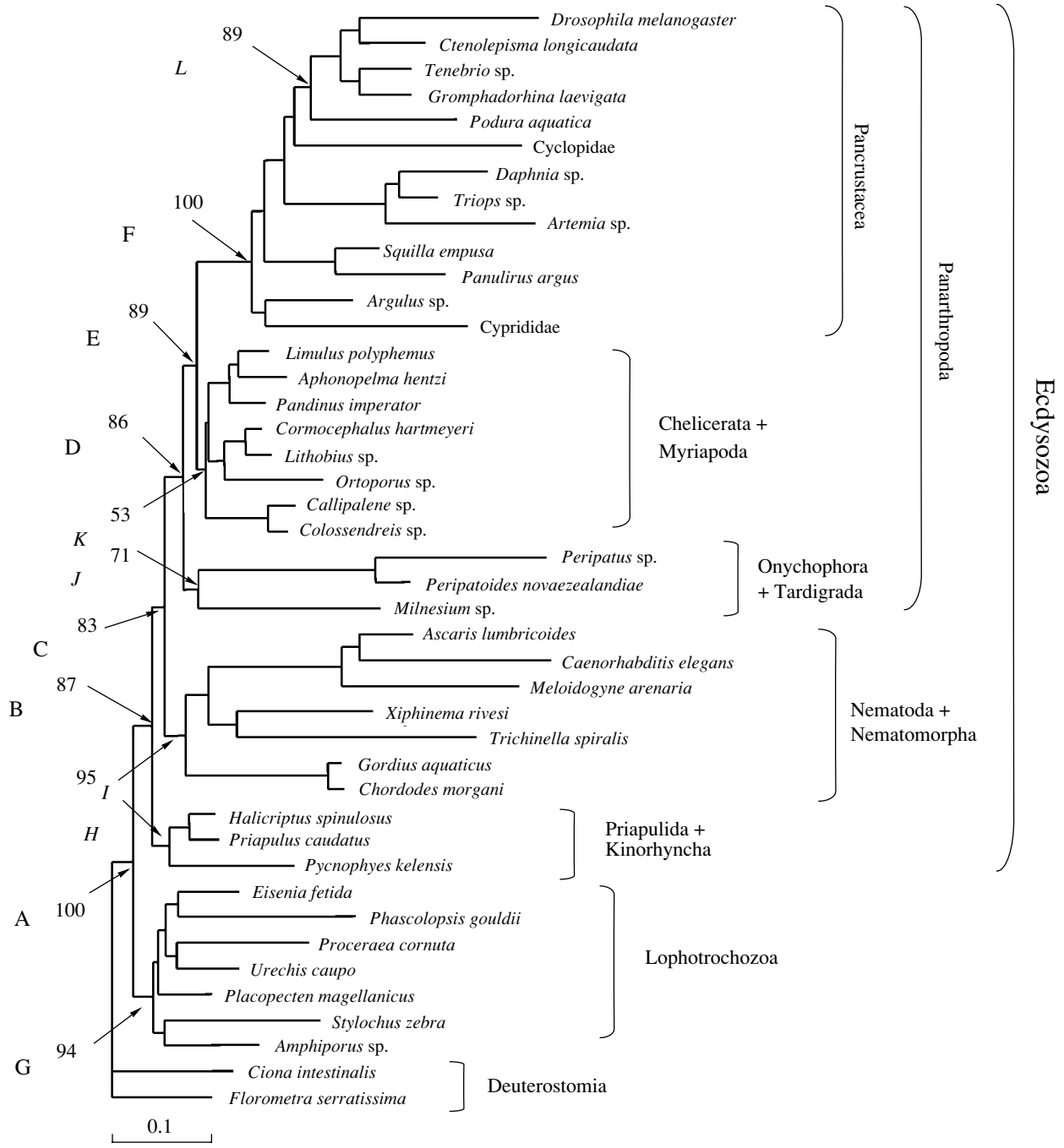

Fig. 4. Consensus phylogenetic tree constructed after bootstrap analysis of combined 18 + 28S rRNA gene sequences by MP, NJ, and ML methods. The tree was optimized as a result of several calculation cycles using the ML method. Arrows indicate the main nodes of the tree joining large groups (designated by capital letters, see table) and smaller groups (designated by italicized capital letters) of animals. Designations of nodes correspond to the following groups: A, Protostomia; B, Ecdysozoa; C, (Nematoda + Nematomorpha) + Panarthropoda; D, Panarthropoda; E, (Myriapoda + Chelicerata) + Pancrustacea; F, Pancrustacea; G, Lophotrochozoa (= Spiralia); *H*, Cephalorhyncha; *I*, Nematoda + Nematomorpha; *J*, Onychophora + Tardigrada; *K*, Myriapoda + Chelicerata; and *L*, Hexapoda (Collembola + Insecta). The main groups of animals are constrained with brackets. Numerals near respective letters and arrows show bootstrap indices, %.

DISCUSSION

The results of this study provide evidence for the phylogenetic hypothesis assuming the existence of Ecdysozoa, which is alternative to the classic coelomate hypothesis of metazoan phylogeny. The main features of the new (ecdysozoan) hypothesis include (1) early segregation of metazoans into protostomes and deuterostomes and (2) segregation of protostomes

into molting Ecdysozoa and nonmolting Lophotrochozoa (= Spiralia), which are characterized by spiral cleavage in embryogenesis. The group of ecdysozoans was proposed as a result of analysis of 18S rRNA gene sequences [20]; it is based on joining pseudocoelomic nematodes and coelomic arthropods. The coelomate hypothesis is based on kinship between metameric coelomic animals, annelids and arthropods, which were united by Cuvier into the group Articulata. The first data testifying against a close kinship between these groups were obtained by cladistic analysis of morphometric traits [30], which joined nemerteans, mollusks, sipunculids, and annelids into an individual clade. After phylogenetic analysis of 18S rRNA genes [15], this clade was transformed into the group Lophotrochozoa, which also included phoronides, brachiopods, and bryozoans. Interestingly, the same morphological analysis that joined nematodes, kinorhynchs, tardigrades, onychophores, and arthropods into one clade gave rise to another group of animals that, after molecular analysis of 18S rRNA genes, was termed Ecdysozoa [20].

To date, molecular analysis of 18S and 28S rRNA genes [6–8, 21, 31–35] and other genes [36, 37], cladistic analysis of morphological traits [8–11, 33], as well as analysis of combined molecular and morphological traits [38], provided ample evidence for the ecdyzoan concept. However, there are morphological [39, 40] and molecular [41–43] data that may be regarded as arguments in favor of the alternative articulate (coelomate) hypothesis. In 2004, the results of studies on a large number of genes encoding proteins were published, although the samples of taxa used in two works [42, 43] were insufficiently representative. For example, nematodes are represented in these samples by only one species, *Caenorhabditis elegans*, which is characterized by a high rate of evolution accompanied by the loss of molecular traits. Taking this factor into account, the results of analysis of 1712 orthologous genes and combinations of 2906 protein domains testify to the ecdysozoan hypothesis [44]. This result is consistent with our earlier data [45] indicating that, when numerous genes are used for analysis, the factor of representativeness of a sample of taxa is important as well [45].

The bootstrap index for the Ecdyzoa on the consensus tree (87%; Fig. 4) may serve as reliable evidence in favor of the ecdysozoan hypothesis [46]. The following monophyletic groups may be distinguished within the group of Ecdysozoa on this tree: Panarthropoda (Onychophora + Tardigrada, Myriapoda + Chelicerata, Pancrustacea), Nematoda + Nematomorpha and Cephalorhyncha (Priapulida + Kinorhyncha). On the basis of morphological traits, the last two groups were formerly joined into the clade Cycloneuralia [47]. However, our data do not corroborate the relevance of such joining, because Cephalorhyncha in the group Priapulida + Kinorhyncha on our trees are situated at the base of all ecdysozoans, forming an individual clade. However, our data reliably support the clade Nematoida (bootstrap index 95%) [9] and contradict the joining of nematomorphs with priapulids and kinorhynchs into one clade of the phylum rank [48]. This clade is also disproved by statistical tests [27–29] when the consensus tree is compared with the trees on which nematomorphs were withdrawn from the clade Nematoida and transferred to other branches (data not shown).

The group Panarthropoda, which includes onychophores, tardigrades, and arthropods, is weakly supported on the 18S trees (bootstrap indices 49 and 31% on the NJ and ML trees; the group is absent from the MP and BA trees); however, it is better supported on the 28S trees and even more reliably supported on the 18 + 28S trees. This accounts for the fact that this group, though accepted by morphologists [47], was not revealed in the reconstructions based solely on 18S rRNA. The relationships between phyla within this group remain obscure. The joining of onychophores and tardigrades with bootstrap support less than 70% is observed on many 18S and 28S trees. On the 18S + 28S trees, this joining has somewhat greater bootstrap support (71 and 92% on the consensus tree (Fig. 4) and BA tree (Fig. 3d), respectively). Thus, the question on the kinship between tardigrades and onychophores remains open.

The question concerning the relationships between the groups of arthropods (Chelicerata, Myriapoda, Crustacea, Collembola + Insecta (= Hexapoda)) deserves special attention. Before the appearance of molecular reconstructions, two hypotheses prevailed in phylogeny: (1) the *mandibulate hypothesis*, which joined groups of animals that have mandibles (Hexapoda + Myriapoda, Crustacea), opposing them to chelicerates, and (2) the *athelocerate hypothesis*, which joined groups of animals with single-branched appendages (Hexapoda + Myriapoda) into the group Uniramia and animals with double-branched appendages into the group Schizoramia. The two hypotheses are based on presumably close kinship between hexapods and myriapods; however, molecular data testify to a close kinship between hexapods and crustaceans within the group Pancrustacea [49–51]. This situation led to the appearance of two new hypotheses, the first of which joins pancrustaceans with myriapods, opposing them to chelicerates [49], and the second joins myriapods and chelicerates into one clade that is sister to pancrustaceans [50–52]. Our data provide evidence for the last hypothesis, reliably supporting the group Pancrustacea (Fig. 4) and less reliably supporting the joining of myriapods with chelicerates.

Our results also confirm the monoplyletic nature of Hexapoda (bootstrap index 89% on the consensus tree, Fig. 4). The monophyletic nature of this group

was questioned judging by the results of analysis of complete mitochondrial genomes [53]; however, it was shown that comparison of nuclear genes is preferable in the cases of deep divergence [54].

On the whole, our results show that the combined 18S and 28S rRNA gene sequences contain a greater number of phylogenetically informative traits than the sequences of these genes taken separately. For this reason, analysis of combined data ensures better bootstrap support for the main nodes of trees and better consistency of the topologies of trees constructed by different methods and allows us to reliably resolve phylogenetic relationships within the group Ecdysozoa. To conclude, the results of this study demonstrate (1) paraphyly of the group Cycloneuralia; (2) basal position of Cephalorhyncha (Kinorhyncha + Priapulida) in the group Ecdysozoa and the absence of close kinship between Cephalorhyncha and Nematoda; (3) monophyly of the groups Nematoida, Panarthropoda, Arthropoda, Pancrustacea (Crustacea + Hexapoda), and Hexapoda (Collembola + Insecta) at a high level of bootstrap support; and (4) better correspondence of our data to the phylogenetic reconstructions that join Myriapoda and Chelicerata into the clade that is sister to Pancrustacea.

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