

The Spermatozoon Structure of *Coptothyris adamsi* (Davidson, 1871) (Brachiopoda, Rhynchonelliformea) and Analysis of the 18S and 28S rRNA Sequences

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Received February 5, 2015

Abstract—The position of brachiopods in the animal system remains controversial, with morphological data often being inconsistent with the results of molecular phylogenetic analysis. In this study, we investigated the structure of the spermatozoa and determined the combined sequences of the 18S and 28S rRNA genes of the articulate brachiopod *Coptothyris adamsi* (Davidson, 1871). The spermatozoa of *C. adamsi* are similar to those of other articulate brachiopods. Two types of sperm structure can be distinguished within Brachiopoda: the first type is characteristic of the articulate brachiopods (Rhynchonelliformea), the second type, the inarticulate brachiopods (Linguliformea and Craniiformea). Rhynchonelliformea spermatozoa are similar to those of the deuterostome animals, in particular to the sperm of the Echinodermata, whereas Linguliformea and Craniiformea spermatozoa are similar to the typical sperm of the Trochozoa, such as annelids and mollusks. Molecular phylogenetic analysis has shown that the brachiopods and phoronids form two monophyletic groups within the group Brachiozoa, where phoronids are united in a separate basal clade, while brachiopods are divided into two clades.

Keywords: Brachiopoda, Rhynchonelliformea, Linguliformea, Craniiformea, spermatozoon ultrastructure, 18S and 28S rRNA, phylogeny, *Coptothyris adamsi*

DOI: 10.1134/S1063074015030037

INTRODUCTION

The phylum Brachiopoda, which is a relict group of marine invertebrates known from lower Cambrian deposits, includes about 390 recent [8, 9] and 30000 fossil species [33]. According to classical concepts, brachiopods were divided into two classes: Articulata (Testicardines) with a hinge between the dorsal and ventral valves and Inarticulata (Ecardines), which have no hinge.

At the end of the last century, inarticulate brachiopods were divided into two systematically equal taxa of high rank [1, 48]. In the latest edition of the six-volume review [46], they were considered as two separate classes, Lingulata and Craniata, along with articulate brachiopods, united in the class Rhynchonellata.

The currently living inarticulate brachiopods lay their eggs in water, while articulate brachiopods can bear them in a curled lophophore or in special brood chambers, where fertilization and development occur [10]. A comparative study of brachiopods shows that the morphology of gametes in articulate and inarticulate brachiopods differ significantly [17, 32]. The sper-

matozoa of the representatives of inarticulate brachiopods are close in their structure to the sperm of bivalves and polychaetes [3, 4], while the sperm of articulate brachiopods is similar to that of echinoderms [5, 6, 21]. The sperm morphology of phoronids that are traditionally regarded as a group that is close to the brachiopods within Lophophorata [15, 16] distinctly differs in structure from the sperm of articulate and inarticulate brachiopods [28, 38].

The positions of brachiopods and phoronids on the molecular trees are unstable. Phoronids are a sister group to articulate brachiopods on some trees [29] and also a sister group to inarticulate brachiopods on others [22–25, 40]. In the second case, phoronids are not an independent group but included in Brachiopoda, i.e., they can be interpreted as brachiopods without shells [23]. In the trees that are constructed on the basis of a large number of genes with a sufficiently representative taxonomic sample of species, phoronids form a separate group as a sister group to brachiopods within the cluster Brachiozoa [14, 27, 30, 31, 43].

The aim of this work is to study the structure of the sperm and the nucleotide sequences of the 18S and

28S rRNA genes in the articulate brachiopods *Coptothyris adamsi* and to analyze on this basis the macrophylogeny and classification of modern brachiopods.

MATERIALS AND METHODS

Mature specimens of *Coptothyris adamsi* (Davidson, 1871) (Brachiopoda, Rhynchonellata, Terebratulida, Terebrataliidae) were collected in July 2010 at depths of 2–10 m in Troitsa Bay (Peter the Great Bay, Sea of Japan) at the biological station of the Pacific Institute of Bioorganic Chemistry, Far Eastern Branch, Russian Academy of Sciences (PIBOC, FEB RAS). For ultrastructural study of the spermatozoa, testicular pieces were fixed in 2.5% glutaraldehyde in a 0.5 M cacodylate buffer supplemented with sodium chloride to seawater tonicity. The material was additionally fixed in 1% OsO₄ in cacodylate buffer, then dehydrated by the standard technique in alcohols and embedded in Epon-Araldite mixture. Ultrathin sections were obtained on an Ultracut Reichert ultramicrotome. For microscopic examination, we selected sections of a silver/gold color. The sections were sequentially stained with uranyl acetate and lead citrate and examined under an electron microscope Zeiss Libra 120.

From animal tissues that were fixed in 95% ethanol, DNA was isolated with the use of a NucleoSpin Tissue Macherey-Nagel kit under the manufacturer's instructions. The 18S genes were amplified by polymerase chain reaction (PCR) using universal eukaryotic primers [34]. For amplification of 28S rRNA gene we used a primer set that was designed on the basis of 13 conserved domains of the 28S rRNA gene sequence [47]. The pairs of primers were selected to amplify with an overlap the almost complete 28S gene sequence from the 5'-end to the 11th conserved domain.

The PCR products were purified by agarose-gel electrophoresis and eluted from the gel using a cytokine kit (OOO Tsitokin, St. Petersburg); the nucleotide sequence of the purified amplification products were determined for both chains on an automated sequencer ABI Prism 3100-Avant Genetic Analyzer in the Center of Shared Use "Genome" (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences).

We obtained the nearly complete sequences of the 18S and 28S rRNA genes and deposited them in the gene bank (GenBank) under the numbers KF038315 and KF038313, respectively; they were aligned together with a set of complete 18S rRNA gene sequences and partial 28S rRNA gene sequences that were extracted from GenBank that represent 62 species of the major Bilateria groups. The bulk of the species in the analyzed set were representatives of the major groups of protostome animals, deuterostomes were represented by three species: *Florometra serratis-*

sima (Echinodermata, Crinoidea), *Ptychodera flava* (Hemichordata and Enteropneusta) and *Hydrolagus colliei* (Vertebrata, Chondrichthyes, and Chimaeriformes). Our analysis also involved the sequences of two species of sponges (Porifera), two species of comb jellies (Ctenophora), one placozoan species (Placozoa) and two species of cnidarians (Coelenterata). One species of collared flagellates (Choanozoa) was included in the analysis as an external group with respect to the above-listed animals. Before phylogenetic analysis, the nucleotide positions that could not be unambiguously aligned, which are related mostly to the variable V4 and V7 regions of the 18S rRNA molecule and also to the variable regions of 28S rRNA gene that is located between the conserved domains, were excised from the alignment. The final version of the alignment of the combined gene sequences contained 63 sequences with 3344 positions and included 11 sequences of brachiopods and 2 sequences of phoronids.

To reduce the possible influence of the nucleotide sequences of the remote external groups upon the branching order within the group of brachiopods and phoronids [23], along with an expanded set of sequences of the 18S and 28S rRNA genes, we also used a reduced set of sequences for the phylogenetic analysis that contain only the genes of brachiopods and phoronids with two sequences of annelids as a close outgroup.

Phylogenetic trees of the combined sequences of 18S and 28S rRNA genes were built by Bayesian analysis (BI) using MrBayes version 3.2 [39], as well as by the method of maximum likelihood (ML) using RAxML v. 7.4.2 [44] with a raxmlGUI v. 1.3 graphical interface [42]. In both cases, the analyses used the general model of the reversible evolution of nucleotide sequences with a gamma correction for the rate of evolution heterogeneity among sites and with the proportion of invariant sites taken into account (GTR + gamma + I). The model parameters were calculated directly in the program during the analysis. Bayesian reconstruction was performed by two independent analyzes of 3000000 generations of four Markov chains, selecting trees for every 300 generations (total 10000), 7000 of which (with low convergence of chains) were discarded, and the remaining 3000 were used for building the consensus tree with estimation of posterior probability of the nodes on the tree. For statistical evaluation of the nodes on the tree that was constructed with RaxML, the algorithm for fast bootstrap analysis was used.

RESULTS

Sperm Structure

Spermatozoa of *Coptothyris adamsi* are $3.3 \pm 0.1 \mu\text{m}$ in length. The sperm head is barrel shaped

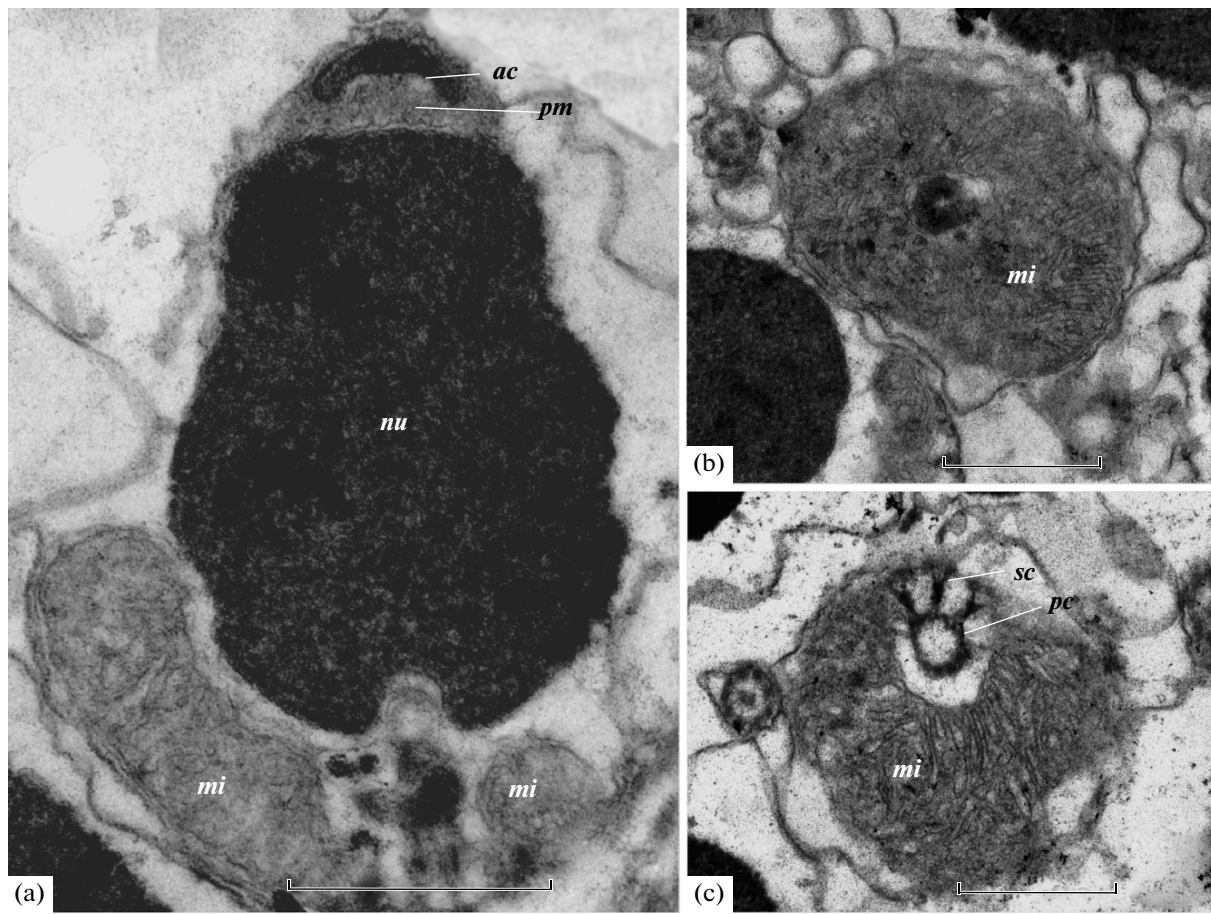


Fig. 1. Longitudinal (a) and transverse (b, c) sections of spermatozoa of the brachiopod *Coptothyris adamsi*. *ac*, acrosome; *mi*, mitochondrion; *nu*, nucleus; *pm*, periacrosomal material; *sc*, satellite complex; *pc*, centriole. Scale: (a) 1 μm ; (b) and (c) 0.5 μm .

(Fig. 1a), crowned with a cap-like acrosome, $0.4 \pm 0.1 \mu\text{m}$ in height and $1 \pm 0.1 \mu\text{m}$ in width, with an electron-dense acrosomal vesicle of a lenticular shape and with periacrosomal material of an average electron density located between the vesicle and the apical part of the nucleus. The apical surface of the nucleus is roundish, the basal surface has a small nuclear fossa. The midpiece of the sperm cell contains a ring-shaped mitochondrion that is $2 \pm 0.1 \mu\text{m}$ in diameter with well-developed lamellar cristae (Figs. 1b and 1c). The centrioles are oriented parallel to each other. The distal centriole serves as a basal body of the flagellum and is surrounded by the pericentriolar complex, connecting it to the cell membrane (Fig. 1c).

A Phylogenetic Analysis of Ribosomal Gene Sequences

We determined that the sequence of the 18S rRNA gene of *C. adamsi* includes 1617 nucleotides, which is almost the complete nucleotide sequence of the gene, except for the missing 45 nucleotides at the 5'-end and approximately 150 nucleotides at the 3'-end. For the preparation of sequence alignment for phylogenetic analysis, missing regions were presented by the corre-

sponding regions of the sequence of the 18S rRNA gene of *C. adamsi* that was obtained from GenBank (GQ275349).

The used set of primers allowed us to amplify and identify 2419 nucleotides of the 28S rRNA gene of *C. adamsi* that belong to the D3–D11 regions of the complete gene. We did not succeed in amplifying and defining the sequence of the 28S rRNA gene of this type for the D1–D3 domains; therefore this region (alignment positions 1–451) was presented during the phylogenetic analysis by the corresponding part of the sequence of this species that was obtained from GenBank (GQ275348), or by a part of the sequence we determined (deposited in GenBank under number KF038314) for the White Sea brachiopod species *Hemithyris psittacea*.

The phylogenetic trees that were constructed by the two methods with the use of the set of the combined sequences of the 18S and 28S rRNA genes have absolutely identical topologies. The trees we obtained (Fig. 2) again confirmed the separation of bilaterally symmetrical animals into the two main lines, viz., deuterostomes and protostomes, as well as the subsequent separation of protostomes into Ecdysozoa

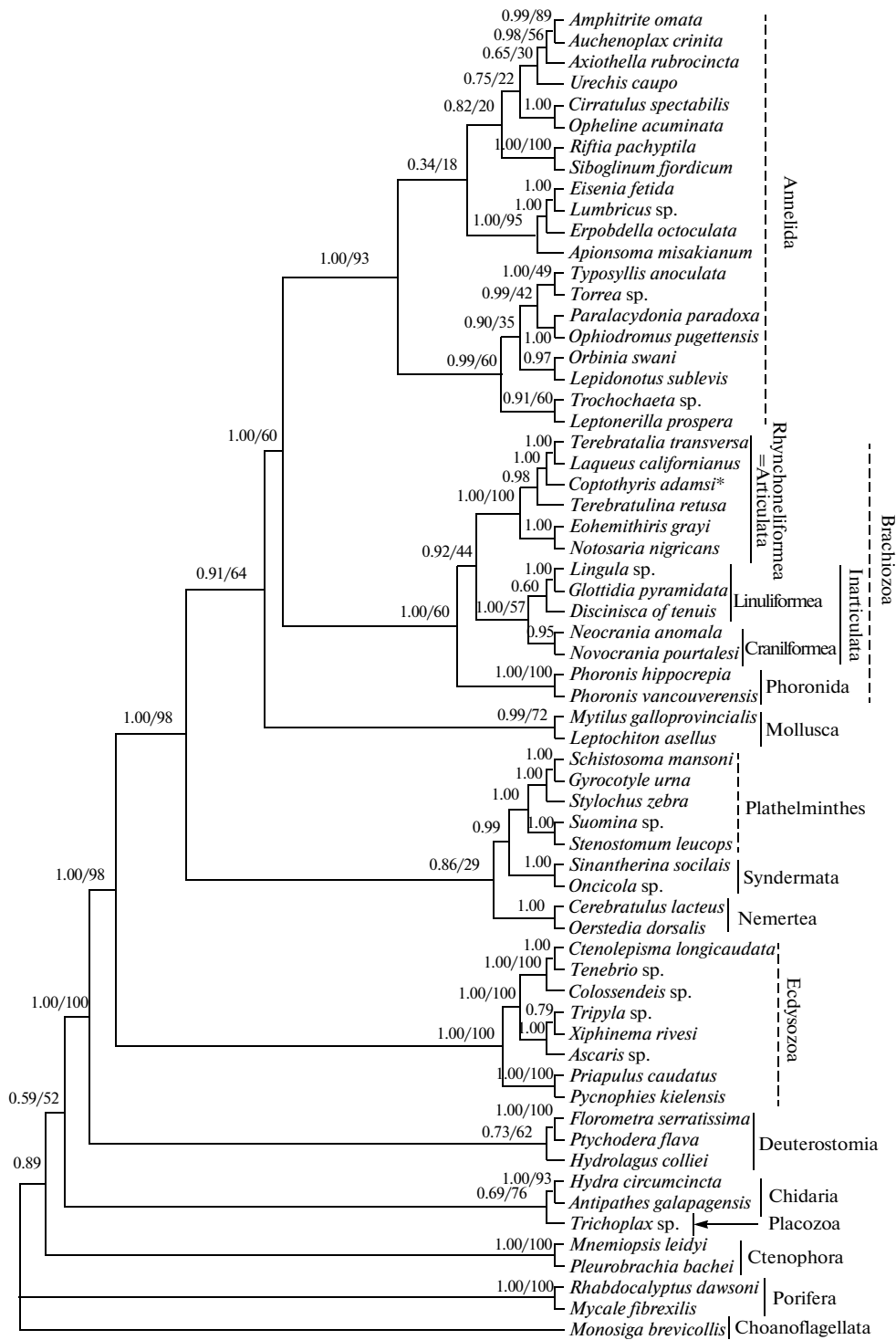


Fig. 2. The phylogenetic tree of the combined sequences of the 18S and 28S rRNA genes of the representatives of major groups of multicellular animals. The figures left of the corresponding nodes is statistical support; the sequence of *Coptothyris adamsi* is defined with asterisk. The tree is unscaled: the length of branches is not proportional to the genetic distance among taxa.

groupings (our tree presents arthropods, nematodes, and priapulids with kinorhyncha), Platyzoa (flatworms, rotifers with acanthocephalans, and nemertines) and Lophotrochozoa (mollusks, brachiopods,

and annelids). The authors of many similar reconstructions previously noted the same branching.

Brachiopods and phoronids take a position on the tree within the group of lophotrochophore animals

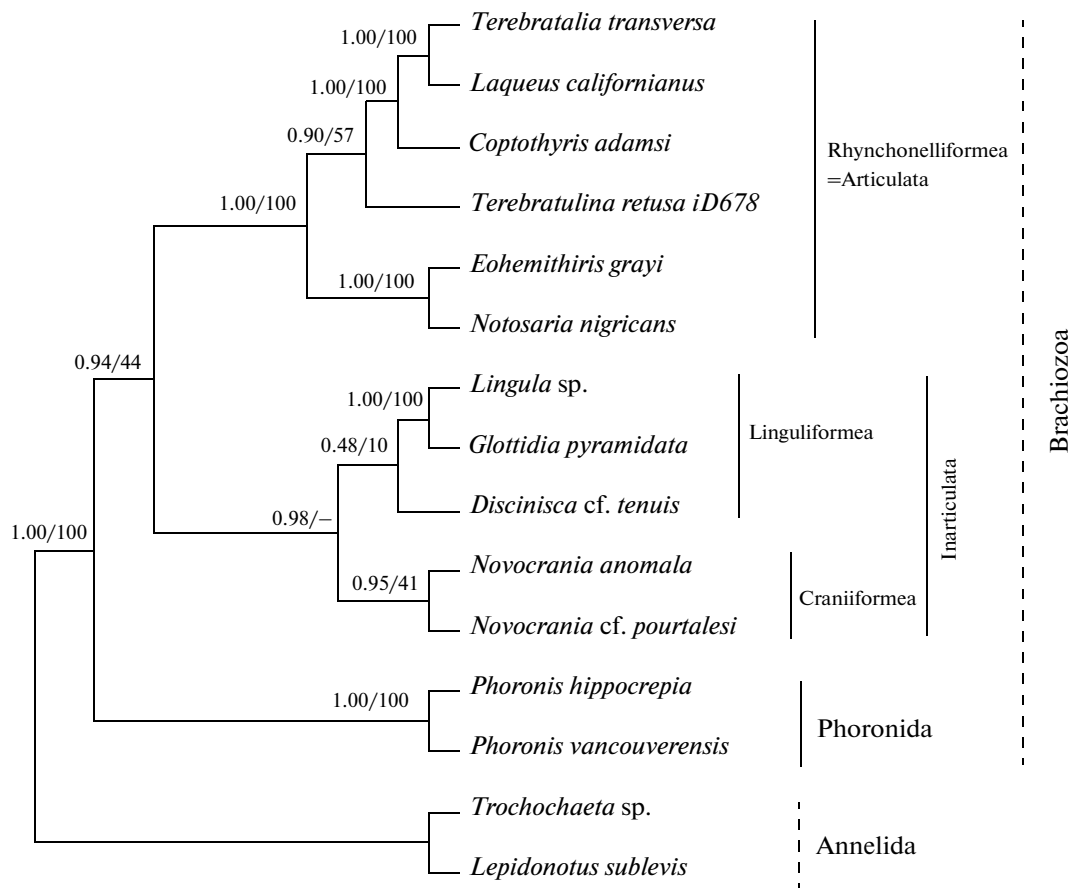


Fig. 3. The phylogenetic tree of the combined sequences of the 18S and 28S rRNA genes of brachiopods and phoronids. The figures left of the corresponding nodes is statistical support. The tree is unscaled: the length of branches is not proportional to the genetic distance among taxa.

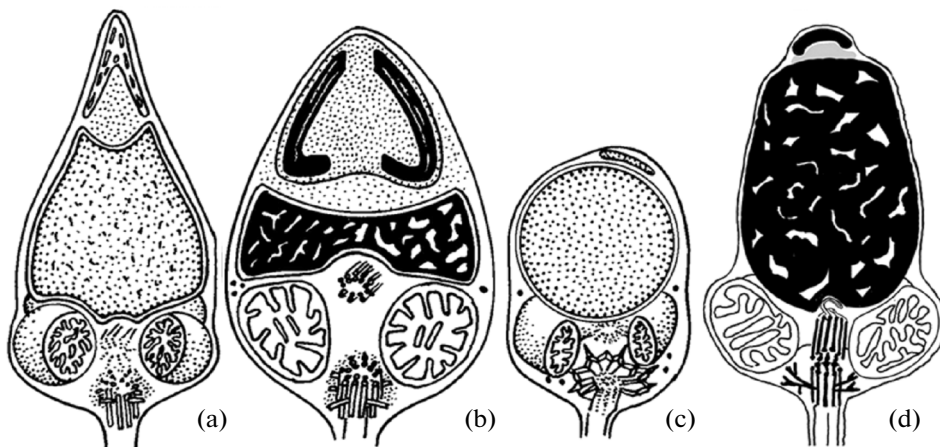


Fig. 4. The schematic structure of brachiopod sperm. (a) *Crania anomala*, (b) *Lingula anatina*, (c) *Terebratulina caputserpentis*, (d) *Coptothyris adamsi*. (a) and (c) after: Afzelius and Ferraguti, 1978; (b) after: Chuang, 1983.

between mollusks and annelids. They form the statistically well-supported group Brachiozoa with phoronids as a sister clade to brachiopods. The association of brachiopods with phoronids has even somewhat stron-

ger statistical support than that for the association of the articulate and inarticulate brachiopods. The corresponding values of the posterior probability (PP) in the Bayesian analysis and bootstrap support (BP) in

RaxML-analysis were 1.00/60 in the first case and 0.92/44 in the second case. Brachiopod sequences with high statistical support divide into two groups, which correspond to articulate Rhynchonelliformea (PP/BP = 1.00/100), as well as to inarticulate Linguliformea and Craniiformea (PP/BP = 1.00/57). In their turn, the sequences of inarticulate brachiopods divide into two clades, which correspond to the groups Linguliformea and Craniiformea.

As a result of phylogenetic analysis using the reduced set of sequences of 18S and 28S rRNA genes, we obtained a tree of the same topology as that during the analysis of the extended set: with the basal position of phoronids relative to brachiopods, with the division of brachiopods into articulate and inarticulate, and with the division of the latter into Linguliformea and Craniiformea (Fig. 3).

DISCUSSION

Mature spermatozoa of brachiopods are of two types (Fig. 4). Inarticulate brachiopods *Lingula unguis*, *L. anatina*, *Discinisca tenuis* and *Crania anomala* belong to the classes Lingulata and Craniata; their sperm cells have cone-shaped heads crowned with an acrosome that consists of two parts: an acrosomal vesicle and periacrosomal amorphous material. The central part of the head is occupied by the nucleus with two interperpendicular centrioles adjoining to the basal part of it; the centrioles are surrounded by spherical mitochondria, which vary in number from 4 to 5 in *Discinisca tenuis* [32] and to 7 in *Lingula unguis* [41] and in *L. anatina* [21]. A flagellum (about 50 μm in length), which is formed by a pattern of microtubules with the formula $9 + 2$, departs from the distal centriole.

Sperm cells of the second type are characteristic of articulate brachiopods. These sperm cells have an anteriorly rounded head, a discoid acrosome, a spherical or oval nucleus, and parallel centrioles surrounded by a single ring mitochondrion. A tail flagellum, which is about 50 μm in length, with a $9 + 2$ pattern of microtubules, grows from the distal centriole. This sperm structure is characteristic of *Terebratulina caputserpentis*, *Terebratula vitrea*, *Kraussina rubra* [17–19, 32] and *Coptothyris adamsi* that we studied.

The spermatozoa of inarticulate brachiopods are similar in their structure to the sperm of typical trochophore animals, such as annelids and mollusks. Their acrosome is relatively large; the nucleus is from oval to elongated, oblong; the midpiece of the spermatozoon has two interperpendicular centrioles surrounded by several spherical mitochondria [3, 7, 13, 36, 37]. The spermatozoa of articulate brachiopods have characteristic traits that bring them close to the sperm cells of deuterostomes that possess external insemination: a relatively small acrosome, the nucleus usually spherical or oval, and one ring mitochondrion

in the midpiece (like in echinoderms and bony fish). The parallel arrangement of centrioles in articulate brachiopods is a rare trait that is also found in the sea urchin sperm [2].

The two types of sperm structure suggest great differences between the inarticulate and articulate brachiopods [17, 26]. Perhaps this is the result of an ancient origin of brachiopods, whose ancestors were probably close to the group of animals that are ancestral to protostome and deuterostome animals; therefore the gametes of inarticulate brachiopods (Lingulata and Craniata) appeared to be closer to Protostomia, while the gametes of articulate brachiopods (Rhynchonella) are closer to Deuterostomia, in particular to sea urchins. Of course, this does not mean that different groups of brachiopods have different origins; the phylogenetic trees based on the gene sequences of 18S rRNA [22, 24, 29], 18S and 28S rRNA genes [23, 25, 40], and a great number of genes of ribosomal proteins [27, 30, 31], and several other genes [43] always place brachiopods and phoronids among Lophotrochozoa within the group of protostome animals.

According to the character of cleavage and the way of development of the coelomic mesoderm, phoronids differ sharply from trochophore animals with their typical spiral cleavage and teloblastic growth [45]. Still, according to Malakhov [11, 12, 15, 16], the contemporary non-classical zoology allows us to combine trochophore and lophophore animals into the single taxon Lophotrochozoa, in particular, based on the presence of such morphological synapomorphies as chaetae, which are typical for Trochozoa and Lophophorata, but do not occur either in Ecdysozoa or in Deuterostomia.

Our tree of the combined sequences of the 18S and 28S rRNA genes (Figs. 2 and 3), as well as the trees that are based on a great number of genes from the representative taxonomic sample [27, 30, 31, 43] show that brachiopods and phoronids form monophyletic groups that join together in grouping Brachiozoa, with phoronids occupying the basal position and brachiopods dividing into two groups corresponding to the articulate (Rhynchonelliformea) and inarticulate (Linguliformea and Craniiformea) species. In this case, brachiopods can be interpreted as phoronids that obtained a shell and several other apomorphic features, such as dorsal and ventral mantles, mantle channels, a double row of tentacles on the lophophore of an adult animal and lophophore with two mesocoelic cavities [20, 35]. Inclusion of phoronids in either of the two groups of brachiopods is only possible upon reduction of these traits in phoronids [29] or at an independent origin of these traits in different groups of brachiopods.

Thus, the results of the molecular-phylogenetic analysis (Figs. 2 and 3) and the structure of the spermatozoa (Fig. 4) support the division of Brachiopoda

into two groups, viz., Inarticulata (with the classes Linguliformea and Craniiformea) and Articulata (with the class Rhynchonelliformea); this corresponds to the traditional notion on the systematic position of brachiopods.

ACKNOWLEDGMENTS

This work was supported by the Far Eastern Program, projects nos. 15-I-6-0140 and 15-I-6-0070 and in its molecular phylogenetic part by the Russian Scientific Foundation, project no. 14-50-00029.

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Translated by I. Barsegova