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Molecular Phylogeny of Gastrotricha on the Basis of a Comparison of the 18S rRNA Genes: Rejection of the Hypothesis of a Relationship between Gastrotricha and Nematoda

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Abstract—Gastrotricha are the small meiobenthic acoelomate worms whose phylogenetic relationships between themselves and other invertebrates remain unclear, despite all attempts to clarify them on the basis of both morphological and molecular analyses. The complete sequences of the 18S rRNA genes (8 new and 7 known) were analyzed in 15 Gastrotricha species to test different hypotheses on the phylogeny of this taxon and to determine the reasons for the contradictions in earlier results. The data were analyzed using both maximum likelihood and Bayesian methods. Based on the results, it was assumed that gastrotrichs form a monophyletic group within the Spiralia clade, which also includes Gnathostomulida, Plathelminthes, Syndermata (Rotifera + Acanthocephala), Nemertea, and Lophotrochozoa. Statistical tests rejected a phylogenetic hypotheses considering Gastrotricha to be closely related to Nematoda and other Ecdysozoa or placing them at the base of the Bilateria tree, close to Acoela or Nemertodermatida. Among gastrotrichs, species belonging to the orders Chaetonotida and Macrodasyida form two well-supported clades. The analysis confirmed monophyly of the families Chaetonotidae and Xenotrichulidae from the order Chaetonida, as well as the families Turbanellidae and Thaumastodermatidae from the order Macrodasyida. Lepidodasyidae is a polyphyletic family, because the genus *Mesodasys* forms a sister group for Turbanellidae; genus *Cephalodasys* forms a separate branch at the base of Macrodasyida; and *Lepidodasys* groups with *Neodasys* between Thaumastodermatidae and Turbanellidae. To confirm these conclusions and to get an authentic view of the phylogeny of Gastrotricha, it is necessary to study more Gastrotricha species and to analyze some other genes.

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Key words: 18S rRNA, molecular phylogeny, Bilateria, Protostomia, Spiralia, Ecdysozoa, Gastrotricha, Gnathostomulida, Nematoda

INTRODUCTION

Gastrotrichs, small and mainly meiobenthic acoelomate worms, form a small invertebrate group that is traditionally included as a class or phylum into pseudocoelomates or aschelminths [1–3]. Phylogenetical relationships of Gastrotricha are still unclear. Initially, they were considered to be close to rotifers or nematodes [2]. Further cladistic analysis of their morphological characters suggested that gastrotrichs occupy a more basal position on the phylogenetic tree of bilaterally symmetrical metazoa (Bilateria) [4]. Gastrotrichs were also considered as a sister group for Introverta (Nematoda + Nematomorpha + Priapulida + Kinorhyncha + Loricifera) [5, 6] or Ecdysozoa (Introverta + Panarthropoda) [7, 8], related with flatworms and Gnathifera (Gnathostomulida + Syndermata) [9], or included into the Platyzoa group together with Gnathostomulida, flatworms, and rotifers [10].

Analysis of the full-length 18S RNA gene sequences from one or three Gastrotricha species has shown that they are close to flatworms [11–13] and/or gnathostomulides, or can be placed to the base of the Bilateria tree right after Acoela and Gnathostomulida [7]. At the same time, analysis of partial sequences of seven species has not shown any close relationships between gastrotrichs and nematodes or rotifers [14];

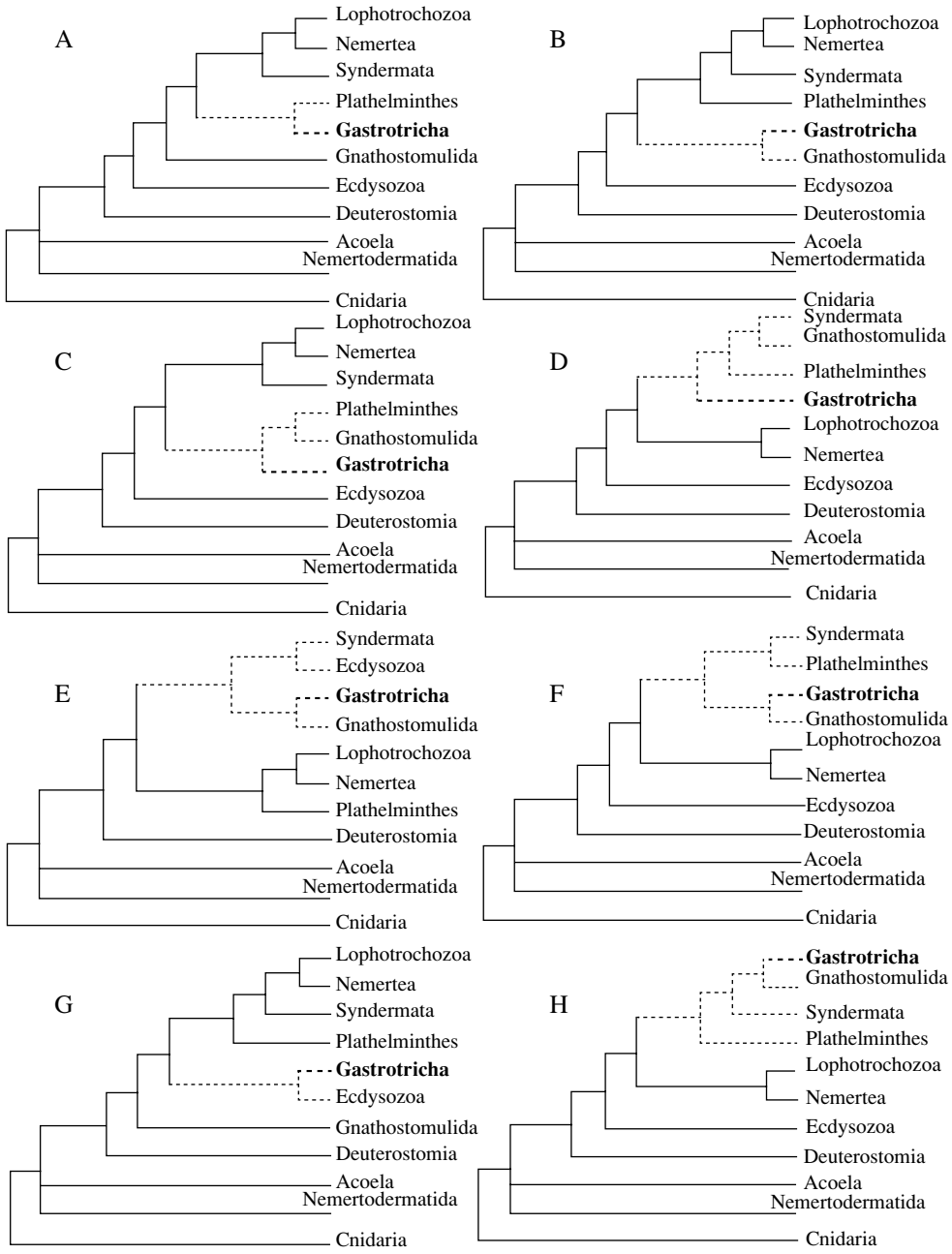

Fig. 1. Possible variants of Gastrotricha phylogeny. Variants A and B place Gastrotricha close to Plathelminthes or Gnathostomulida (Monoconta, or Neotrichozoa), respectively. Variant C combines Gastrotricha with Plathelminthes and Gnathostomulida into one group. Variants D, F, and H reflect the variants of the Platyzoa concept, combining Gastrotricha with Gnathostomulida, Plathelminthes, or Rotifera, respectively. Variants E and G place Gastrotricha close to the Ecdysozoa group. The groups containing gastrotrichs are marked with dotted lines.

however, when the number of partial sequences has been increased (up to 14 species), their possible relationships with Gnathostomulida, Plathelminthes, and Syndermata group, uniting the Rotifera and Acanthocephala, have been demonstrated [15].

Thus, phylogenetic analysis of morphological and molecular data obtained by different authors allows different hypotheses about the relationships of gastrotrichs. Some of these hypotheses are shown in Fig. 1, where eight possible variants of phylogenetic relations of gastrotrichs with other Bilateria phyla are shown. The figure does not show variants placing the gastrotrichs to the base of the Bilateria tree, near Acoela and Nemertodermatida [7, 16].

The internal phylogeny of gastrotrichs is also still unclear. Large differences between two Gastrotricha

orders regarding their morphology and ultrastructure suggest this group to be paraphyletic in relation to nematodes [1, 17], but recent cladistic analysis of morphological characters testifies to its monophyly [18, 19]. At the same time, some doubts remain concerning the monophyly of the families Lepidodasyidae and Planodasyidae of the order Macrodasyida, as well as the position of the unusual genus *Neodasys*, which has several morphological features typical of the order Macrodasyida [3] but is assigned to the separate suborder Multitubulatina of the order Chaetonotida.

Thus, analysis of molecular data produces conflicting results regarding both monophyly of the group and its internal phylogenetic relationships [14, 15, 20] and does not clarify the above-mentioned questions. In this study, we investigated the phylogenetic relationships of gastrotrichs, using an extended set of full sequences of the 18S rRNA genes. We determined eight full sequences, pooled them with the seven already known sequences, and carried out phylogenetic analysis, using a set of similar sequences obtained from species belonging to different invertebrate groups. The results obtained testify to the monophyly of gastrotrichs and their close relation to the Plathelminthes + Syndermata + Nemertea + Lophotrochozoa group and allow us to reject some hypotheses about their relationships.

EXPERIMENTAL

Gastrotrichs were collected in the neighborhood of the Marine Biological Station of St. Petersburg State University (Chupinskaya Bay of Kandalaksha Gulf, White Sea) and fixed with 95% ethanol (5–30 worms of every species). We investigated the following Gastrotricha species: order Chaetonotida: *Xenotrichula* sp. (family Xenotrichulidae) and *Neodasys* sp. (family Neodasydae); order Macrodasyida: *Tetranchyroderma* sp. (family Thaumastodermatidae), *Lepidodasys* sp., *Mesodasys* sp., *Cephalodasys* sp. (family Lepidodasyidae), *Macrodasys buddenbrocki* (family Macrodasyidae), *Turbanella lutheri*, and *T. cornuta* (family Turbanellidae).

DNA isolation. To amplify the 18S rRNA genes, DNA was isolated using modified alkaline lysis with subsequent neutralization and without any additional purification [21]. Several animals were placed into 20 μl of 0.25 M NaOH for 3–16 h at room temperature. The lysate was heated for 3 min at 95°C, combined with 10 μl of 0.5 M Tris-HCl (pH 8.2), neutralized with 12 μl of 0.4 N HCl, combined with 5 μl of 2% Triton X-100, heated again at 95°C for 3 min, and stored at –20°C. To carry out the amplification, we used 0.5–2 μl of the lysate.

Amplification of the 18S rRNA genes was carried out by PCR with universal eukaryotic primers [22]. The PCR products were purified by agarose gel electrophoresis. To determine the nucleotide sequences of the 18S rRNA genes, we used either purified PCR products or the products of their cloning in pBluescript KS+.

Sequence alignment. The 18S rRNA gene sequences obtained were added to an alignment containing the full sequences of these genes from seven other Gastrotricha species, species from the main Bilateria groups, and also a sequence obtained from *Anemonia sulcata* (Cnidaria), used as an outgroup (Table 1). Prior to phylogenetic analysis, we excluded the nucleotide positions that resisted unambiguous alignment; such positions occurred mainly in variable regions V4 and V7 of the 18S rRNA. The final variant of the alignment contained 49 sequences, each including 1634 positions.

Phylogenetic trees of the 18S rRNA genes were constructed using the maximum likelihood (ML) method with the PAUP software package, version 4.b10 [23]; the mlsearch and DNArates programs [24]; and the MrBayes program, version 3.01 [25].

Parameters of a sequence evolution model necessary for the ML analysis were computed using the Modeltest program [26]. The parameters obtained in the first cycle of calculations were used in the appropriate PAUP block to construct a tree by the neighbor-joining (NJ) method; the best tree was selected according to the minimal evolution criterion. The tree obtained was used in the next calculation cycle of the Modeltest program to specify the parameters of the evolution model; the parameters were used for reconstruction and selection of the best tree by the ML method. The analysis has been repeated until a stable topology of the tree was obtained.

Bayesian analysis was carried out using a general time reversible model of sequence evolution with a γ correction for the rate heterogeneity of substitutions among sites and taking into account the proportion of invariable sites (GTR + G + I); parameters of the model were directly calculated by the MrBayes program. In analysis of 400,000 generations for four Markov chains, we selected 40,000 trees, of which 15,000 were rejected as having not reached convergence of the chains. For the other 25,000 trees, we constructed a consensus tree and evaluated the posterior probability (PP) of its nodes.

Statistical significance of differences between the trees was evaluated using improved Shimodaira's AU-test [27] and the CONSEL program [28].

RESULTS

All reconstruction methods yielded trees of the same topology (Fig. 2). The following basic characteristics are typical for all these trees: (1) the two first Bilateria branches are represented by Acoela and

Table 1. Sequences of the 18S rRNA genes used in the study

Sequences determined in the study		
taxonomic position	species	GenBank accession no.
Gastrotricha, order Chaetonotida	*Xenotrichula* sp. aff. *velox*	AY963686
Gastrotricha, Neodasys	*Neodasys* sp.	AY963687
Gastrotricha, order Macrodasyida	*Macrodasys buddenbrocki*	AY963692
	Mesodasys sp.	AY963690
	Lepidodasys sp.	AY963689
	Cephalodasys sp.	AY963691
	Tetranchyroderma sp. aff. *paradoxa*	AY963688
	Turbanella luteri	AY963693
Other sequences used in this study		
Gastrotricha, order Chaetonotida	*Lepidodermella squammata*	U29198
	Chaetonotus sp.	AJ001735
	Xenotrichula intermedia	AY228128
Gastrotricha, order Macrodasyida	*Paraturbanella dohrni*	AY228139
	Turbanella cornuta	AF157007
	Tetranchyroderma papii	AY228137
	Pseudostomella etrusca	AY228136
Annelida, class Polychaeta	*Nereis pelagica*	AF474279
class Oligochaeta	*Eisenia fetida*	X79872
Pogonophora	*Siboglinum fiordicum*	X79876
Vestimentifera	*Ridgeia piscesae*	X79877
Mollusca, class Polyplacophora	*Acanthopleura japonica*	X70210
class Bivalvia	*Mytilus edulis*	L33448
Brachiopoda	*Lingula anatine*	X81631
	Terebratalia transversa	U12650
Phoronida	*Phoronis ijimai*	AY202113
Nemertea	*Prostoma eilhardi*	U29494
	Lineus sp.	X79878
Acanthocephala	*Neoechinorhynchus crassus*	AF001842
Rotifera	*Brachionus plicatilis*	U49911
Plathelminthes, “Turbellaria”	*Stenostomum* sp.	U95947
	Discocelis tigrina	U70078
	Coelogynopora gynocotyla	AJ243679
	Nemertinoides elongatus	AY078381
	Meara stichopi	AF119085
	Paratomella rubra	AF102892
class Trematoda	*Schistosoma mansoni*	U65657
Gnathostomulida	*Haplognathia* sp.	AF119084
	Gnathostomula sp.	AF119083
Nematomorpha	*Gordius aquaticus*	X87985
Priapulida	*Priapulus caudatus*	X80234
Kinorhyncha	*Pycnophyes kielensis*	U67997
Arthropoda, class Insecta	*Tenebrio molitor*	X07801
class Pycnogonida	*Nymphon* sp.	U88338
Nematoda	*Enoplus brevis*	U88336
	Paracanthonchus caecus	AF047888
	Longidorus elongatus	AF036594
Hemichordata	*Balanoglossus carnosus*	D14359
	Ptychodera flava	AF278681
Cnidaria, Anthozoa	*Anemonia sulcata*	X53498

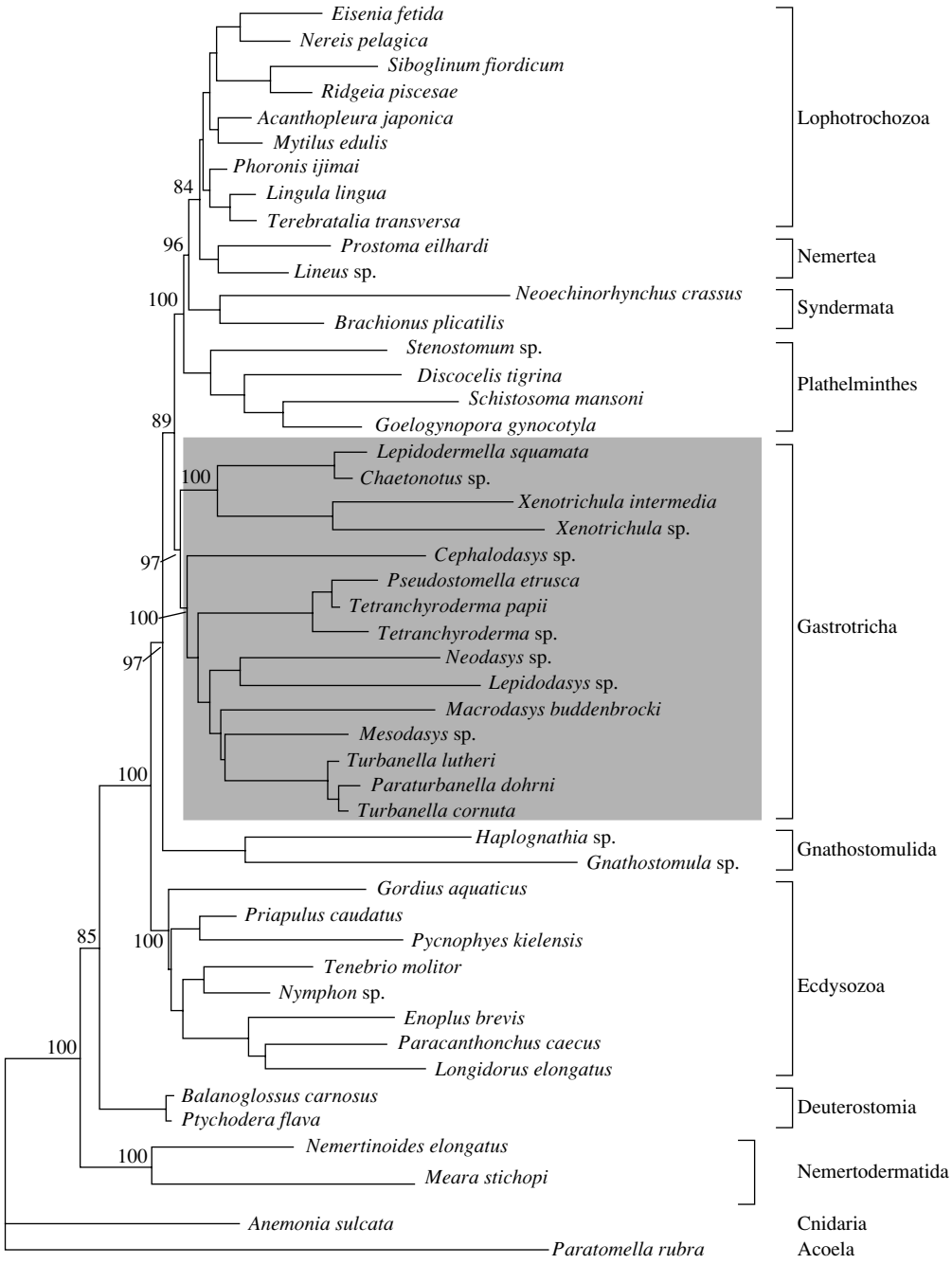

Fig. 2. Phylogenetic tree of the 18S rRNA genes of Bilateria. The tree was constructed using the Bayesian analysis of the set of 48 Bilateria sequences and the sequence from *Anemonia sulcata* as an outgroup. The topology of the tree corresponds to that of the trees obtained by the ML method. The monophyletic group of gastrotrichs is shaded. Arrows point to the joining nodes of the main monophyletic groups; the statistical support of the nodes (indicated) is the percent posterior probability obtained by the Bayesian analysis.

Nemertodermatida; (2) the division of Bilateria into Deuterostomia (represented by Hemicordata) and Protostomia takes place right after the separation of the first two branches; (3) the primary protostomian clade is formed by Ecdysozoa, molting animals with chitinous shells (represented by Nematomorpha, Priapulida, Kinorhyncha, Arthropoda, and Nematoda); (4) Gastrotricha are separated as an independent branch right after the separation of Ecdysozoa and Gnathostomulida and belong to a monophyletic group, which also includes Plathelminthes, Rotifera, Syndermata, Nemertini, and the Lophotrochozoa group, including Brachiopoda, Mollusca, Vestimentifera, Pogonofora, and Annelida.

Table 2. Statistical evaluation of the reliability of differences between the best tree obtained and other trees reflecting other variants of the phylogeny of Gastrotricha*

Phylogeny variant[1]	Difference[2]	Indices of reliability of differences for different test variants							
		Au	Np	Bp	Pp	Kh	Sh	Wkh	Wsh
1	–5.4	0.907	0.669	0.674	0.996	0.852	0.983	0.798	0.986
3 (B)	5.4	0.239	0.072	0.073	0.004	0.148	0.741	0.148	0.539
7 (F)	10.0	0.326	0.157	0.068	5e–005	0.202	0.436	0.202	0.573
9 (H)	10.0	0.325	0.157	0.086	5e–005	0.202	0.436	0.202	0.573
2 (A)	10.3	0.066	0.023	0.021	3e–005	0.075	0.436	0.075	0.240
4 (C)	11.9	0.172	0.058	0.054	7e–006	0.127	0.369	0.127	0.401
5 (D)	15.7	0.071	0.019	0.020	2e–007	0.118	0.244	0.109	0.301
8 (G)	19.4	0.007	0.003	0.002	4e–009	0.030	0.156	0.030	0.074
10	19.5	0.004	0.009	0.011	4e–009	0.031	0.076	0.031	0.045
6 (E)	31.4	0.001	2e–004	2e–004	2e–014	0.014	0.029	0.009	0.037
11	59.5	1e–004	5e–005	0	1e–026	1e–004	3e–004	3e–004	0.001

Notes: * See text for explanations.

[1] The phylogeny variants are placed in order of increasing difference from the best tree. Variants 2–9 correspond to topologies A–H shown in Fig. 1; variants 10 and 11 correspond to topologies with the basal location of Gastrotricha (not shown).

[2] The values reflect the difference in maximum likelihood logarithm (–Ln ML) between the topologies of the best tree and the trees reflecting other variants of Gastrotricha phylogeny.

The main monophyletic groups (indicated with arrows pointing to the joining nodes) have a high statistical support (percent PP is shown at the arrows). Thus, PP is 100% for Bilateria as a whole, Nemertodermatida, Ecdysozoa, the whole protostomian clade, and the Plathelminthes + Syndermata + Nemertea + Lophotrochozoa clade. On the whole, the monophyletic group of gastrotrichs (PP = 97%) forms two equal well-supported clades (PP = 100%) corresponding to the orders Chaetonotida and Macrodasyida. The monophyletic group including Gastrotricha and the Plathelminthes + Syndermata + Nemertea + Lophotrochozoa clade has a weaker support (PP = 89%); the support of the Gnathostomulida + Gastrotricha + Plathelminthes + Syndermata + Nemertea + Lophotrochozoa clade is slightly higher (PP = 97%).

The resulting best tree was compared with the trees reflecting other possible variants of phylogenetic relationships of gastrotrichs. In addition to the variants shown in Fig. 1, we included two variants with the basal position of gastrotrichs. The results of the comparison carried out with the use of the improved AU-test [28] are shown in Table 2.

Tree 1 is the best tree according to the ML test (Fig. 2); numbers 2–9 correspond to phylogeny variants designated as A, B, C, D, E, F, G, and H (Fig. 1); numbers 10–11 represent the variants with a more basal position of Gastrotricha. All variants are arranged in the order of increasing difference from the best tree by the probability logarithm (second column). The next eight columns show the probability (p) indices for the difference of each of the variants from the best variant. These indices were obtained using different statistical tests realized in the CONSEL program [28]. Significant differences correspond to $p < 0.05$.

All statistical tests indicate that the best tree significantly differs from variant E, which unites Gastrotricha with Gnathostomulida, Ecdysozoa, and Syndermata, and from variants 10 and 11 (not shown), which place Gastrotricha to the base of the Bilateria tree, right after Acoela and Nemertodermatida or between them. Topology G, uniting Gastrotricha with molting Ecdysozoa, also differs from the best variant in most of the indices (excepting Shimodaira–Hasegawa index (column Sh) and weighted Shimodaira–Hasegawa index (column Wsh)). Topology D (one of the variants of the Platyzoa concept) differs in three indices (columns Np, Bp, and Pp) [10]. All other variants, including two other variants of the Platyzoa concept (Fig. 1, F and H), differ from the best tree only in the mildest PP index (Pp column).

DISCUSSION

On the whole, the results of our analysis of the 15 full-length sequences of the 18S rRNA genes coincided with the results of many other molecular reconstructions based on the 18S rRNA genes, which show the early division of Bilateria into Protostomia and Deuterostomia and the division of Protostomia into two clades, molting Ecdysozoa and nonmolting Spiralia, and place Acoela to the base of Bilateria [7, 29–32].

According to our results, Gastrotricha, along with Gnathostomulida, take a border position in the well-supported monophyletic Spiralia group. All phylogeny variants that do not differ significantly from the phylogeny presented by the best tree assign gastrotrichs to this group. On the contrary, the variants that suppose Gastrotricha to be closely related to Ecdysozoa (Fig. 1, E and G) sufficiently differ from the best tree in many parameters. None of the molecular reconstructions shows close relationships of gastrotrichs to nematodes or other Ecdysozoa groups [11, 14, 15, 20, 33, 34]. Thus, our results obviously conflict with the hypotheses suggesting sister relationships of gastrotrichs with nematodes [1] or Ecdysozoa [5, 35] on the basis of morphological data analysis.

Evidence of the proximity of Gastrotricha to Gnathostomulida is less unequivocal. The possibility of such proximity has already been supposed by authors who combined Gastrotricha and Gnathostomulida into one group, named Monokonta [10] or Neotrichozoa [33]. According to our analysis, the variant combining gastrotrichs with flatworms (Fig. 1a) differs from the best tree by three statistical tests (Table 2, row 5, columns Np, Bp, and Pp). Variant B, combining Gastrotricha with Gnathostomulida (Fig. 1b), differs from the best tree by only one and the mildest statistical test (Table 2, row 2, column Pp). Thus, it seems more preferable to combine Gastrotricha with Gnathostomulida, rather than with Plathelminthes, whose proximity to gastrotrichs has been inferred from early molecular analyses [11]. Among the three topology variants reflecting the Platyzoa concept (Fig. 1; D, F, and H), topologies F and H, which place Gastrotricha and Gnathostomulida together, differ to a lesser extent from the best tree topology as compared to topology D, where these groups are placed far from each other (Table 2). On the whole, the variants with a close proximity of Gastrotricha and Gnathostomulida differ from our tree (Fig. 2) by at least one statistical test, but neither of them differs by all tests performed. Therefore, based on our analysis, these phylogeny variants cannot be rejected with certainty.

As for the monophyly of gastrotrichs and their intragroup phylogeny, then, contrary to some hypotheses [17, 36] and the results of our earlier analysis of the partial 18S rRNA sequences [20], the tree of full-length sequences demonstrates the well-supported (PP = 97%) monophyly of gastrotrichs. The Gastrotricha clade splits into two statistically reliable (PP = 100%) clades corresponding to the orders Macrodasyida and Chaetonotida. This fact quite agrees with the results of other molecular analyses [15, 20] but conflicts with earlier data [14]. Our data on the position of the genus *Mesodasys* are also in conflict with the results of the study [14]. In our tree, as in a 18S rRNA gene tree constructed by other authors [15], this genus groups, although with a weak support (PP = 45%), with species of the family Turbanellidae within the Macrodasyida clade, evidencing a polyphyly of the family Lepidodasyidae, to which it is assigned. All these facts confirm our supposition [20] that there were some mistakes in the study [14] concerning specific identification of gastrotrichs or the labeling of DNA preparations, which caused the above contradictions. In our tree, the sequence isolated from worms of the genus *Neodasys* groups with the sequence from the genus *Lepidodasys*, included into the Macrodasyida clade; this fact conflicts with the results of the morphological analysis [18] and the position of this genus within the order Chaetonotida accepted by most zoologists. Our results confirm the monophyly of the families Xenotrichuldae and Chaetonotidae within the Chaetonotida clade (PP = 100%). The support of other internal nodes of the Gastrotricha clade is much weaker.

On the whole, our results show that gastrotrichs are a monophyletic invertebrate group positioned at the base of the Spiralia group on the Bilateria tree, in the immediate proximity of Gnathostomulida and Plathelminthes. This conclusion allows us to reject the hypotheses about the relationships of gastrotrichs with nematodes or the Ecdysozoa group but does not exclude some alternative variants of their relationships with other groups. Thus, the question of the phylogenetic relationships of gastrotrichs with other invertebrate groups and within themselves still remains unclear; neither morphological nor molecular data on the 18S rRNA genes do finally solve this problem. Obviously, it is necessary to significantly increase the sample of species and to use the sequences of other genes in order to answer this question.

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