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Organisms Diversity & Evolution

Kinetid in larval cells of Spongillida (Porifera: Demospongiae): tracing the ancestral traits

--Manuscript Draft--

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Abstract:	<p>The kinetid (flagellar/ciliary apparatus) of eukaryotic cells is an important source of phylogenetic information. It was found to be a prospective morphological phylogenetic marker in sponges, since its arrangement in choanocytes is congruent with the topology of the phylogenetic trees. But investigation of the kinetid of sponge larval cells remains fragmentary. Here, we report the results of an ultrastructural study on larval kinetid of the freshwater sponges <i>Eunapius fragilis</i> and <i>Lubomirskia baikalensis</i> (Demospongiae: Spongillida). Their kinetids were found to comprise a kinetosome associated with an accessory centriole and linked to the nucleus by simple fibrillar root. The kinetosome bears a transverse cytoskeleton: filamentous train and microtubules which radiate from microtubule organizing center (MTOC) shaped as a large hollow foot. In the short transition zone, between the central axonemal microtubules and kinetosome, a transverse plate with an axosome (central thickening) occurs. Reviewing known kinetids of different sponge larvae, we conducted ancestral state reconstruction. We suggest that spongillids retain the plesiomorphic characteristics of roots and an accessory centriole. But they possess the peculiarities of the transition zone, transverse cytoskeleton and MTOC structure.</p>	
Response to Reviewers:	<p>Dear Editor and Reviewer, Please, find our revised manuscript. We followed your advice and performed an ancestral state reconstruction analysis. To do this, we invited our colleague Dr. Schepetov who has a pertinent skill. This greatly benefited the manuscript. We also</p>	

dealt with other issues raised and hope that the manuscript became clearer.
Sincerely yours,
Agniya Sokolova

Thanks a lot for your useful review!

- The text is not very well written. I'm not a native English speaker and I usually don't like to complain about the language used in the text. However, I had problems to understand the text in many different points of the text. This characteristic makes hard to understand several important messages of the manuscript. Therefore, I would like to reinforce the need to send the final version of the manuscript for a more "English-speaking skilled" colleague or a company that proofreads scientific manuscripts. You will see, in my specific comments, that I started suggesting some changes in the text, but later I gave up.

Language was improved.

- I have also to complain that the conclusions about the state (plesiomorphic, derived, etc) of the characters is difficult to believe with the current methodology used in the text. I suggest the use of proper character mapping software after a good tabulation of the characters in different groups. Please, refer to a specific comment below (under the "Discussion" subsection). Currently, the "ancestral" characteristics of the kinetid of Porifera could be related to personal opinion, as you usually don't show enough results to support the hypothesis.

Conclusions were supported by character mapping.

- I also suggest avoiding to "generalize" the use of Larval and adult kinetid. Larvae and adults are multicellular organisms with several cell types and not all of them have kinetids. I think that a better term (flagellated cells of the larvae, or choanocytes) should be used all along the text.

'Larval kinetid' can be found only in those cells of larvae which bears a flagellum, i. e. the cells under discuss. However, we replaced this term, leaving it only in few cases.

- Finally, Please, enumerate the pages to facilitate the communication of the reviewers and editors with the authors.

Done

- Abstract
Page 1, Line 33-35: I believe that "as the arrangement of flagellar apparatus in choanocytes is congruent with the topology of the phylogenies of sponges" is better than the current sentence: "since the arrangement of the flagellar apparatus in choanocytes fits the phylogenetic tree of sponges".

Ok, done

- Line 44-45: Please, change the sentence "In short transition zone between central microtubules of the flagellum and kinetosome a transverse plate..." for "In the short transition zone, between the central microtubules of the flagellum and the kinetosome, a transverse plate...".

Done

- Line 48: Consider changing the sentence starting at "Analyzing known data..." for "Compared to the larval kinetids of other sponges, we suggest..."
I liked the last sentence of the Abstract, but I would expect that you show what are these characteristics of the "ancestral" kinetid, rather than "indicate" that you are discussing

this issue in the text. I'm inclined to think that a descriptive abstract is better than an informative one.

Rewritten

- Key words: three of the five key words are already present in the title. Maybe, you should choose other key words to help readers finding your work in the future. For instance: freshwater sponges; choanocytes; cilia/ flagella

Done

- Introduction
I think that the first paragraph needs a reference to confirm that the sponges are the most ancient extant animals.

Was rewritten, references were added

- Page 3, Line 7: I can't agree with the "stable morphological organization" of sponges. Actually, if there's one thing that sponges are, is that they are plastic and not stable. What do you mean in this sentence? Do you want to say that the bauplan didn't change over the millions of years of their existence in the planet? Or do you mean a morphological stability during the life cycle of the organism? This sentence might be reviewed to make it clearer.

Rewritten

- Line 11: Systema Porifera is not exactly a citation. You should refer to a specific chapter of the book that was edited by Hooper and van Soest. // The modern classification of sponges is not only based on genetic data. Furthermore, when the previous works using molecular markers to construct the phylogeny of different groups were compiled together by Morrow and Cardenas (2015), more than a new classification scheme for DEMOSPONGES or Calcarea (in this case, Voigt et al 2012), they proposed a more stable phylogenetic topology for the classes. This is more important for the following sentence than a "modern classification".

Rewritten

- Line 15-17: I think that you should use "seems to be", rather than "tuned out to be" in this part of the text. Following this part of the paragraph, I think that you should make a case for why kinetids are a good feature to be studied on the light of the sponge phylogeny. It's fine to say that this structure is important for unicellular eukaryotes, but you break the train of thought here, as you were talking about sponges, about the possibility to investigate the evolution of morphological traits in the group and to understand the relationship of these animals. Maybe, you should rearrange this paragraph to explain what is a kinetid, than show its usability for sponge evolution (the following paragraph) and finally testify that the kinetid is really useful to understand phylogenetic relationships not only in sponges, but also in unicellular eukaryotes.

Rewritten

- Line 28: Please, cite who defined what is a kinetid.

Reference was added

- Line 50-52: The sentence "Kinetids of larvae and adults differ in more or less extant" is not good. First, kinetids are of larval cells and choanocytes of adults. I don't think you should say that kinetids are of larvae and adults.

Ok, corrected

- Second, what makes them different? You should point these characteristics here. Just saying that they are more or less different is not scientifically accurate.

We are not sure that it is a proper place for such a specialized discussion. But we inserted references to confirm our words.

- Finally, "extant" is wrongly used here. I believe you wanted to use "extent".

Corrected

- Life 52-55: I also don't like this sentence. I don't think that is good to state that the scientist was wondering something to justify carrying out the work. What are the evidences that made you wonder that the kinetid of larval cells evolved independently? That they could have evolved in their own way? And so on... I mean, it is fine to wonder about these questions. This is what motivate us in the real world, but this should be better justified in the paper.

Ok, we tried to clarify it

- Line 59: What do you mean by "frames" here? I would like to see some citations here and that either this paragraph is better developed (currently it comprises a single sentence) or joined to the following one. I like it and I think that it shows in the text that you have the tools to develop your work. Maybe, you should transfer it to the Material and Methods.

Rewritten

- Page 4, line 9: what do you mean by "system Demospongiae"? I think that the first paragraph in this page needs a thorough review of the English language (as said before, this is a problem in many parts of the work). Moreover, I think that it needs a better framing to justify why you choose to investigate their kinetids. The following paragraph is also difficult to understand (despite being a single sentence paragraph, which should be avoided!). Which "same scheme"?

Rewritten

- Line 48-49: Do you mean water containing the larvae? Why did you fix a fragment of the sponge? Is it a piece of the adult that spawn the larvae? I can't understand this sentence. "Sponge fragments" is used repeatedly in the text. It might be just an error related to copy the protocol from a previous work; or, maybe, they did fix the sponge and observed the larval cell from larvae still being incubated in the parental tissue. Please, clarify this.

Yes, that was a mistake. Thanks.

- Page 5, line 1-3: Please, provide the concentration of the uranyl acetate and lead citrate.

Done

- Line 4: Did you use grids to support the sections? Or have you used FORMVAR (for instance) in order to observe the consecutive sections?

We used formvar-coated blends. This information was added.

- Lines 13-35: I understand that the protocols were different for the different species. However, it's preferable that the way in which the protocols are presented is consistent between them. In the protocol for *Lubomirskia*, the reader will find references for the brands of the products, units are in mM (something that should be avoided, as the correct one suggested by the SI is mol.L⁻¹), and the number of washes is showed clearly. The protocol for *Eunapius*, on the other hand, lacks all this information. Please, uniformize this part of the text.

Uniformised

- Results
Page 5, Line 42: Please, use Fig. instead of fig. I also suggest to you to put the letter ("a") with the numeral ("1") without space between them. Do this, please, in all figure "calls" along the manuscript.

Done

- Line 46: What this "External layer" refers to? The larvae?

Yes, external layer refers to larval 'epithelium'. Clarified.

- Line 8: I can't observe the transition fibers in figure 3k.

Removed

- Line 11: The basal foot tube is closed at the end. Which end? The one opposite to the kinetosome?

Yes, distal end. Clarified.

- Line 22-24: What are the evidences that fibrous trains and transition fibers are different structures? Looking at the images, in many of them, I couldn't distinguish between these two structures. Moreover, I suggest using other abbreviation in the figures, as tf and ft might be misleading.

Transition fibres (=alar sheets) are the structure characteristic of eukaryotic cells. They are nine short fibres radiating from the apical part of kinetosome (as seen in fig. 2e, 2h, 2o). They form a specific radial structure on the transverse sections, which are somewhat oblique in our figures. So, you can observe only half of this structure (fig. 3 a, e, f, j).

Filamentous train is a characteristic of spongillids. It is situated below the fibres (see fig. 2 e, h and a series on fig. 3 e-g). We changed the abbreviation 'tf' and add signs on the figures to make it clearer.

- Discussion
I believe that a figure showing presence/absence of the structures in different orders of demosponges and the other classes is pivotal for your discussion. As you are not "mapping" the evolution of the characters in a phylogenetic tree of Porifera, this figure (you could use Riesgo et al. 2014, doi:10.1093/molbev/msu057 Fig 2B as an inspiration) would help the reader to see what is present, what is absent, and is unknown way easier

that currently presented in the discussion. The way in which you compare the presence of the kinetid elements is hard to follow, because many taxa and citations are intermingled with the good information provided there.

However, I think that present this comparison using a phylogenetic tree and a proper character mapping would be more desirable to propose evolutive scenarios, like plesiomorphic or derived states for a given character. In Lanna & Klautau 2016, for instance (doi:10.1017/S0025315415001290), the authors mapped the evolution of some characters related to the oogenesis of *Calcinea*. There, an oversimplified phylogenetic tree of *Calcarea* was used to map the data using the Mesquite software. This approach provides a more reasonable scenario for the evolution of the characters. On the other hand, I understand that there many 'missing data' due to the difficulties to observe these structures, but maybe the use of character mapping could help to improve the reliability of your hypotheses.

Thanks for your recommendation. We performed character mapping. Hope that new figures will clarify our statements.

- I also think that such mapping would need to have the data of choanoflagellates, as well. This could work as a good argument for your conclusion that sponge kinetids are very similar to those of the ancestral (choano)flagellates.

We included choanoflagellates into analysis, but actually they are not a good group for it. Choanoflagellates are not sponge ancestors, they are specialised animals with specialised kinetids. We added a paragraph on this issue.

- Page 8, line 13: What do you mean by "fluent" here?

We deciphered it after a semicolon.

- Page 10, line 4-6: This sentence is completely non understandable.

Rewritten

- Line 51-52: Why an ancestral flagellate? Which evidences in flagellates do you have to support this hypothesis? Please, present it here. How is the kinetid of choanoflagellates?

There are no doubts that sponge ancestor had a flagellum, so was a flagellate. We compared the kinetids of choanocytes and choanoflagellates earlier (Pozdnyakov et al., 2017). The discussion was however rewritten, may be this issue became clearer.

- Page 11, line 1: What is an axosome? I found that axosomes are related to synapses.

It is a central thickening of a transverse plate. Explained in Abstract and Results.

- Line 4: Please, provide a citation to the information that the flagellum is disassembled during the metamorphosis.

Provided

- Line 11-14: I'm not sure if I could understand what is being said here. Please, improve this argument.

Impoved

- The final conclusion of the study is not related to the objective of the work. Please, add a proper conclusion for your study.

Thanks. We formulated the objectives in other way.

- Figures legends. Figure 1: Please, add the scale bars for both parts of the figures.

Added

- The legend fv is not present in the figure [...]

Please, find it in the upper left corner of 'b' figure.

- [...] and the meaning of the n, present in the figure, is not present in the legend.

Added

- Figure 2: It's a matter of style, but I can't find the legend of this figure informative. What is being shown in a-d, e-g, h, i-j and so on? I think that it is fine to say that they are consecutive sections of a given cell, but what is the main message in this series of figures? I think that the legend is too abbreviated.
Figure 3: The same comment presented above fits here. The meaning of "lmt" is missing in this legend.
Figure 4: The same comment about the legend of Fig. 2 applies here. Please, organize the abbreviations in alphabetical order.

Improved

- The scale bar is hidden in figure "e". Please, put it in a place that is easier to find or, better, with a higher contrast with the background.

There is a single scale bar in 'a' image. It is common for all the images of the figure.

- Figure 5: The meaning of "cm" is missing in the abbreviation list. Please, list the abbreviations in alphabetical order.

Done

Kinetid in larval cells of Spongillida (Porifera: Demospongiae): tracing the ancestral traits

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2056-1310

Abstract

The kinetid (flagellar/ciliary apparatus) of eukaryotic cells is an important source of phylogenetic information. It was found to be a prospective morphological phylogenetic marker in sponges, since its arrangement in choanocytes is congruent with the topology of the phylogenetic trees. But investigation of the kinetid of sponge larval cells remains fragmentary. Here, we report the results of an ultrastructural study on larval kinetid of the freshwater sponges *Eunapius fragilis* and *Lubomirskia baikalensis* (Demospongiae: Spongillida). Their kinetids were found to comprise a kinetosome associated with an accessory centriole and linked to the nucleus by simple fibrillar root. The kinetosome bears a transverse cytoskeleton: filamentous train and microtubules which radiate from microtubule organizing center (MTOC) shaped as a large hollow foot. In the short transition zone, between the central axonemal microtubules and kinetosome, a transverse plate with an axosome (central thickening) occurs. Reviewing known kinetids of different sponge larvae, we conducted ancestral state reconstruction. We suggest that spongillids retain the plesiomorphic characteristics of roots and an accessory centriole. But they possess the peculiarities of the transition zone, transverse cytoskeleton and MTOC structure.

Key words: freshwater sponges, sponge larvae, cilia/flagella, ultrastructure

Introduction

Sponges (the phylum Porifera) are among the most ancient invertebrates on our planet (Simion et al., 2017; Nielsen 2019). They are crucial to interpreting the nature of the earliest

1 animals. Researchers peer into their simply organised bodies seeking features inherited from
2 the dawn of metazoan history.

3 Sponges have only a few morphotraits available for comparison and classification. The main
4 trait, skeleton structure, was evaluated somewhat differently and led to different conclusions
5 (compare Hartman 1958; Bergquist 1978; Hooper and van Soest, 2002). When genetic analysis
6 provided topological stability at the level of classes (Cárdenas et al. 2012; Voigt et al. 2012;
7 Morrow and Cardenas 2015), the opportunity arose to trace informative phylogenetic features.

8 Kinetid (flagellar/ciliary apparatus) structure is a suitable feature for analysis. The kinetid
9 consists of three main parts: 1) a free part (the flagellum/cilium); 2) a basal intracellular part,
10 normally comprising two kinetosomes (the non-flagellated one is often called an accessory
11 centriole) with attached microtubular or fibrillary rootlets; and 3) a transition zone connecting the
12 free and basal parts of the flagellum (Lynn and Small 1981; Moestrup 1982 and others).

13 Kinetid structure is diverse in choanocytes. Our studies showed that choanocyte kinetid structures
14 of sponges can be successfully superimposed on the phylogenetic tree (Pozdnyakov et al. 2018).
15 Comparing kinetid structures in different sponge species, led us to propose a scheme of the
16 ancestral choanocyte's kinetid type (Pozdnyakov et al. 2017, 2018).

17 In addition to choanocytes, sponges have other essential flagellated cells – the covering cells
18 of larvae. Sponge larvae are motile and relatively sophisticated organisms. Their morphological
19 and behavioural complexity, which brings them closer to Eumetazoa, has long fascinated
20 researchers. Larva flagellum serves for moving it through the water column, while choanocyte
21 flagellum establishes water flow through the sponge body. During metamorphosis, larval
22 flagellated cells in calcareans and demospongians lose the flagellum and dedifferentiate
23 (Ivanova, 1997b; Amano and Hori 2001; Ereskovsky and Wilenz 2008; Gonobobleva and
24 Ereskovsky 2004; Usher and Ereskovsky 2005; Sogabe et al. 2016). Kinetid in larval and adult
25 cells differs in varying degrees (Amano and Hori 1996; 2001; Gonobobleva and Maldonado
26 2009; Maldonado 2009; Sokolova et al. 2019). These facts raise the questions of whether larval
27 kinetid could have evolved in its own way and what ancestral kinetid looked like?

28 It is not easy to obtain a comprehensive collection of sponge larvae, since many species
29 have a short breeding period (often with unknown time frames) or produce few larvae
30 (Maldonado 2006; Ereskovsky et al. 2007). But data on the accessible species are accumulated
31 and this study will complement them. The present work explores the larvae of Spongillida, the
32 only freshwater order of Porifera. This taxon was traditionally classed with the marine order
33 Haplosclerida. In *Systema Porifera*, it was assigned to Haplosclerida as the suborder Spongillina
34 (Manconi and Pronzato 2002). Later, genetic data supported changing its status to a separate
35 order, Spongillida, within the subclass Heteroscleromorpha. Haplosclerida were recently shown
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1 to stand apart from the rest of Heteroscleromorpha (Morrow and Cardenas 2015). Accordingly,
2 the majority of data place Spongillida and Haplosclerida distant from one another (Morrow et al.
3 2012, 2013; Morrow and Cardenas 2015; Simion et al. 2017). But data in some works imply a
4 joining of the Spongillida and Haplosclerida branches (Sperling et al. 2009; Hill et al. 2013).
5 These works were not widely recognised due to small sample size (Morrow and Cardenas 2015),
6 but the same outcome has continued to emerge in recent years (Schuster et al. 2017). Therefore,
7 the relationship between Haplosclerida and Spongillida remains uncertain. Interestingly, the
8 choanocyte kinetids of haplosclerids and spongillids are similar to each other and different from
9 the rest of the sponges (Pozdnyakov et al. 2018). Studying the kinetids of larval cells might
10 provide additional information on their relationship.

11 We studied the ultrastructure of the kinetid in larval cells of freshwater sponges *Eunapius*
12 *fragilis* (Leidy, 1851) (family Spongillidae) and *Lubomirskia baikalensis* (Pallas, 1776) (family
13 Lubomirskiidae). In addition to describing their kinetids, we intend to: 1) suggest which
14 characteristics of the kinetid resemble the ancestral kinetid, 2) estimate similarities between kinetid
15 structures in the proposed ancestral larva and adult, and 3) compare kinetids of spongillids and
16 haplosclerids.

17 **Materials and Methods**

18 **Sample preparation**

19 *Eunapius fragilis* specimens with mature larvae were collected in the Moscow Channel
20 (Moscow region, Russia) in the middle of June, 2017. Larvae were sampled by means of a glass
21 Pasteur pipette, after putting sponge in the tank with stagnant water. Larvae were fixed and
22 prepared for electron microscopy, according to the following protocol. For prefixation, 1 ml of 1%
23 osmium tetroxide in cacodylate buffer (0.1 mol l⁻¹, pH7.4) was added to 3 ml of water containing
24 the larvae. Next, 4 ml of 4% glutaraldehyde in the same buffer was added and the larvae was kept
25 in this mixture for 15 min on ice without light. Then, the fixative mixture was replaced by 2%
26 glutaraldehyde for 1 h on ice. Afterwards, the larvae were rinsed in the buffer twice and postfixed
27 in 1% osmium tetroxide for 1 h at room temperature.

28 The samples were then washed twice for 10 min in the same buffer, dehydrated in a graded
29 ethanol series and embedded Spurr resin. After polymerization, the resin blocks with larvae were
30 trimmed and treated with 10% hydrofluoric acid for 5 min to remove siliceous skeletal elements.
31 Ultrathin sections (60 nm) were cut with a Leica EM UC6 ultramicrotome using a glass knife. The
32 sections were mounted on formvar-coated blends, double stained in 2% aqueous uranyl acetate
33 (15 min) and afterwards in 2% lead citrate (3 min) and put under a JEM 1400 electron microscopes
34 equipped with an Olympus Veleta digital camera. Serial consecutive sections were studied.

1 The parts of the branches of *Lubomirskia baikalensis* were collected in Lake Baikal near Cape
2 Listvenichny at a depth of 10-15 m using SCUBA in March 2017. The samples were immediately
3 placed in containers with Baikal water where larvae were sampled by means of a glass Pasteur
4 pipette. Fixation for semi-thin sections and TEM was performed according to the following
5 protocol: pre-fixation in 1 % OsO₄, 10 min; washing in cacodylate buffer, 10 min; fixation in 1.5
6 % glutaraldehyde solution in cacodylate buffer, 1 h; washing in cacodylate buffer, 30 min; post-
7 fixation in 1 % OsO₄ solution in cacodylate buffer, 2 h; washing distilled water 3 time for 15
8 min at room temperature; dehydration in increasing concentrations of ethanol (30, 50, 70, 90 and
9 100 %, 20 min in each) at room temperature; and finally embedding in Araldite resin (Electron
10 Microscopy Sciences). Semi-thin sections (60–80 nm) were cut with an Ultramicrotome
11 PowerTome XL and contrasted with 4% aqueous uranyl acetate. Digital photos were taken with a
12 Leica DMLB microscope using the Evolution LC color photo capture system.

13 Descriptions follow the terminology used by Andersen et al. (1991) and Woolacott and Pinto
14 (1995).

15 **Ancestral character state reconstruction**

16 The phylogeny of taxa represented in this study was reconstructed using the maximum
17 likelihood method implemented in RaxML (Stamatakis 2014) and Bayesian inference
18 implemented in MrBayes, (Ronquist and Huelsenbeck 2003) on 18S data from the GeneBank
19 database aligned using MUSCLE multiple alignment algorithm (Edgar 2004) (Suppl. Fig. 1, 2). A
20 generalised phylogram based on recovered phylogenies was built for further manipulation. We
21 used Mesquite (Maddison and Maddison 2019) to trace eight standard categorical characters
22 (Suppl. Table 1) under the most parsimonious model for polymorphic terminal taxa data.

23 We took into account available data on most (Table 1). *Crambe crambe* (Schmidt, 1862) and
24 *Cacospongia mollior* Schmidt, 1862 (Uriz et al. 2008) were excluded from the analysis as these
25 genera's position on the tree is not known. *Haliclona indistincta* (Bowerbank, 1866) was excluded
26 because it is plausible it was misassigned to Haplosclerida (for details see Sokolova et al. 2019).
27 *Scopalina lophyropoda* Schmidt, 1862 (Uriz et al. 2008), *Chondrosia reniformes* (Lévi and Lévi
28 1976) and *Halichondria moorei* Bergquist, 1961 (Evans, 1977) were not clearly illustrated for our
29 purposes. Since information was not complete in some cases, we had to extrapolate the species
30 data to the entire clade (Table 1). A total of 38 sponge species from 15 monophyletic clades were
31 included in the analysis. The names of clades are given in accordance to current classification (Van
32 Soest et al. 2020), which is however not fully corresponds with the molecular phylogeny at the
33 family level.

34 **Results**

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The larva of spongillid sponges is a peculiar parenchymella, often with significant internal cavity in the anterior part (Fig. 1a). It is covered by slightly, relatively short elongated cells.

Kinetid structure of larval cells in *Eunapius fragilis*

Cells of the external layer in *Eunapius fragilis* larva have the irregular shape, but tightly contact each other in the very distal zone (Fig. 1b). They are generally elongated in apical-basal direction, being more or less wide (Fig. 1b). The flagellum emerges from a deep apical pit and bears one to four flagellar vanes (Fig. 1b). The axoneme is typical, with microtubules formula 9×2 .

The transition zone of the flagellum is short. The central microtubules reach the cell surface and connect there to axosome, a thickening in the middle of the transverse plate located on the distal end of kinetosome (Fig. 2c, f, h, i, k, n, o).

A so-called dark zone of the flagellum, which seems to be characteristic for all cilia and flagella of sponges, locates at about 100 nm distal to the transverse plate (Fig. 2m, n, o). It is filled with electron-dense material at a distance of approximately 150 nm. It includes transitional cylinder distinguishable on the inside of peripheral microtubules (Fig. 2m, n). Outside of the axoneme, we observed Y-linkers: electron-dense fibres radiating from the microtubules to the flagellar membrane (Fig. 2m, n, o).

Nine transition fibres (=alar sheets) start from the apical edge of the kinetosome (Fig. 2b; Fig. 3a, e, f, j). A single microtubule organizing center (MTOC) on the kinetosome surface is a basal foot of an unusual shape. It looks like a fairly long (200 nm length) expanding tube with electron-dense walls (Fig. 2b, e, f, h, i, k, m; Fig. 3a, b, f, g, h, i). This tube is hollow, closed at the distal end and has no distinct head. The lateral microtubules start from the basal foot and radiate in different directions (Fig. 2b, g, k, m; Fig. 3 f, g).

Opposite to the foot, the kinetosome produces a filamentous train that expands and extends parallel to the plasma membrane for quite a long distance (up to 1.1 μm) (Fig. 2d, h, j; Fig. 3a-g). It consists of several bundles of the filaments (Fig. 2c, d, e, h, i, j, k) arising from several points of kinetosome. Two of them are prominent in longitudinal sections: apical and basal bundles (Fig. 2c, d, e, j). Right near the kinetosome, where the bundles arise, the filamentous material is so dense that sometimes it looks like a small dense body (Fig. 2c).

Broad fibrillar root connects the proximal end of the kinetosome to the nucleus (Fig. 2 d, h, i, j, k). The nucleus occupies apical, though in some cases rather middle position in the cell, and has a beak connected to the fibrillar root (Fig. 2l).

The accessory centriole is located mostly at the side of the foot, slightly basal to the kinetosome at a sharp angle to it (Fig. 2b-e, h, i, k). The centriole is connected with the kinetosome by the bridge of fibrillar material that looks like common with the fibrillar root mass (Fig. 2 h, i, j, m, n).

The angle between the centriole and kinetosome axis varies from 30 to 60° depending on the nuclear position and shape of the cell.

Kinetid structure of larval cells in *Lubomirskia baikalensis*

The kinetid of flagellated cells in *Lubomirskia baikalensis* larvae is similar to that in *Eunapius fragilis* (Fig. 4). However, some quantitative differences are noteworthy.

In *L. baikalensis* more filamentous bundles are attached to the kinetosome; that is why the filamentous train coming from the kinetosome is larger and denser than in *E. fragilis* (Fig. 4a-d, g).

The fibrillar roots of *L. baikalensis* are generally longer than those in *E. fragilis* (Fig. 4b, c, e vs. Fig 2.d, h, i, j). The centriole orientation relative to the kinetosome in *L. baikalensis* varies from nearly orthogonal to almost parallel in this species (Fig. 4e, g). Its position is more variable than in *E. fragilis* and less often associated with the position of the basal foot, but the top of centriole is also mostly directed towards the basal foot. As in *E. fragilis*, the arrangement of centriole apparently depends on the position of nucleus in the cell.

The principal scheme of kinetid structure in *L. baikalensis* and *E. fragilis* larval cells is summarized at Fig. 5.

Ancestral state reconstruction

Character state reconstructions showed following ancestral features in sponges: the accessory centriole presence, MTOC shaped as a simple basal foot, absence of additional cytoskeletal structures such as lateral bundles or lateral arms and striated root (Fig. 6; Supl. Fig. 3).

The accessory centriole disappeared twice: in Mycalidae group and Haplosclerida order. MTOCs transformations occurred for three times: in Spongillida order it took the shape of a large hollow tube, while in mycalids and some haplosclerids it became a multicomponent structure. The additional lateral bundle is found in Irciniidae family, Spongillida order and Calcarea+Homoscleromorpha lineage. This bundle is of microtubular nature in Homoscleromorpha, filamentous in Spongillida and unclear, possibly mixed, in Irciniidae and Calcarea. Lateral arms popped up twice: single in some Haplosclerida and double in the only investigated Dendroceratida (for description see Woolacott and Pinto 1995; Sokolova et al. 2019).

Ancestral larval roots are shown to be striated, but larva of the common ancestor of Demospongiae had simple unstriated roots. In order Poecilosclerida and subclass Verongimorpha complicated laminar roots appeared independently (for description see Woolacott and Pinto 1995). In order Haplosclerida the roots are also complicated, shaped as hollow large tubes (for description see Sokolova et al. 2019).

1 The kinetid linked to the beak of the pear-shaped nucleus may be either derived or ancestral. It
2 was found in subclass Verongimorpha, orders Spongillida and Suberitida, class Calcarea and in at
3 least some cells of the class Homoscleromorpha. The roundish nucleus separated from the kinetid
4 was found in orders Poecilosclerida and Haplosclerida, subclass Keratosa and class
5 Homoscleromorpha.
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8 The outgroup (choanoflagellates) shares with sponges striated roots and the accessory centriole,
9 but show differences in MTOC structure and pattern of microtubules divergence, and lack the dark
10 zone (Suppl. Table 1, Suppl. fig. 3).
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14 Discussion

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17 The kinetid in the observed spongillid larvae has these main elements: fibrillar root connecting
18 the kinetosome and nucleus, accessory centriole located at a sharp to blunt angle to the kinetosome,
19 long hollow basal foot (MTOC) with radiating lateral microtubules, filamentous train deriving
20 from the kinetosome, transitional fibres, and short transition zone with the transverse plate and
21 axosome. Comparing kinetid composition in different branches of the sponge evolutionary tree
22 allows us to trace the fate of kinetid elements. Then, we can seek to identify the apomorphic
23 (derived) and plesiomorphic (ancestral) states of spongillid larva's kinetid. To meet this goal, we
24 performed an ancestral state reconstruction analysis. Here, we will discuss the kinetid elements
25 according to their position in the cell, proceeding from basal toward apical direction.
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38 Our results imply that the ancestral root of sponge larval cells could be striated. This is
39 plausible, because striated roots are present in larvae of Calcarea and Homoscleromorpha (Boury-
40 Esnault et al. 2003; Ereskovsky and Willenz 2008, Pozdnyakov et al. 2020 and others) and in
41 embryos of Demospongiae: the chondrillid *Halisarca dujardini* Johnston, 1842 (Gonobobleva
42 2007) and the poecilosclerid *Lycopodina occidentalis* (Lambe, 1893) (Riesgo et al. 2007). In *H.*
43 *dujardini* (Gonobobleva 2007) the striation is shown to be 'fluent': it appears in embryos,
44 disappears in larvae and reappears in adults. Striated roots are also common in Eumetazoa and
45 diverse protists (e. g. Dingle and Larson 1981; Flammang et al. 1994; Tamm and Tamm 2002;
46 Karpov 2000). Further studies will enable us to estimate the actual distribution of root structures
47 among Porifera. Our results show the simple fibrillar root replaced a striated one in Demospongiae.
48 Spongillid roots, therefore, must be an ancient structure too. This structure is simple and looks
49 unspecific, unlike the long, complicated roots of Poecilosclerida (Woolacott and Pinto 1995) or
50 Haplosclerida (Sokolova et al. 2019).
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1 The accessory centriole is a common structure in sponge larvae, and its presence in the ancestral
2 state reconstructed seems not surprising. Its position may vary in different cells within the same
3 larva of spongillids and other sponges, but is fixed under the kinetosome in Homoscleromorpha
4 larvae (Pozdnyakov et al. 2020).
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7 The kinetosome of spongillids is anchored in the cells not only by the root, but also by a
8 transverse cytoskeleton of distinctive appearance. As in other sponges, it consists of lateral
9 microtubules radiating from the MTOC, which has an unusual shape. A large, homogeneous
10 hollow foot replaces the typical simple basal foot, which consists of a distal head attached to the
11 kinetosome by stalks. Moreover, the additional filamentous train occurs on the other side of the
12 kinetosome. This train was previously observed in larva of another freshwater species,
13 *Baikalospongia bacillifera* Dybowsky, 1880 and thought to be a microfilament bundle (Efremova
14 et al. 1988). Structures resembling this train are seen in sections of calcarean larvae (Borojevic
15 1969; Amano and Hori 2001; Ereskovsky and Willenz 2008; Lanna and Klautau 2012). They are
16 considered by authors to be a microtubule bundle. In some cases, they resemble microtubules due
17 to their diameter and arrangement (see fig. 17 in Borojevic 1969), but other images do not exclude
18 their filamentous nature (Amano and Hori 2001; Ereskovsky and Willenz 2008; Lanna and
19 Klautau 2012), as was also supposed earlier (Efremova et al. 1988). More detailed studies are
20 needed to confirm or deny the similarity of the transverse skeleton in Spongillida and Calcarea.
21 Here, we consider lateral bundle of Calcarea to be of unclear, probably mixed nature, as well as in
22 the dictyoceratid *Ircinia oros* (Schmidt, 1864) (Ereskovsky and Tokina 2004); in
23 Homoscleromorpha it was shown to be microtubular (Pozdnyakov et al. 2020). Thus, the
24 filamentous train of Spongillida is either rare or unique among Demospongiae.
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41 As in other eukaryotes, the apical part of the kinetosome is attached to the plasma membrane
42 by transitional fibres. Above it, the kinetosome transits to the axoneme. The transition zone
43 between the kinetosome and central microtubules is considered to weakly depend on the conditions
44 of flagellum function, so it is evolutionarily conservative (Moestrup 1982, 2000; Melkonian 1982;
45 Andersen et al. 1991; Karpov and Fokin 1995; Karpov 2000, 2016). Aside from spongillids, a
46 short transition zone with transverse plate in larvae is described in Haplosclerida (Sokolova et al.
47 2019) and distinguishable in dendroceratid *Aplysina aerophoba* (Nardo, 1833) (Maldonado 2009).
48 Thus, this feature occurs in sponges that are not closely related. A variant of transition zone
49 structure is a long zone without a transverse plate but with an axial granule in the lumen of the
50 kinetosome apex. This was found in also distantly related demospongian taxa (subclasses Keratosa
51 and Verongimorpha, orders Suberitida and Poecilosclerida) and occurred in larval cells of calcarean
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1 and homoscleromorph sponge (Gonobobleva 2007; Uriz et al. 2008; Sokolova et al. 2019; personal
2 unpubl. data on *Hymedesmia irregularis* Lundbeck, 1910). This thin structure is rarely clear in
3 published illustrations since it requires good fixation and high-resolution imaging mode. More
4 data are needed to estimate its phylogenetic signal.
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7 Structures of the so-called dark zone above the transversal plate are usually not recognisable on
8 the sections because of unsuitable fixation, but this zone is present in all investigated sponge
9 flagella (Pozdnyakov et al. 2017). Previously, we demonstrated that this dark zone is composed of
10 a transition cylinder connected to peripheral doublets and a sheath encircling central microtubules
11 (Sokolova et al. 2019). Here, we add that the dark zone includes Y-linkers that connect the
12 axoneme with the flagellar membrane (seen also in Sokolova et al. 2019, Figs. 2e-h).
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19 Kinetid in sponge cells can be associated with the nucleus. In these cases, the nucleus is pear-
20 shaped and its beak contacts the roots. In absence of such a link, the nucleus is roundish and usually
21 situated in the basal part of the cell. This character may be prone to homoplasy. We cannot yet
22 conclude which state was initial in larvae of the entire sponge lineage. But we suggest it was more
23 likely the presence of the link at least in demosponge larvae, since their simple ancestral non-
24 striated root is always associated with the nucleus. The link lacks only in the when long laminar
25 or tubular root appears. Disconnection of the nucleus and kinetosome therefore correlates with the
26 derived state and probably is a derived state itself.
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35 Thus, we suggest spongillid larva's kinetid acquired its hollow tubular foot and filamentous
36 lateral train during the evolutionary process, and that these characteristics should be considered as
37 apomorphies. Spongillids share with the proposed demospongian ancestral larva the following
38 features: accessory centriole, simple fibrillar root, absence of lateral arm and, probably, a
39 kinetosome-nucleus connection.
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46 Comparison of larval cell kinetids in Haplosclerida and Spongillida shows that haplosclerid
47 kinetid has more traits that have diverged far from the ancestral state of demosponges. While the
48 kinetid modifications in spongillids are the unusual hollow basal foot and long fibrillar bundle, in
49 haplosclerids they are centriole loss, tubular roots and, in some cases, a complex basal foot and
50 lateral arm. Unlike spongillids, haplosclerids have a disconnected kinetid and nucleus. Thus,
51 kinetid structures of larval cells in these orders differ notably, although they share the transverse
52 plate in the transition zone. Likewise, choanocytes of Spongillida and Haplosclerida are related by
53 the absence of a kinetosome-nucleus connection and accessory centriole, and the presence of the
54 transverse plate; this feature set that distinguishes them from the rest of sponges (Pozdnyakov et
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al. 2018). If the structure of the choanocyte kinetid can challenge the separation of these orders inferred from molecular data, then the structure of the larvae's kinetid confirms this conclusion.

After metamorphosis, the flagellar vanes are maintained in choanocytes, in spite of resorption of the flagellum (Ivanova, 1997b). The newly assembled kinetid of spongillids' choanocytes retains the transverse plate with the axosome in the transitional zone of flagellum. But it loses nucleus connection, permanent accessory centriole, and its MTOC becomes an electron-dense ring around the kinetosome. Thus, the choanocyte kinetid of spongillid adults differs from that in both their larvae and the suggested ancestral choanocyte (Pozdnyakov et al. 2018).

Comparing the inferred larval and choanocyte kinetids of the ancestral sponge reveals similarities: both have a simple basal foot, accessory centriole and no extra cytoskeleton structures. But the ancestral larval cell possessed a striated root, which was replaced by the simple fibrillar root in the Demospongiae lineage. In choanocytes the simple root apparently emerged in most basal nodes (Pozdnyakov et al. 2018). The kinetid of the supposed ancestral choanocyte was connected to the kinetosome, although it lost link in several lineages (Pozdnyakov et al. 2018; 2020). So, it might also be the same in larval cells. In this case, the main difference between the ancestral kinetid of larvae and adults is the root structure. Generally, kinetids in sponge larvae are prone to strengthen the transverse skeleton – cytoskeletal bundles or lateral arms that have never been encountered in choanocytes. Their longitudinal skeleton (roots) also tends to be longer and stronger. This may result from the higher load that larval flagellum faces.

Future studies of sponge cell ultrastructure will complete and specify the scheme of evolutionary transformation of the kinetid elements. It will be worthwhile to compare ancestral kinetids of larval cells and choanocytes with kinetids in unicellular relatives of Metazoa, when sufficient pertinent images are available. Such comparison will be useful for understanding early metazoan evolution. For today, we can operate only with choanoflagellates, whose kinetid structure is specialised, with apomorphies in the MTOC structure and transition zone; it differs from both ancestral sponge choanocytes and larvae (Pozdnyakov et al. 2017).

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Data availability statement

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Fig. 1 General views in longitudinal sections: a) spongillid larva (*Lubomirskia baikalensis*),
 longitudinal section. Scale bar 100 µm; b) flagellated (ciliary) cells of *Eunapius fragilis*
 larva. Scale bar 600 nm.

Abbreviations: c, cavity; ic, internal cells; fv, flagellar vane; lfc, layer of flagellated cells,
 n, nucleus.

Fig. 2. Ultrastructure of the kinetid in larval cells of *Eunapius fragilis*, longitudinal plane.
 Consecutive sections of three cells (a-d, e-g, i-j) and separate sections of six cells (h, k, l,
 m, n, o). Scale bars: a-l) 200 nm, m-o) 250 nm.

Abbreviations: afb, apical filamentous bundle; axs, axosome; bfb, basal filamentous bundle; bf,
 basal foot; fb, fibrillar bridge between the kinetosome and the centriole; c, centriole; cm, central
 microtubules; dz, electron-dense zone; fb, fibrillar bundle; fr, fibrillar root; ftr, filamentous train;
 k, kinetosome; lmt, lateral microtubules; n, nucleus; pc, points of connection of transition fibres to
 the plasma membrane; tc, transition cylinder; tf, transition fiber; tp, transverse plate; Y-l, Y-
 linkers.

Fig. 3. Ultrastructure of the kinetid in larval cells of *Eunapius fragilis*, transverse plane.
 Consecutive sections of three cells (a-d, e-g, i-k) and a section of another cell (h). Scale bar:
 200 nm.

Abbreviations: bf, basal foot; c, centriole; ftr, filamentous train; lmt, lateral microtubules;
 k, kinetosome; tf, transition fiber.

Fig. 4. Ultrastructure of the kinetid in larval cells of *Lubomirskia baikalensis*, longitudinal

plane. a-c) series of consecutive sections through a cell; d-g) sections through the kinetid of different cells. Scale bars: a-d, f-g) 200 nm; e) 250nm.

Abbreviations: afb, upper filamentous bundle; axs, axosome; bf, basal foot; bfb, basal filamentous bundle; c, centriole; cm, central microtubules; fb, fibrillar bridge; fr, fibrillar root; ftr, filamentous train; k, kinetosome, pc, points of connection of transition fibres to the plasma membrane.

Fig. 5. Schematic reconstruction of spongillid larva's kinetid from lateral view.

Abbreviations: afb, apical fibrillar bundle; bfb, basal filamentous bundle; bf, basal foot; c, centriole; cm, central microtubules; dz, dark zone; fl, flagellum; fb, fibrillar bridge between the kinetosome and centriole; fr, fibrillar root; ftr, filamentous train; Ga, Golgi apparatus; k, kinetosome; lmt, lateral microtubules; n, nucleus; pr, cell protrusion; tc, transition cylinder; tf, transition fiber; tp+axs, transverse plate with axosome.

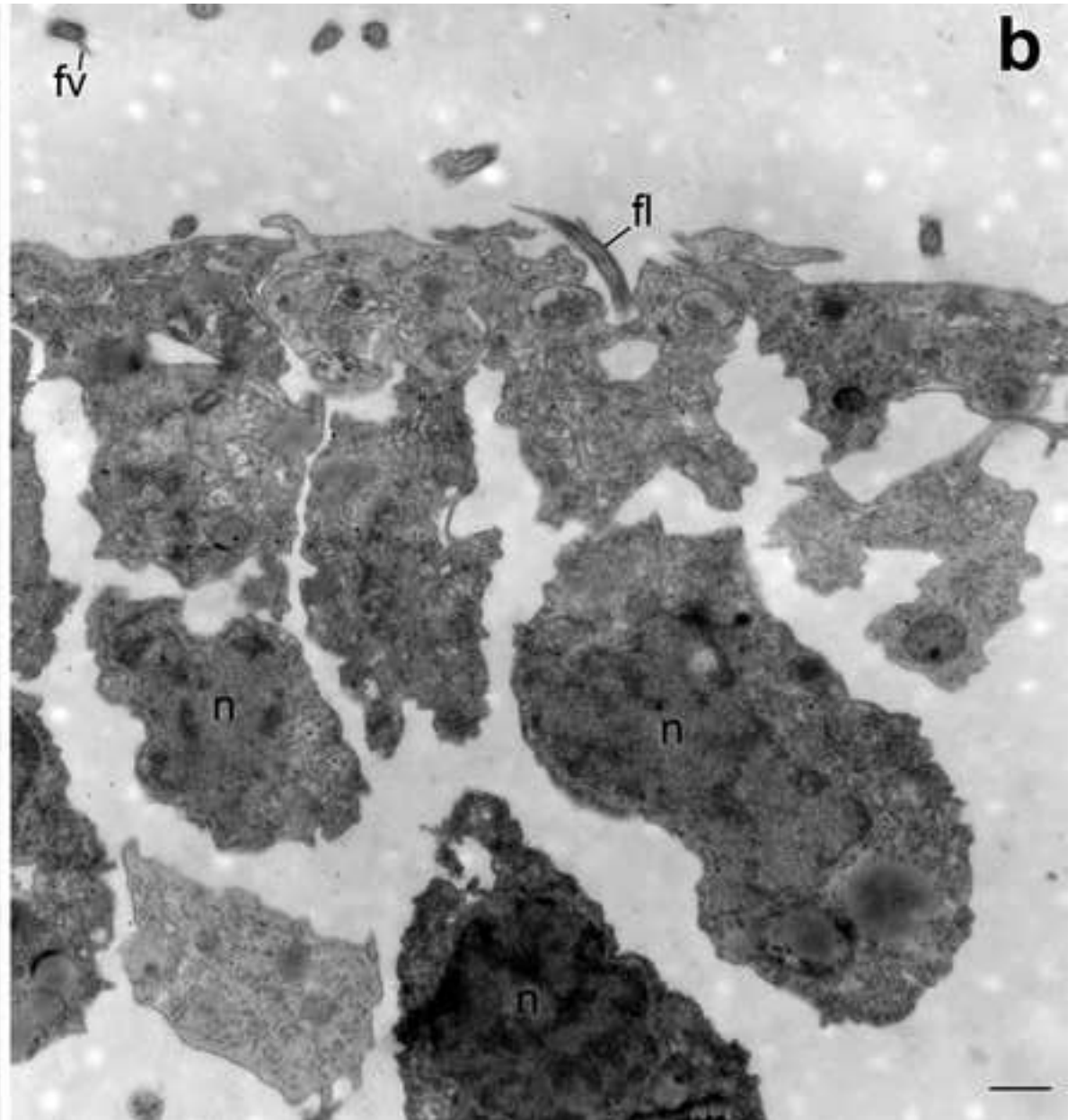
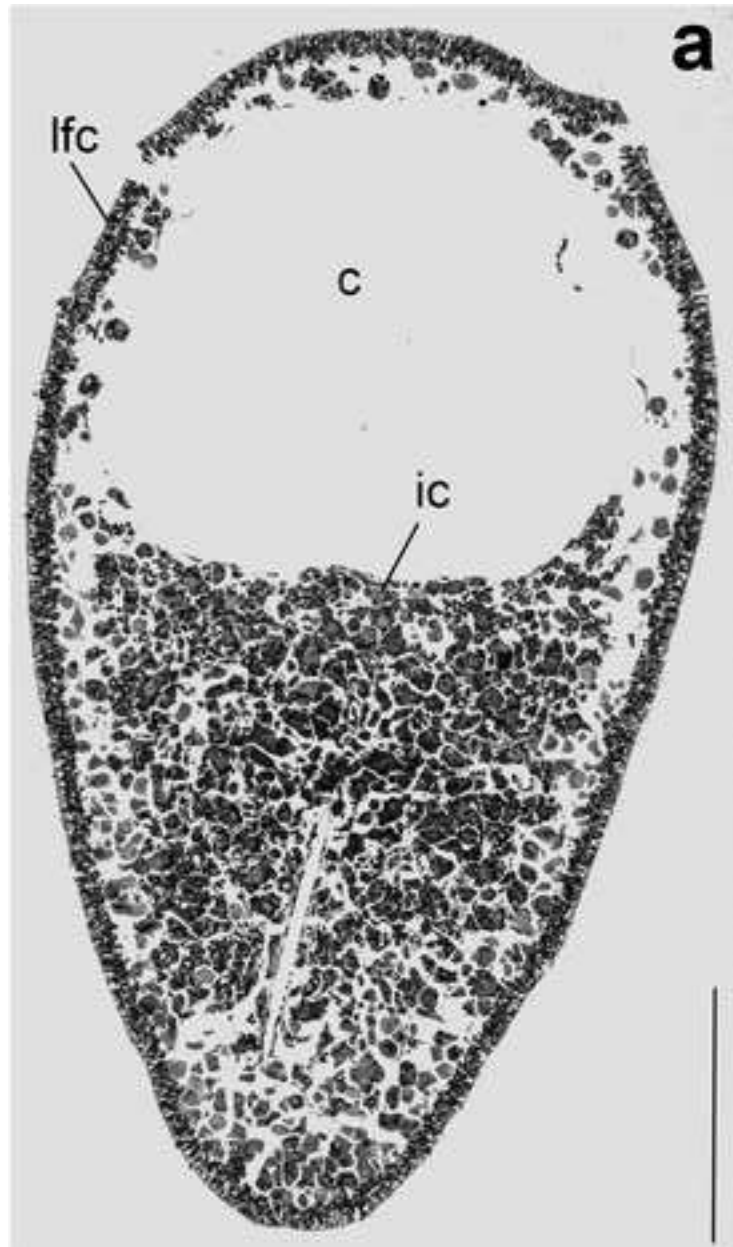
Fig. 6. The scheme of possible evolution of kinetid elements in sponge larval cells. Lateral bundle: a – filamentous; b – microtubular, c – absent. Roots: a – striated, b – simple unstriated, c – tubular, d – laminar. MTOC: a – simple basal foot, b – complex, c – large hollow foot. Accessory centriole: a – present, b – absent. Kinetosome-nucleus-link: a – absent, b – present. Lateral arm (imaged as a transverse section through the kinetosome): a – double, b – single, c – absent.

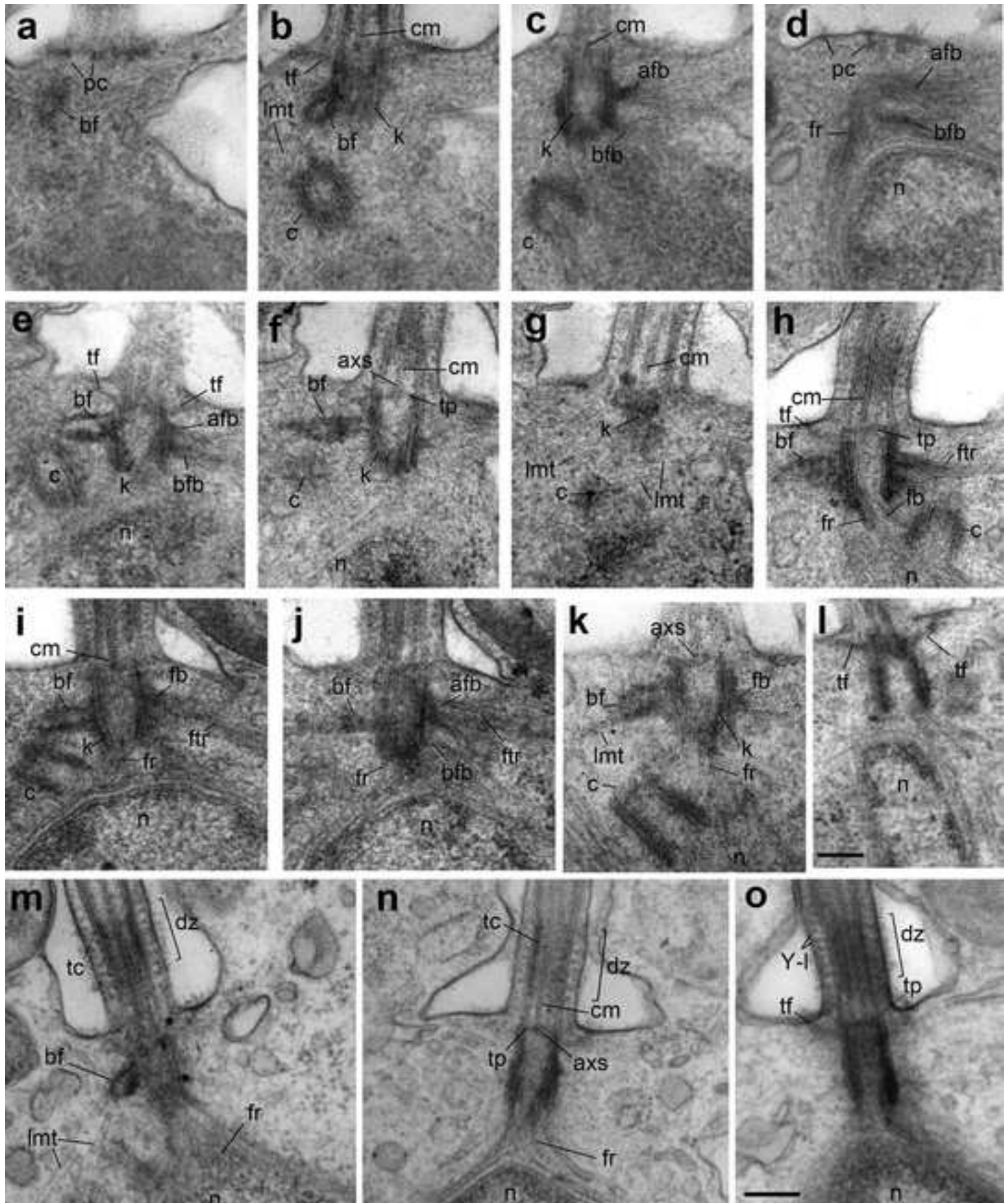
Supplementary Material

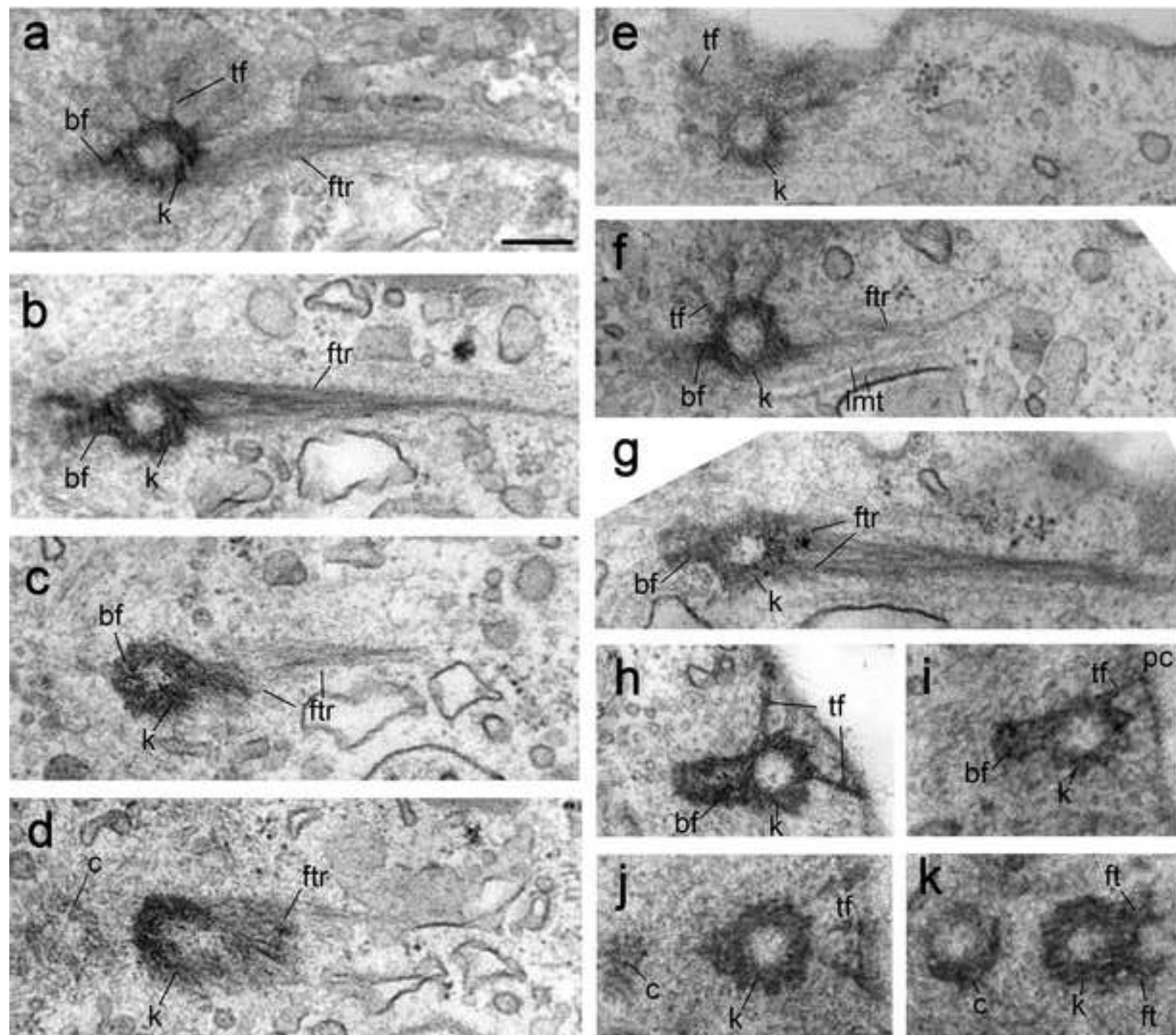
Fig. 1. Bayesian phylogenetic tree of the sponges based on the 18S dataset. Node support is indicated as branch labels, scale according to GTR+G+I model distances.

Fig. 2. Maximum likelihood phylogenetic tree of sponges based on the 18S dataset. Node support is indicated as branch labels, scale according to GTR+G+I model distances.

Table 1. Standard Categorical matrix of morphological characters used in the analysis. Polymorphisms are listed separated by the '&' symbol; uncertain states are indicated by the '/' symbol. Clades included in analysis are highlighted in blue.







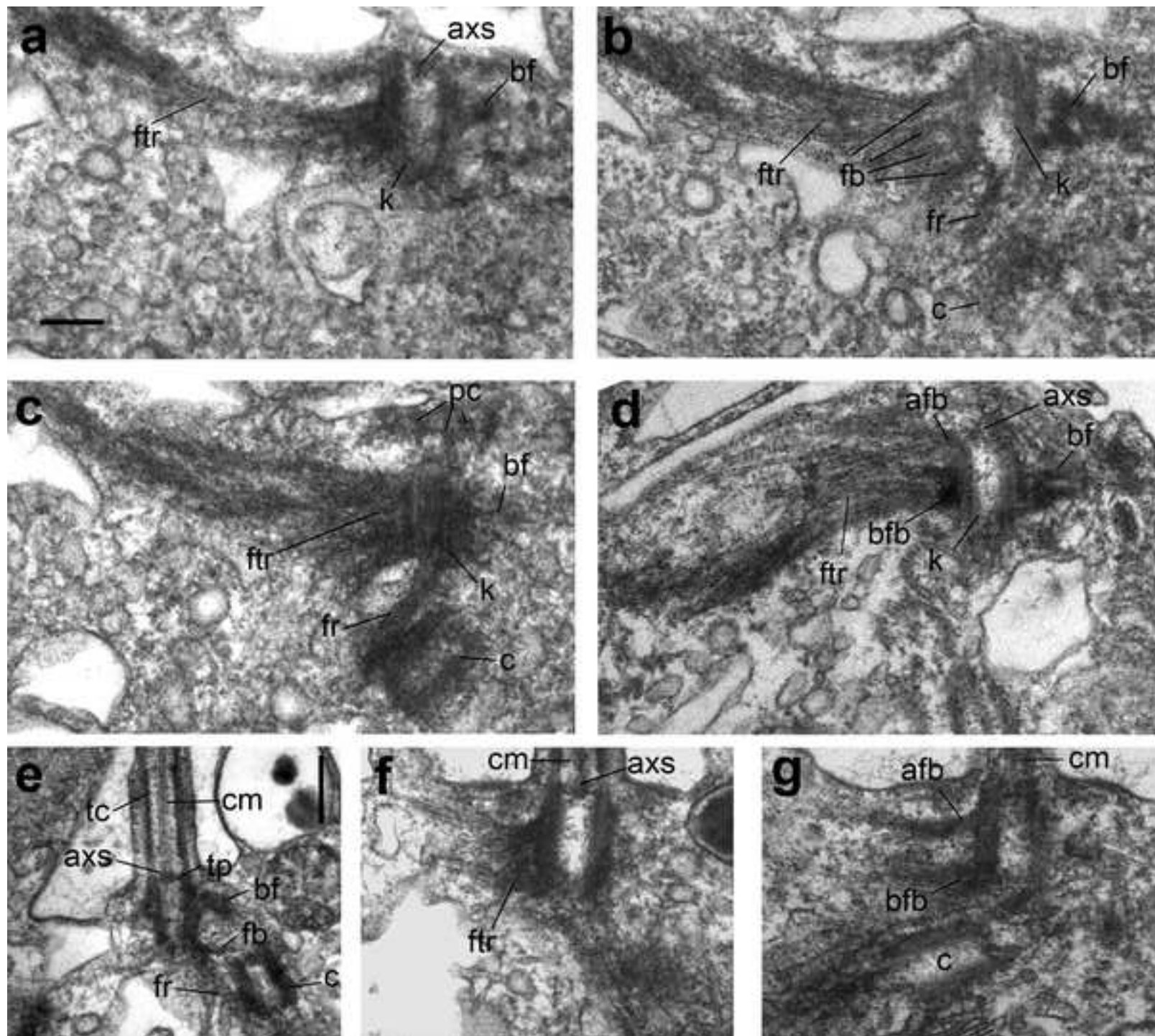
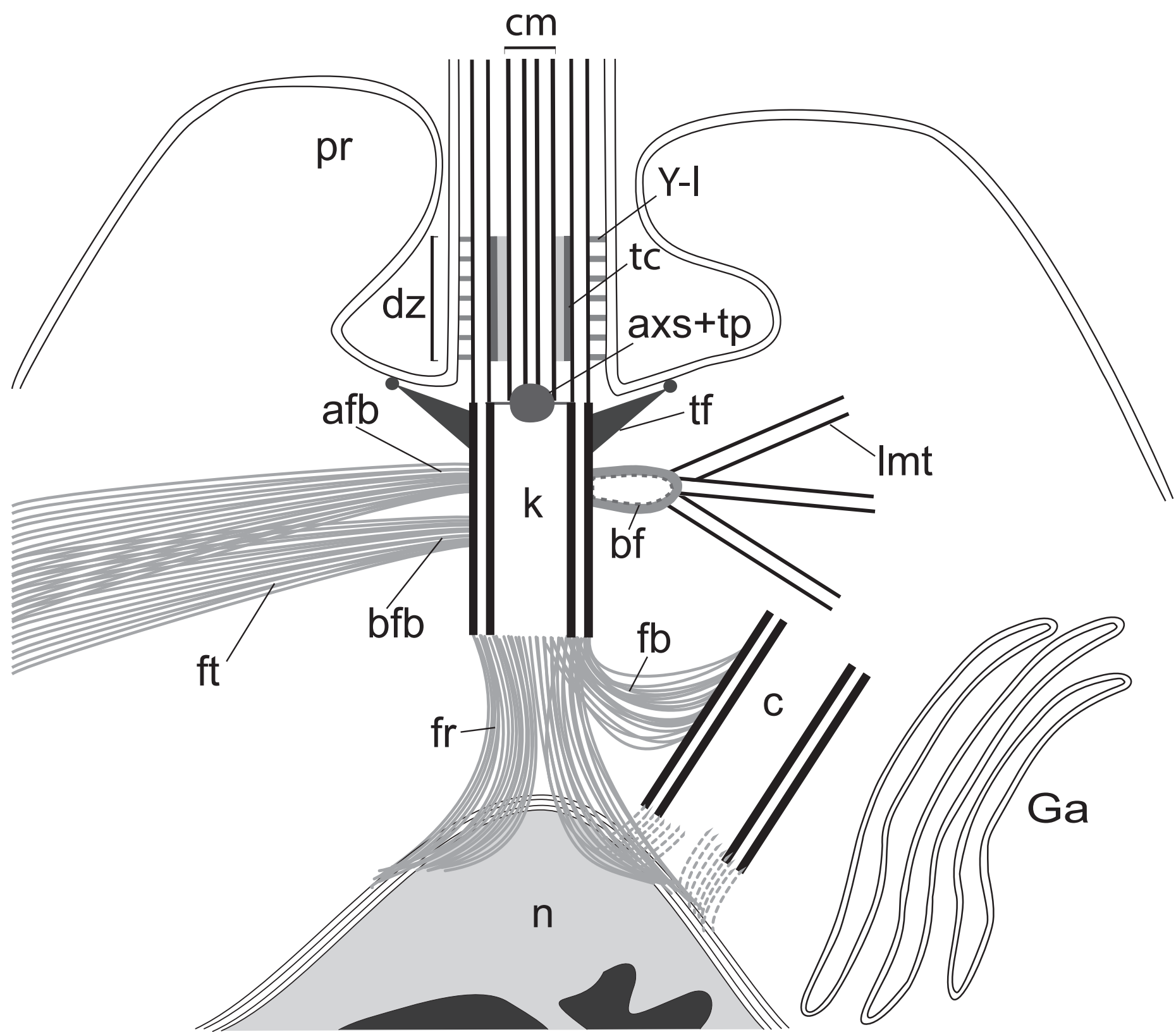
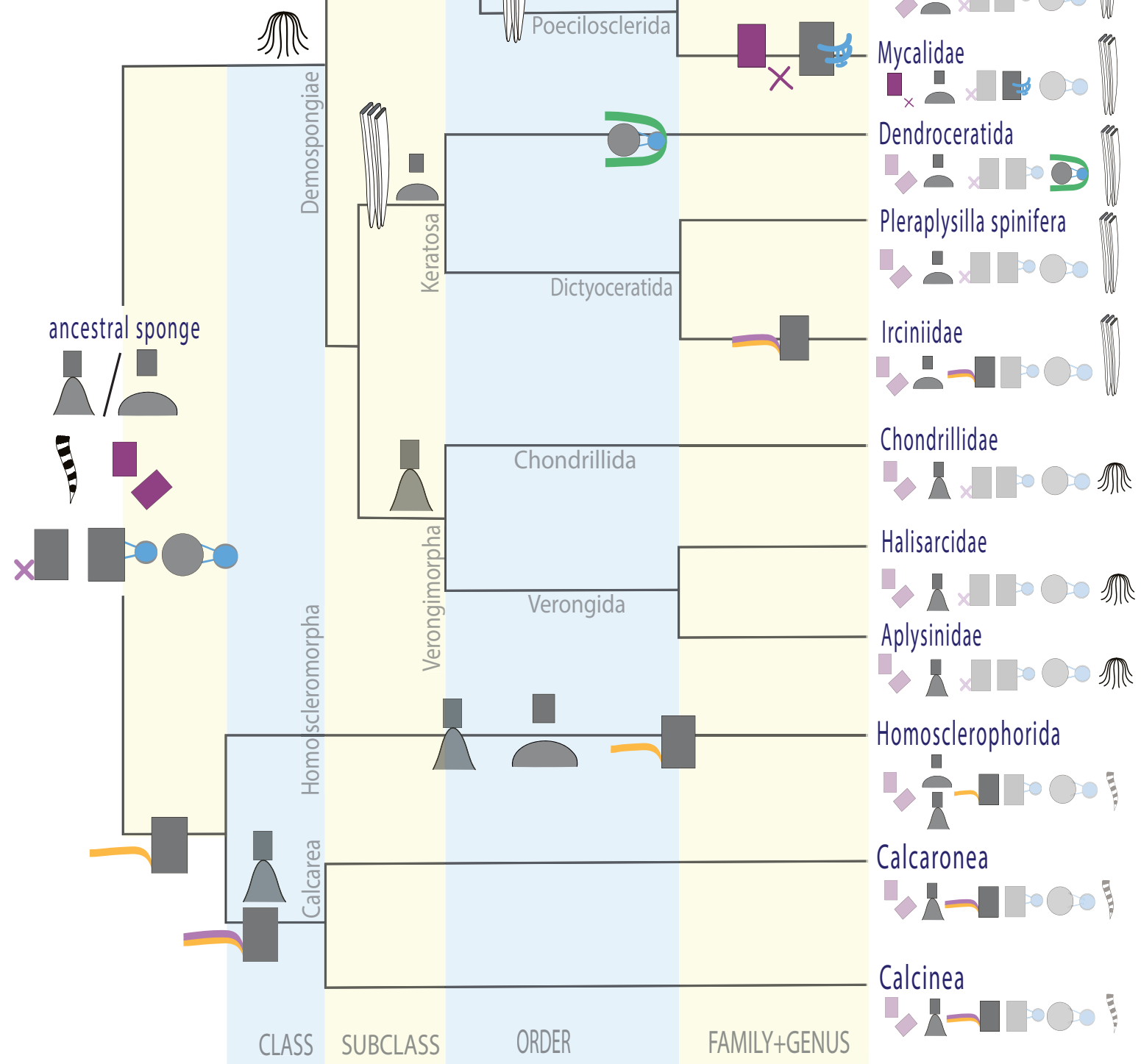
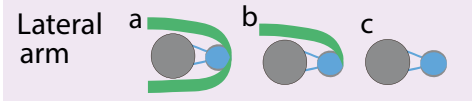
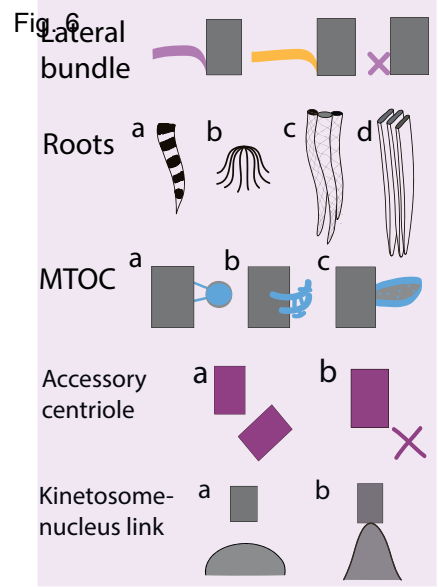
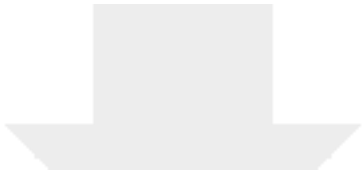



Fig. 5








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