

5S rRNA sequences of 12 species of flatworms: implications for the phylogeny of the Platyhelminthes

B. I. Joffe¹, K. M. Valiejo Roman², V. Ya. Birstein³ & A. V. Troitsky²

¹ Zoological Institute RAN, St-Petersburg 199034, Russia

² A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia

³ N. K. Koltzov Institute of Developmental Biology RAN, Moscow 117334, Russia

Key words: 5S rRNA, Platyhelminthes, Turbellaria, parasitic flatworms, phylogeny

Abstract

5S rRNAs from 12 species of free living and parasitic platyhelminthes were sequenced. In the phylogenetic analysis, attention was focused on the statistical estimates of the trees corresponding to existing phylogenetic hypotheses. The available 5S rRNA data agree well with widely accepted views on the relationships between the Acoela, Polycladida, Tricladida, and Neorhabdocoela; our analysis of the published 18S rRNA sequences also demonstrated good correspondence between these views and molecular data. With available 5S rRNA data the hypothesis that the dalyelliid turbellarians is the sister group of the Neodermata is less convincing than the hypotheses proposing the Neodermata as the sister group of the Neorhabdocoela, or of the Seriata, or of the branch uniting them. A relatively low rate of base replacement in parasitic flatworms, probably, accounts for the uncertain position of the Neodermata, while a relatively high rate in planarians may explain a relatively too early divergence of the Tricladida in several published phylogenetic trees constructed from various rRNA data.

Introduction

The phylogeny of the Platyhelminthes is a promising field for application of molecular methods because, while some problems have already found reasonable solutions and may serve as a 'control' (see Ehlers, 1985; Rieger *et al.*, 1990; Rohde, 1990), many others remain unsolved. By now, portions of 18S rRNA have been sequenced from various platyhelminthes (Baverstock *et al.*, 1990; Ruitort *et al.*, 1992 a,b). Yet, additional sequence data are still necessary, in particular, from other molecules. With the 12 new sequences presented here, a total of 14 sequences of 5S rRNA may now contribute to this goal of elucidating platyhelminth phylogeny.

The shortness of the 5S rRNA limits its phylogenetic usage (Hendriks *et al.*, 1986; Hori & Osava, 1987). Therefore, we focused our attention not on inferring phylogenetic trees, but on the statistical estimation of the existing phylogenetic hypothesis, *i.e.*, on their compatibility with 5S rRNA sequence data. This aspect of the problem is important irrespective of the length of

the analyzed sequences. Indeed, a tree computed from molecular data is a statistical estimate, and one must know whether there are statistically significant reasons to prefer it (at least, under the assumptions of the used tree constructing method) to some other topology, e.g. one supported by morphological evidence.

Material and methods

The species studied, their systematic positions and collection sites are listed in Table 1. The total RNA was isolated by the hot phenol extraction procedure with 4M guanidinium isothiocyanate; 5S rRNA was purified by electrophoresis on 8% polyacrylamide gel and sequenced by the Peattie's chemical method after 3'-end labelling with (5'-32P)pCp as described earlier (see Troitsky *et al.*, 1991 for the protocol used).

The sequences used for rooting the platyhelminth tree were taken from the 'Berlin RNA databank' (Specht *et al.*, 1990) with the exception of 5S rRNA of *Echinorhynchus gadi* which was sequenced by

Table 1. Systematic positions and sites of collection of the platyhelminth species used in this study

'Turbellaria'	
Archoophora	
Acoela	
<i>Convoluta convoluta</i> (Abildgaard, 1806)	(1)
Polycladida	
<i>Notoplana humulis</i> (Stimpson, 1857)	(2)
* <i>Planocera reticulata</i> (Stimpson, 1855)	
Neophora	
Tricladida	
Fam. Dugesiidae	
*Dugesia (<i>Dugesia</i>) <i>japonica</i> (Ichikawa et Kawakatsu, 1964)	
<i>Dugesia</i> (<i>Girardia</i>) <i>tigrina</i> (Girard, 1850)	(3)
Fam. Planariidae	
<i>Planaria torva</i> O. F. Muller, 1773	(4)
Fam. Dendrocoelidae	
<i>Dendrocoelium lacteum</i> (O. F. Muller, 1773)	(4)
Neorhabdoocula	
Typhloplanoida	
<i>Bothromesostoma esseni</i> (Braun, 1885)	(5)
Kalyptorhynchia	
<i>Macrorhynchus crocea</i> (O. Fabricius, 1826)	(1)
Dalyellioidea	
<i>Graffilla graffi</i> Mitin, 1970	(6)
<i>Pseudograffilla arenicola</i> (Meixner, 1938)	(7)
Monogenea	
<i>Discocoryle sagittata</i> (Leuckart, 1842)	(8)
Cestoda	
<i>Khavia sinensis</i> Hsu, 1935	(9)
Trematoda	
<i>Fasciola hepatica</i> Linne, 1758	(10)

* Species sequenced by other authors. For the species sequenced by us, the numbers in parentheses indicate the collection sites, as well as the hosts for the parasitic species. (1) Dalnezelenetskaya Inlet, Barentz Sea; (2) Biological station 'Vostok', Sea of Japan; (3) laboratory stock; (4) a pond near St-Petersburg; (5) a pool in Borok (Yaroslavl District, Russia); (6) from the marine gastropod, *Neptunea despecta*, Biological station 'Kartesh', White Sea; (7) Biological station 'Kartesh', White Sea; (8) from the fish *Coregonus albula*, Ladoga lake; (9) from the fish *Cyprinus carpio*, fish-farm in Estonia; (10) from cows from a slaughter house.

us and will be published elsewhere. All the platyhelminth sequences wholly fitted to the alignment universally used for 5S rRNA (see Specht *et al.*, 1990). 3'-terminal uracil residues were not used in the analysis because variation in number of 3'-terminal uracils (2 or 3) was observed in practically all studied species. Phylogenetic analysis was done using

PHYLIP 3.3 package (Felsenstein, 1990). Maximum likelihood (ML) method was always implemented with empirical estimation of base frequencies and a transition/transversion ratio = 2. Distances were calculated using the 2-parameter Kimura's model, also with a transition/transversion ratio = 2. Lower probability for fixation of transversions is suggested by a very high level of conservation of the secondary structure of rRNAs, which is always affected by transversions, while the influence of transitions is moderated by non-canonical pairing (GU). The value of 2 (rather than, e.g., 1.5 or 3) was empirically chosen based on a series of preliminary experiments with various rRNA data sets. Computation of the ML trees was repeated three times with different species input order (one starting with the root sequence and two random ones) for each species set with global rearrangements. Being mainly interested in the statistical estimates of given tree topologies, we omitted bootstrapping; moreover, with the ML method, branch lengths which are not significantly positive themselves indicate the places where changes in the tree are most probable (Felsenstein, 1990).

Results and discussion

Twelve 5S rRNA sequences of the species studied are presented in Fig. 1 together with the sequences from two species studied earlier (Ohama *et al.*, 1986; Hori *et al.*, 1988).

Approach to the phylogenetic analysis

The first question we had to answer was whether the tree(s) constructed from 5S rRNA data reproduced the relations between the main groups of turbellarians represented in our material (Fig. 2A) which had been convincingly established from morphological evidence (Ehlers, 1985; Rieger *et al.*, 1990; yet, see Rohde, 1990 and present volume for different views — these views are not discussed below because no tree topology suitable for statistic estimation has been published).

In view of the fact that changes in the set of species are the most important test for the consistency of the tree topology (see, e.g. Wainwright *et al.*, 1993), we constructed the trees for several sets of species. Each of them included all the 14 platyhelminth sequences and differed from the others in the single outgroup (rooting) sequence. This explicitly showed the effect of each outgroup on the tree topology and provided a

C.co
P.re
N.hw
cD.j
D.ti
D.la
P.to
M.cr
B.es
G.gr
P.ai
D.sa
K.s.
F.h

CC
Pr
Nh
Dj
Dt
D1
Pt
Mc
Be
Gg
Pa
Ds
Ks
Fh

Fig. 1
seque
P. tor
long.

seve
regi
seq
we
the
ed
cati
cep
lati
sus
bra
spe
nel
(w
bu
ity

		10	20	30	40	50		
<i>C. convoluta</i>	(Cc)	GCCUACGACCAUACGAUGUUGAAUACACCGGUUCGUCCGAUCACCGAA-GU						
<i>P. reticulata*</i>	(Pr)	.AUAG..U.....C.CAC....A.....				C...		
<i>N. humulis</i>	(Nh)	..UA..UG.....UC.YAC....A.Y.....						
<i>cD. japonica</i>	(Dj)	.U.G...CU.....U.G...GG.....C.A.....U.....U.G...U..						
<i>D. tigrina</i>	(Dt)	.U.A.U.G.....UG...GG.....A.....U.....U.....U.....						
<i>D. lacteum</i>	(Dl)	.U.....GU.....AUCUG.G.A.UG..U.A.....U.....U.A.....						
<i>P. torva</i>	(Pt)	.U.A.G..U.....UGCCG.G.A.U..UU.A.....U.....U.A...C..						
<i>M. crocea</i>	(Mc)	.U.AG.....C.CCG....A.U.....						
<i>B. esseni</i>	(Be)	.U.A...G.....UC..AG..GCA.....				U.....C...		
<i>G. graffi</i>	(Gg)	.AG.G...G.....UC.....G.A.....				U.....		
<i>P. arenicola</i>	(Pa)	.AG.G...G.....UC.C.....GCA.....						
<i>D. sagittata</i>	(Ds)	...AG.....U.C.....						
<i>K. sinensis</i>	(Ks)	N.GAG.....U.C..A....GU.....						
<i>F. hepatica</i>	(Fh)	YG.CG.....U.C....G.A.U.....U.....						
		u iiii	ui uiu i iiii	iiii i i i i				
		60	70	80	90	100	110	120
<i>Cc</i>	UAAGCAACAUUGGCCUGGUAGUACUJUGGAUGGGUGACCGCUUGGGAACACCGGGUGUUGUAGGCUUU							
<i>Pr</i>GUG.C.....CA.....					C.....U..U.....C.CUAC....		
<i>Nh</i>GUG.C.A...YA..U.....					C.....U..U.....A..UA....		
<i>Dj</i>G.C..A.....C.....		A...C.....G..UC.....U..GA.....G...C.A....					
<i>Dt</i>U...UAAC...UGC.....		AU.....A.....U..GCA.....CCA.U.A....					
<i>Dl</i>GUAGAAU..UAC.....		U..U.....UA.GC.....U..GUA.....C...A....					
<i>Pt</i>GCGCAA..UAC.....		C..A.....U.GC.....U..GUA.....C.U.A....					
<i>Mc</i>CGG.....UC..A.....		C.....UG.....U..A.....CU.A....					
<i>Be</i>CUG.C.A..G.....		C.....UG.....U..UAC...C...U.A...U					
<i>Gg</i>C.A..G.....		UC.....UG.....U..UAC...C..C.CU...U					
<i>Pa</i>G.C.A..ACU.....		C.....UG.....U..A.U.....C.CU....					
<i>Ds</i>G.....C.....		C.....GC.....U.....CU.....					
<i>Ks</i>U..G.....C.....		C.....GC.....U.....CUC.A....					
<i>Fh</i>G.C.....C.....		C.....GC.....U.....CGCCG....					
	iiiiu	ii	iuiuiiiii	ii uiui	iiiiiiuii	iii	ii	

Fig. 1. 5S rRNA sequences known from the platyhelminths. The sequence of *P. reticulata* (*) was published earlier; the majority consensus sequence for *D. japonica* is compiled from three sequences known from three populations (Ohama *et al.*, 1986; Hori *et al.*, 1988). Insertion in *P. torva* is proved by presence of a minor rRNA fraction which was partially sequenced and has in the same position an insertion 3 nucleotides long. Dots indicate the nucleotides identical to those in *C. convoluta*. i, invariant position; u, unique state.

severe test for its consistency, especially, in the root region.

To provide maximal homogeneity of the set of sequences in the analysis (see, e.g., Felsenstein, 1990), we used for rooting all the sequences known from the animals generally considered more closely related to the Platyhelminthes, that is *Brachionus pilicaris* (Rotatoria), *Echinorhynchus gadi* (Acanthocephala), *Embletonema gracile*, and *Lineus geniculatus* (Nemertini). In addition, the majority consensus sequences for four more distant lower invertebrate groups were composed and used: cPorifera (3 species), cCoelenterata (6), cNematoda (3), and cAnnelida (3). The rooting sequences were rather diverse (with distances between them of 0.071–0.348 K_{nuc}), but did not seem to markedly increase the heterogeneity of the analyzed data sets — at least, the distances

between the outgroup and platyhelminth sequences (0.090–0.597 K_{nuc}) varied within the same range as the distances between the platyhelminth sequences themselves (0.093–0.625 K_{nuc}).

Phylogenetic trees

Figure 2 shows that six of the eight used rooting sequences inferred maximum likelihood (ML) trees (Figs 2B,C) which correspond well to recent views on the phylogeny of the Turbellaria (Fig. 2A) and differ only in the position of the parasitic flatworms. The seventh tree was very similar, though *Echinorhynchus* ‘branched off together with polyclads’ instead of rooting the platyhelminth tree (Fig. 2B, arrowhead). The tree rooted with *Lineus* was very different (Fig. 2D), though this sequence in itself did not seem peculiar

Table 2. Comparison of several tree topologies applied to eight species sets rooted with different outgroup sequences

Tree topology	Rooting sequence							
	<i>Brachionus</i>		<i>Emplectonema</i>		cCoelenterata		<i>Echinorhynchus</i>	
	Ln L	t	Ln L	t	Ln L	t	Ln L	t
ML	-1038.459	-	-1044.60	-	-1053.06	-	-1041.01	-
C	-1045.945	1.06	-1051.56	0.96	-1060.18	1.00	-1057.90	1.68
D	-1047.508	1.49	-1052.96	1.17	-1061.75	1.23	-1059.54	1.91
B	-1047.020	1.37	-1053.20	1.49	-1061.71	1.49	-1059.82	1.99*
A1	-1059.044	1.66	-1065.21	1.61	-1073.40	1.64	-1071.49	2.07*
A2	-1064.366	1.94	-1068.82	1.76	-1076.89	1.75	-1074.84	2.09*
A3	-1067.434	2.13*	-1071.52	2.09*	-1080.14	2.13*	-1077.85	2.37*
Tree topology	Rooting sequence							
	cAnnelida		cPorifera		cNematoda		<i>Lineus</i>	
	Ln L	t	Ln L	t	Ln L	t	Ln L	t
ML	-1026.779	-	-1049.85	-	-1078.22	-	-1047.73	-
C	-1030.887	0.53	-1054.75	0.63	-1083.03	0.62	-1058.37	0.74
D	-1032.716	0.73	-1056.61	1.02	-1085.00	1.03	-1060.05	0.87
B	-1032.177	0.89	-1056.66	0.95	-1084.80	0.93	-1059.99	0.85
A1	-1042.349	1.21	-1068.74	1.41	-1097.17	1.42	-1071.06	1.24
A2	-1046.519	1.39	-1073.18	1.59	-1102.65	1.70	-1074.87	1.42
A3	-1050.101	1.82	-1077.08	2.05*	-1105.89	1.89*	-1078.69	1.70

Ln L, logarithm likelihood, t - Student's t-ratio for the difference in Ln L between given tree (see Fig. 3) and the ML tree for corresponding data set. * denotes that difference is statistically significant (>1.96 for $p<0.05$).

(distances to the other rooting sequences 0.101–0.244 K_{nuc}, those to the platyhelminth species 0.142–0.475 K_{nuc}; see the previous paragraph). This confirms that our series of outgroup sequences was an effective test for the consistency of the dominant tree topology. Yet, two details of this topology are probably erroneous: *Dugesia* is definitely a monophyletic taxon, and the Cestoda is generally considered more closely related to the Monogenea, than to the Trematoda. This questions the reliability of other branching patterns and outlines the importance of statistical estimation of the tree topologies (see below).

On the position of the Neodermata

Parasitic flatworms could be grouped together in the same way in all ML trees, though two branches with zero length are to be taken in account here (Fig. 2). Maximum parsimony (MP) and Fitch trees nearly always support monophyly of the Neodermata. The

phylogenetic relationships of the Neodermata remain controversial. The Neodermata is most often considered either the sister group for the Dalyellioidea, rhabdocoels with doliform pharynx (see, e.g., Ehlers, 1985; Brooks, 1989), or as an earlier branch of rhabdocoels which retained the less specialized rosulate pharynx (see Joffe *et al.*, 1987, Joffe & Chubrik, 1988; Kotikova & Joffe, 1988 for review and evidence from the morphology of the pharynx and nervous system). Two other hypotheses which could be tested with our data would consider the Neodermata the sister group either for the Neorhabdocoela + Seriata (c.f. Ruitort *et al.*, 1992b), or for the Seriata (note again that the hypotheses suggested by Rohde, (1990 and present volume) cannot be discussed here).

Phylip allows a statistical test of whether the difference between logarithm likelihoods or lengths of two given trees is significant. We estimated 6 topologies representing these four hypotheses (Fig. 3) in each of our 8 species sets. Statistical estimates (Student's t-

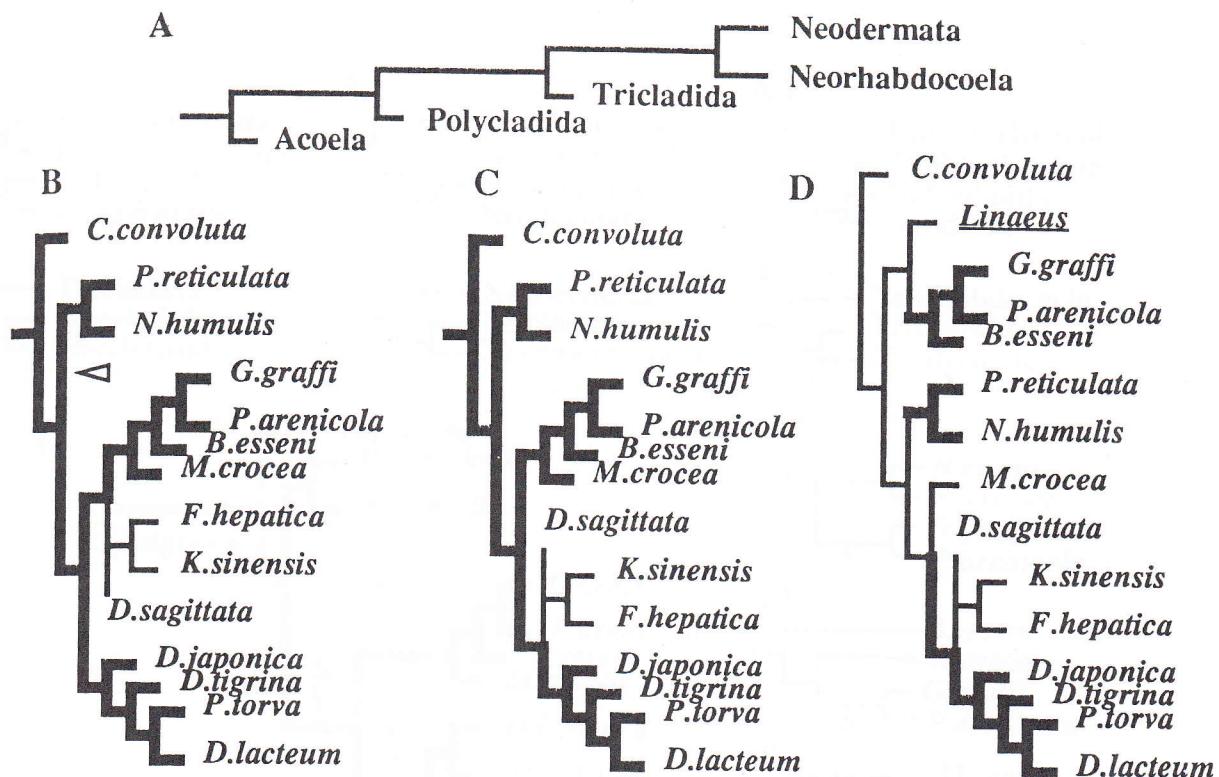


Fig. 2. A. Phylogenetic tree representing recent views on the evolution of the Turbellaria and one of widely accepted views on the position of the Neodermata. B-D. Topologies of maximum likelihood trees for eight sets of species with different outgroup sequences. B. *Brachionus*, cCoelenterata and *Echinorhynchus*; the last occupies a position shown with arrowhead (and not in the root). C. cAnnelida, cPorifera, cNematoda. D. *Lineus* (the branches present in the trees on Figs B and C are shown by the wide lines).

ratio) for the trees based on the 'dalyelliod' hypothesis were 1.15–2 fold higher than for three other hypotheses, and its variant (Fig. 3,A3) was significantly worse than ML tree in 6 of 8 data sets (Table 2). Thus, the 'dalyelliod' hypothesis fits poorly with the available 5S rRNA data compared with other tested assumptions. None of the 3 other hypotheses may be preferred.

Compatibility of recent hypotheses on the phylogeny of the Turbellaria with rRNA molecular data

Statistical estimates described in the previous paragraph also demonstrated that the tree topologies representing widely accepted recent views on the phylogeny of the Turbellaria, with Neodermata not branching with the Dalyellioda (Figs 2A, 3E, b,c,d), fit well with 5S rRNA data (Table 2).

The maximum parsimony (MP) method returned 1–10 shortest trees, depending of the species set. Estimates of various hypotheses about the relationships of Neodermata were the same as obtained from ML

method. The trees corresponding to recent views on the platyhelminth phylogeny, provided that the Neodermata were not derived together with the Dalyellioda, were less than 5% longer than MP trees, and the difference was far from being statistically significant.

In addition, we united the data published by Ruitort *et al.*, 1992a and Baverstock *et al.*, 1991, corrected alignment and, thus, obtained two data sets including 16 and 13 platyhelminth species with 443 and 536 well aligned positions correspondingly. For the sake of comparison, they were rooted as in Baverstock *et al.* (1991). Studied both by ML and MP methods, these data also demonstrated no statistically significant disagreement with phylogenetic concepts corresponding to the tree shown on Fig. 2A.

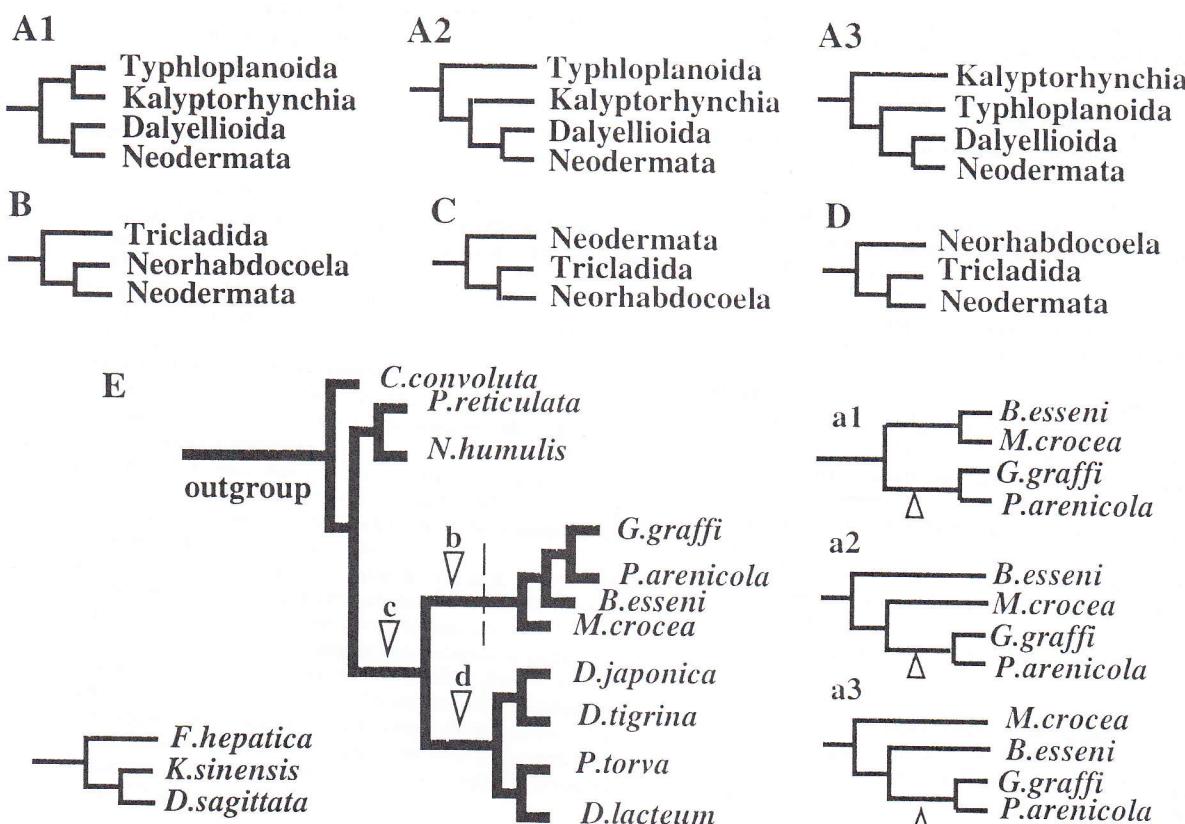


Fig. 3. A–D. Trees, representing four hypotheses about the relationships of the Neodermata with the Turbellaria, the ‘dalyelliod’ hypotheses is shown in 3 variants, A1–A3. E, topologies of the trees used for the statistical estimation of these hypotheses. To make trees corresponding to hypotheses shown in B,C,D the neodermate subtree (bottom, left) was grafted to the base turbellarian tree (bottom, center) at the positions marked with arrowheads b,c,d accordingly. To compose the trees corresponding to A1–A3, the neorhabdocoel subtree (on the right of the dashed line) was replaced by subtrees a1–a3 correspondingly, with the neodermate subtree grafted at the positions shown by arrowheads.

The rates of nucleotide base replacement and phylogenetic trees

The relative rate of change may be approximated from the lengths of the branches in the trees (ML, MP, or Fitch-Margoliash) or directly from a distance matrix. With any method, the rate of base replacement in 5S rRNA of planarians was estimated as high, and that of parasitic platyhelminths as very low. These results completely agree with the estimates obtained from 18S rRNA by Ruitort *et al.* (1992b). A high rate of evolution may cause inappropriate early divergence of a group in the tree or incorrect clustering of the ‘quick clocks’ together (see, e.g., Lake, 1991). Therefore, the uncertainty of the position of the Neodermata and/or a relatively too early position of the planarian branch in various trees constructed from 5S and 18S rRNA data (see Hendriks *et al.*, 1986; Hori & Osava, 1987; Baver-

stock *et al.*, 1991; Ruitort *et al.*, 1992a,b) may result, correspondingly, from too low and too high rates of base replacement. The influence of the heterogeneity in the rates of base replacement is important for it can drastically affect the topology of phylogenetic trees independently of the sequence lengths.

Acknowledgments

We are very grateful to Dr O. N. Yunchis (Research Institute for Lake and River Fishery, St-Petersburg) and to Dr T. A. Timofeeva (Zoological Institute, St-Petersburg) who collected for us two parasitic species. Dr E. N. Grusov from the same Institute let us use his computer, though it notably hindered his own work. We are also very grateful to our colleagues from Moscow State University: to Dr V. K. Bobrova for the help in

experi
and wil
Antono
remark

Refere

Baversto
endg
site J
RNA
Brooks,
helm
J. Pa
Ehlers,
Fish
Felsens
num.
Hendri
1986
pod
latte
Hori, I
198
RN
Hori, I
ded
Jotte,
mo
ym
tio
21
Jotte,
ph
str

experimental work, to Miss Katya Drusina for perfect and willing technical assistance, and to Prof. A. S. Antonov for constant attention to this work and critical remarks to the manuscript.

References

- Baverstock, P. R., R. Fielke, A. M. Johnson, R. A. Bray & I. Bevridge, 1991. Conflicting phylogenetic hypothesis for the parasitic platyhelminths tested by partial sequencing of 18S ribosomal RNA. *Int. J. Parasitol.* 21: 329-339.
- Brooks, D. R., 1989. The phylogeny of the Cercoherdaria (Platyhelminthes: Rhabdocoela) and general evolutionary principles. *J. Parasitol.* 75: 606-616.
- Ehlers, U., 1985. Das phylogenetische System der Plathelminthes. G. Fisher, Stuttgart - New York, 317 pp.
- Felsenstein, J., 1990. PHYLIP 3.3. University of California Herbarium, Berkley, California.
- Hendriks, L., E. Huysmans, A. Van den Berghe & R. De Wachter, 1986. Primary structure of the 5S ribosomal RNAs of 11 arthropods and applicability of 5S RNA to the study of metazoan evolution. *J. Mol. Evol.* 24: 103-109.
- Hori, H., A. Muto, S. Osawa, M. Takai, K.-Y. Lue & M. Kawakatsu, 1988. Evolution of turbellaria as deduced from 5S ribosomal RNA sequences. *Fortschritte Zool./Progress Zool.* 36: 163-167.
- Hori, H. & S. Osawa, 1987. Origins and evolution of organisms as deduced from 5S rRNA sequences. *Mol. Biol. Evol.* 4: 445-472.
- Joffe, B. I., G. S. Slusarav & T. A. Timofeeva, 1987. Stroenie glotki mongenei i ih filogeneticheskie sviasi s turbellariami. [Pharynx structure in the monogeneans and their phylogenetic relationships with the turbellarians.] *Parasitologia* (St-Petersburg) 21: 472-481.
- Joffe, B. I. & G. K. Chubrik, 1988. Stroenie glotki trematoda i filogeneticheskie sviasi mezhdu Trematoda i Turbellaria. [The structure of the pharynx in trematodes and phylogenetic rela-
- tions between Trematoda and Turbellaria]. *Parasitologia* (St-Petersburg) 22: 297-303.
- Kotikova, E. A. & B. I. Joffe, 1988. On the nervous system of the dalyellloid turbellarians. *Fortschritte Zool./Progress Zool.* 36: 191-194.
- Lake, A. J., 1989. Origin of the eukaryotic nucleus determined by rate-invariant analyses of ribosomal RNA genes. In B. Fernholm, K. Bremer & H. Jornvall (eds), *The hierarchy of life*. Elsevier Science Publishers, Amsterdam: 87-101.
- Ohama, T., T. Kumazaki, H. Hori, S. Osawa & M. Takai, 1983. Fresh water planarians and a marine planaria are relatively dissimilar in the 5S rRNA sequences. *Nucleic Acids Res.* 11: 433-436.
- Rieger, R. M., S. Tyler, J. P. S. Smith & G. E. Rieger, 1990. Platyhelminthes: Turbellaria. In F. W. Harrison & B. J. Bogitsh (eds), *Platyhelminthes and Rhynchocoela*, vol. 3 in F. W. Harrison (ed.), *Microscopic anatomy of invertebrates*. Wiley-Liss, New York: 7-140.
- Rohde, K., 1990. Phylogeny of the Plathelminthes, with special reference to parasitic groups. *Int. J. Parasitol.* 20: 979-1007.
- Ruitort, M., K. G. Field, J. M. Turbeville, R. A. Raff & J. Baguñà, 1992a. Enzyme electrophoresis, 18S rRNA sequences, and levels of phylogenetic resolution among several species of freshwater planarians (Platyhelminthes, Tricladida, Paludicola). *Can. J. Zool.* 70: 1425-1439.
- Ruitort, M., K. G. Field, R. A. Raff & J. Baguñà, 1992b. 18S rRNA sequences and phylogeny of Platyhelminthes. *Biochem. Syst. Ecol.* 21: 71-77.
- Specht, T., J. Wolters & V. A. Erdmann, 1990. Compilation of 5S rRNA and 5S rRNA gene sequences. *Nucl. Acids Res.* 18: 2215-2230.
- Troitsky, A. V., Yu E. Melekhovets, G. M. Rakimova, V. K. Bobrova, K. M. Valiejo-Roman & A. S. Antonov, 1991. Angiosperm origin and early stages of seed plant evolution deduced from rRNA sequence comparisons. *J. Mol. Evol.* 32: 253-261.
- Wainwright, P., G. Hinkle, M. L. Sogin & S. K. Stickel, 1993. Monophyletic origins of the Metazoa: an evolutionary link with Fungi. *Science* 260: 340-342.