Small Bilaterian Fossils from 40 to 55 Million Years Before the Cambrian

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Small Bilaterian Fossils from 40 to 55 Million Years Before the Cambrian

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Ten phosphatized specimens of a small (<180 micrometers) animal displaying clear bilaterian features have been recovered from the Doushantuo Formation, China, dating from 40 to 55 million years before the Cambrian. Seen in sections, this animal (*Vernanimalcula guizhouena* gen. et sp. nov.) had paired coeloms extending the length of the gut; paired external pits that could be sense organs; bilateral, anterior-posterior organization; a ventrally directed anterior mouth with thick walled pharynx; and a triploblastic structure. The structural complexity is that of an adult rather than a larval form. These fossils provide the first evidence confirming the phylogenetic inference that Bilateria arose well before the Cambrian.

The highly elaborated bilaterian animals of the Early Cambrian (*1*) imply a complex prior evolutionary process that has, however, remained enigmatic. The earliest unequivocal bilaterian forms in the fossil record are from the Ediacaran biota, e.g., the mollusc-like *Kimberella* (*2*). Ediacaran fossils are estimated to extend down to about 20 to 30 million years before the Lower Cambrian boundary (*3*, *4*), which is now dated at 543 (+/–1) million years before the present (*5*). A recent phylogenetic analysis of metazoan protein evolution (*6*) indicates that the last common bilaterian ancestor (LCBA) and the divergence of the main bilaterian branches occurred between the end of a long period of intermittent worldwide glaciation about 600 million years ago (*7*–*9*) and the Cambrian boundary, although this divergence time is more recent than concluded in previous analyses [reviewed in (*6*)]. The LCBA had to possess sufficient complexity to display the definitive shared characters of the bilaterians: bilateral symmetry (more or less); anterior-posterior (A-P) body plan organization with dorsoventral specializations; gut with oral and anal openings; localized sense organs; and body composed of mesodermal, endodermal, and ectodermal layers. The LCBA required the genomic apparatus to erect such complex body plans in the process of development. Thus, it must have possessed the genetic "tool kit" for development shared by all bilaterians (*10*), as well as the type of complex gene regulatory program that underlies all bilaterian developmental processes (*11*). The LCBA was predicted to have been microscopic in dimension and could well have lacked any very distinctive external features (*10*).

Here, we describe microscopic bilaterian fossils from the Doushantuo Formation, Southwest China, dating from 580 to 600 million years ago (*4*, *12*). The fossils derive from a stratum containing the earliest multicellular animal forms yet recovered, all microscopic in scale. Thus far, this assemblage has consisted of adult and embryonic sponges, some cnidarian-like forms, and diverse eggs and embryonic stages that could include some of bilaterian provenance (*13*–*16*).

The Neoproterozoic Doushantuo Formation at Weng'an, Guizhou Province, overlies a tillite demarcating perhaps the latest glaciation in this region (*13*, *14*). The dolostones and phosphorites of the lowermost 30 m represent deposition under anoxic environmental conditions (*17*, *18*), within which no animal fossil remains have been found, either here or in contemporaneous strata elsewhere in China. These rocks are capped by a karstic exposure surface. This unconformity is overlain by a phosphorite 15 m thick (Weng'an Phosphorite Member) containing phosphoclasts that include very well preserved microfossils. The fossils discussed here, as well as many additional complex animal forms [e.g., (*16*)], derive from a basal black bituminous phosphorite layer of the lower Weng'an Phosphorite Member that was deposited in a shallow marine environment, coincident with a dramatic swing to a more oxygenated environment (*19*, *20*). Dolostones of the Dengying Formation overlie the Doushantuo Formation and contain Cambrian small shelly fossils near the top (*21*).

Specimens. Ten specimens were examined in this study. The three best preserved specimens (together with color-coded interpretation) of *Vernanimalcula guizhouena* gen. et sp. nov. are shown in Fig. 1, and close-ups of two additional well-preserved specimens are in Fig. 2. We consider the specimen reproduced in Fig. 1, A1, the holotype. Measurements on all studied specimens, including two that may be a closely related species, are in Table 1. The salient features of the fossils follow.

Size. The images considered are interpreted as roughly coronal sections at different levels from the ventral surface of the animal and at slightly different angles to this surface. Given their more or less similar orientation, the measurements are comparable: The fossilized animal had the shape of a broad oval, although in every specimen the external wall is deformed in one region or another. The longer axis, i.e., the A-P or oral-anal axis, is 124 to 178 μm in those examples that are relatively well preserved and of consistent dimensions in the other specimens as well (Table 1). The left/right axis averages about 10% less than the long axis in each individual specimen (about 20% in the related species) (Table 1). However, unlike the A-P dimension, the width consists partly of coelomic space and thus is subject to local deformation, as well as expansion due to squashing and/or decay processes. The microscopic dimensions of the original animal were only about the size of a modern indirectly developing marine larva, although its multilayered anatomical structure is much more complex.

General bilateral A-P organization and triploblastic structure. The fossils are bilaterally symmetrical, in respect to their major features (Fig. 1). The holotype specimen displays the structure of the animal toward its ventral side, with the mouth and pharynx at the top of the image (Fig. 1, A1 and A2). The mouth opened ventrally. The second specimen probably represents a more dorsal coronal section, which passes above the ventrally located mouth and through the top wall of the pharynx (Fig. 1, B1 and B2). Both of these sections traverse the lumen of the gut, although in the holo-

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type some portions of the ventral wall of the gut are included. The third section (Fig. 1) displays the lumen of the pharynx (Fig. 1, A1 and A2) but apparently tilts ventrally toward the posterior so as to display mainly the lower endodermal wall. The digestive tract of this animal extends from the mouth to where it joins the posterior body wall, as seen most completely in the specimen in Fig. 1, B1 and B2. The digestive tract is flanked by paired coeloms, clearly lined both medially and distally with a heavy walled epithelium-like layer of uniform thickness. On each side, the medial and distal walls can be seen to join at both anterior and posterior ends. These bilateral coelomic structures are prominent and obvious in every one of the 10 specimens included in Table 1. Their topology requires that the coelomic wall be considered a mesodermal layer (orange in Fig. 1), peripheral to the endodermal pharynx and gut (mauve and tan in Fig. 1). The whole can be seen to be surrounded by another separate

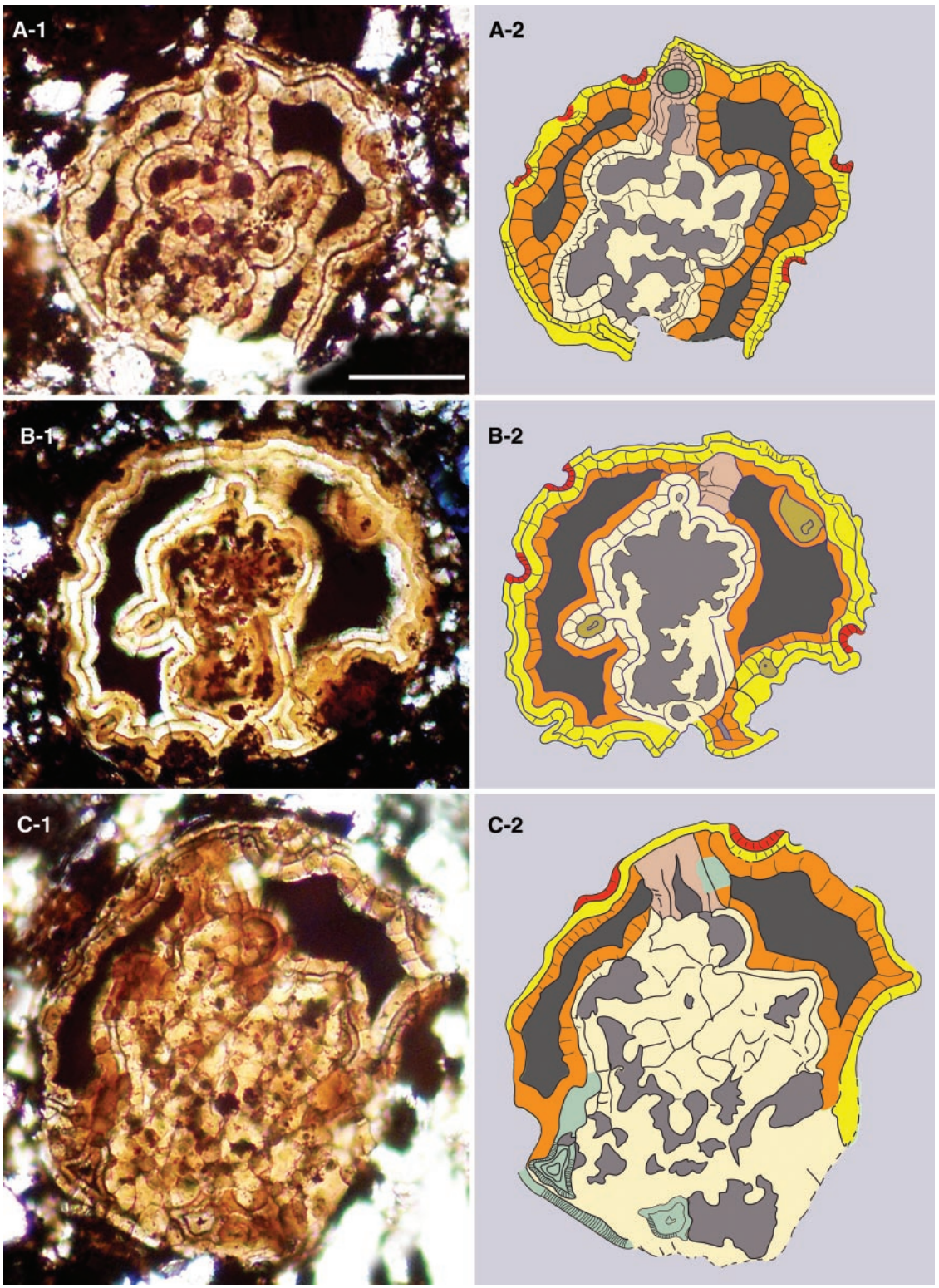

Fig. 1. Images of three different, fairly well preserved specimens of the bilaterally organized fossil animal *Vernanimalcula guizhouena*. Left panels show digitally recorded, transmitted light images of sections about 50 μm thick, which had been ground from larger rock samples, mounted on slides, and viewed through a light microscope. Right panels show color-coded representations of the images on the left. These were prepared by digital image overlay. Yellow, external ectodermal layer; ochre, coelomic mesodermal layer; red, surface pits; mauve, pharynx; light tan, endodermal wall of gut; gray-green, lumen of mouth; dark gray, paired coelomic cavities; lighter gray, lumen of gut; brown, "gland-like" structures, with central lumen (B); light green, mineral inclusions (C). The scale bar represents 40 μm in (A), 55 μm in (B), and 46 μm in (C). (**A**) Holotype specimen, X00305, slightly tilted, almost complete ventral level coronal section, passing through the ventrally located mouth. (**B**) Coronal section of second specimen, X08981, passing through dorsal wall of pharynx and displaying complete A-P length of digestive tract, including posterior end [not visible in (A)]. (**C**) Tilted coronal section of third specimen, X10475, possibly slightly squashed, passing through dorsal wall of pharynx and through the dorsal wall of the gut. For dimensions, see Table 1.

layer of phosphatized tissue, i.e., by an external epithelium (yellow in Fig. 1). The body plan of the fossilized animal is therefore triploblastic in organization.

The digestive tract. Additional features from the fourth and fifth relatively well preserved specimens of Table 1 confirm the structure of the digestive tract apparent in the holotype (Fig. 2, A to C). The mouth has a distinctive internal circular lumen surrounded by an outer collar (Fig. 2, A and B). A multilayered pharynx that gives the appearance of muscularity follows posteriorly (Fig. 2A). Here, the wall consists of a symmetrical (with respect to the axis of the pharyngeal passageway), thick, laminar structure composed of three or possibly four concentric layers separated by thin reddish partitions. The pharynx opens into a large stomach or intestine (Fig. 1, A to C; Fig. 2, A to C). The lumen of the digestive tract is filled with indistinct globular and other matter, much of it dark red in color, in contrast to the phosphatized endodermal walls tangentially included in the sections, which appear tan. There are also in the stomach or intestine some dark regions similar in hue to the lumens of the mouth and the coeloms and to the external matrix surrounding the specimen (Fig. 1, A1 and B1). The wall of the stomach or intestine is composed everywhere of two tightly apposed, thin layers of phosphatized tissue (Fig. 1, A to C). In the holotype, the two layers are separated by continuous reddish partitions, perhaps the remains of basement membranes. In their dimensions and organization, the endodermal epithelial layers are entirely distinct from the single, thick mesodermal wall lining the coeloms. The outer layer of the endoderm wall is very closely applied to the medial coelomic wall. The endodermal layers bounding the stomach or intestine can be seen to be continuous, with a thickened region of the external body wall at the posterior end, i.e., the anus (Fig. 1, A and B). The posterior portion of the specimen in Fig. 1C is not as well preserved.

Bilateral surface pits. Regularly spaced pits, lined by concave pockets, are seen on the external surfaces of all the specimens in Fig. 1. In the holotype, there is one located anteriorly near the mouth (seen in close-up in Fig. 2A). (A close-up view of two pits from the specimen in Fig. 1B is also shown in Fig. 2D.) The holotype specimen displays on each side a further pair of these pits, which appear to be symmetrically positioned with respect to the A-P body axis. The symmetrical arrangement of the anterior pair of surface pits on either side of the pharynx is particularly clear in the specimen of Fig. 1C. [The right anterior pit of this pair in the holotype specimen is not included in the section, (Fig. 1A.)] The pits display a discrete structure and evidently represent surface specializations; a speculative interpretation is that they are the remains of external sensory structures.

Three-dimensional form. As an aid in understanding the morphology, we developed a three-dimensional computational reconstruction of the animal (Fig. 3). This was based on the interpretations discussed above, with the additional assumption that it had a flattened ventral surface that would allow it to browse along the benthic surface, feeding through its ventrally oriented mouth.

Taphonomy. The great majority of the holotype specimen is the result of early phosphatization of primary biological structures, and its morphology cannot be the product of secondary diagenetic processes (Fig. 1, A1.). (The same is true of the specimen in Fig. 1, C1.) Later diagenesis in the holotype specimen does account

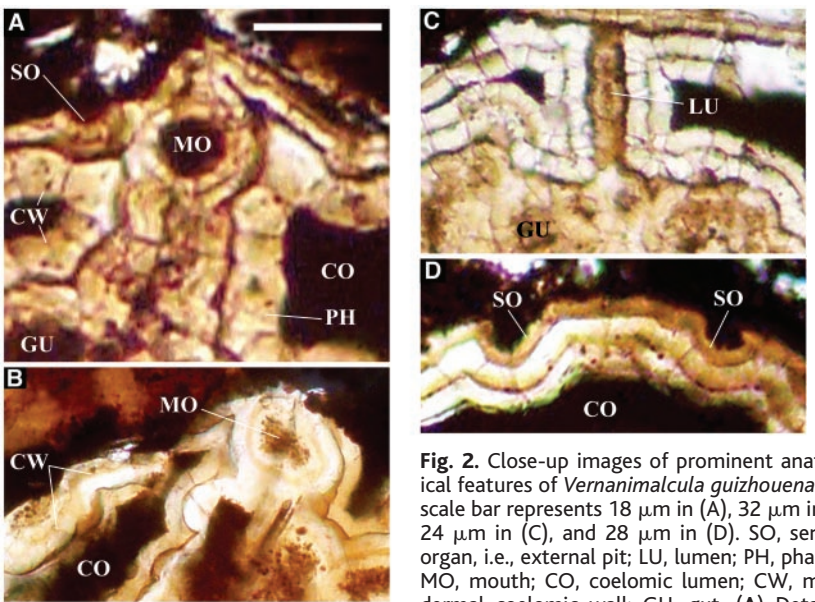

Fig. 2. Close-up images of prominent anatomical features of *Vernanimalcula guizhouena*. The scale bar represents 18 μm in (A), 32 μm in (B), 24 μm in (C), and 28 μm in (D). SO, sensory organ, i.e., external pit; LU, lumen; PH, pharynx; MO, mouth; CO, coelomic lumen; CW, mesodermal coelomic wall; GU, gut. (**A**) Detail of collared mouth, multilayered pharynx, and one anterior surface pit. In this image, which is from the holotype specimen (Fig. 1A), the floor of the pit can be seen to be composed of a specialized concave layer. Note the coelomic wall, which here as elsewhere in these specimens has a thickness of about 5 to 6 μm. (**B**) Mouth of a fourth specimen, Q3105, displaying collared mouth and pharynx, ventral view. (**C**) Lumen of pharynx from a fifth specimen, X10419, secondarily encrusted but revealing morphology of opening of pharynx into gut similar to that seen in the specimens shown in Fig. 1. (**D**) Close-up of spaced external pits, interpreted as possible sensory organs, from the same specimen as shown in Fig. 1B [compare (A)].

Table 1. Characters and measurements of studied specimens of *Vernanimalcula*. "No" indicates that this region of the specimen is not well preserved enough to display the character. GP, good preservation; MP, moderate preservation; C, putative cellular structure preserved.

Slide number	Quality	Length§	Width	Pharynx‖	Pits¶	Anus	Mouth**	Coeloms	Gut
X00305*	GP, C	124	115	35	5	yes	21	yes	yes
X08981†	GP, C	178	157	36	3	yes	no	yes	yes
X10475†	GP, C	155	150	26	2	no	no	yes	yes
Q3105†	GP	145	130	34	1	no	14	yes	yes
X10419†	GP, C	167	162	27	no	no	no	yes	yes
Q3045	MP	95	92	11	2	no	no	yes	yes
X08978	MP	143	117	19	no	no	no	yes	yes
X08980	MP	129	103	19	no	no	no	yes	yes
J9830‡	MP	204	199	28	no	no	no	yes	yes
J2851‡	GP	167	145	37	no	no	no	yes	yes

*Holotype †Illustrated specimen ‡Unillustrated specimen of closely related species §Length in mm of anterior posterior axis ‖Length of pharynx ¶Number of sensory pits **Diameter if present

for the thin white external coating on the outermost surface of the epidermis toward the posterior end, as well as the destructive dolomitization near the posterior end. Although an internal laminar encrustation is evident in some other specimens (e.g., Fig. 1, B1), it was evidently deposited on the substrate provided by the original morphological structures and cannot be responsible for the morphological topology of the specimens. Many arguments contribute to this conclusion and preclude the alternative that the specimens are entirely diagenetic artifacts: (i) multiple independent specimens display the same overall morphological organization (Fig. 1, Fig. 2, and Table 1); (ii) the tissue-specific features noted above, that is, the special character of the gut wall, the coelomic mesodermal walls, the external paired pits, and the pharynx, are consistently observed in independent specimens; (iii) the specimens are all bilaterally symmetrical around the A-P axis of the body plan; (iv) all the specimens display distinct local soft tissue deformations; therefore, although biologically symmetrical, they are not geometrically symmetrical; and (v) the specimens are all approximately of the same dimensions and proportions (Table 1).

Rarity. From many thousands of rock thin sections, we identified as specimens of this animal only one out of every 5,000 to 10,000 microfossils that were examined. However rare the animal is, the conditions for the fossilization of its internal structures were certainly rare, and its preservation must have depended on local taphonomic factors. Primary fossilization in this milieu occurs by the rapid replacement of cellular structural elements by phosphate, the mechanism of which remains yet ill defined (*17*, *22*, *23*).

Identification. We have named the genus represented by the fossils reproduced in Figs. 1 and 2, and reconstructed as in Fig. 3, *Vernanimalcula*, for "small spring animal" (i.e., following the "winter" of Snowball Earth) (*24*).

Phylogenetic and evolutionary implications. *Vernanimalcula* is by far the earliest organism of bilaterian form so far found. It appears in strata that overlie the last of several extreme glacial or proposed Snowball Earth episodes, and this age is consistent with the general chronological predictions of phylogenetic analysis in (*6*), given that it belongs in the stem group of one or another bilaterian clade. Its small size, its simple external morphology, and its lack of obvious appendages also conform to the morphological and developmental predictions of (*10*). Beyond this, however, these fossils are in many ways surprising. *Vernanimalcula* is complex in structure and represents an almost textbook example of the body plan of an adult, triploblastic coelomate, yet its dimensions are minute. As noted elsewhere (*10*, *20*), some aspect of the Doushantuo environment was not consistent with the existence of macroscopic animal forms.

To understand the evolutionary implications, *Vernanimalcula* must be considered in the context of bilaterian phylogeny, but, unfortunately, that remains an incompletely resolved problem. The phylogeny in Fig. 4A is supported as a majority solution in the most recent and complete protein and ribosomal RNA molecular evolution analyses (*6*), but not overwhelmingly. According to the conventional current view (Fig. 4A), the deepest split within Bilateria is between the protostomes and deuterostomes. This produces three possibilities: *Vernanimalcula* could be related to a stem-group precursor of Bilateria other than acoels, to a precursor of the deuterostomes, or to a clade that includes some of the lophotrochozoans, i.e., spiralians and other groups that display a complete coelomic mesoderm extending the whole length of the gut. In the first case, it would follow that a complete coelomic mesoderm was lost in the ecdysozoans, although it had been present in their ancestor; in the second case, that a complete coelomic mesoderm evolved at least one other time as an independent convergence in an ancestral branch of some lophotrochozoan groups; in the third, that a complete coelomic mesoderm evolved independently in the deuterostomes. An alternative phylogeny (Fig. 4B), which appears as a minority solution in some phylogenetic analyses, would require that coelomate lophotrochozoans and deuterostomes are more closely related to one another than either is to the ecdysozoans and that the classical superphylum Protostomia is actually not monophyletic. The model in Fig. 4B would place *Vernanimalcula* in the stem lineage leading to those modern clades that display a complete coelomic mesoderm, i.e., the deuterostomes and some major lophotrochozoan groups. In this case, the complete coelomic mesoderm would be an ancient pleisiomorphy that did not evolve multiple times in independent clades.

The morphology of *Vernanimalcula* demonstrates that the evolutionary appearance of developmental programs required to generate a multilayered bilaterian body plan preceded the entrainment of the growth programs required for macroscopic body size. Further-

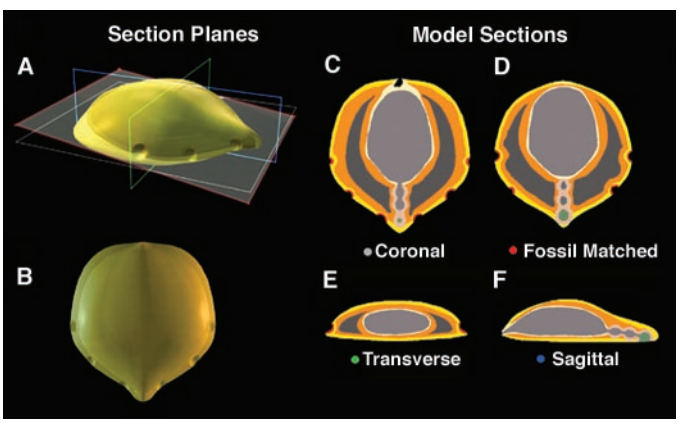

Fig. 3. Three-dimensional reconstruction of *Vernanimalcula*. The model was constructed with FormZ, version 4.0, a software from Auto-Des-Sys Inc. (Columbus, OH), and with proportions derived from the holotype specimen in Fig. 1A. (A) and (B) display computed external views of the reconstruction, (C) to (F) the computed sections. (**A**) Perspective view indicating planes of section shown in (C) to (F), as indicated by the colored dots. (**B**) Dorsal view. (**C**) Perfect coronal section. (**D**) Slightly tilted coronal section similar to the image shown in Fig. 1A. (**E**) Transverse section. (**F**) Sagittal section. (C to F) Yellow, ectoderm; ochre, coelomic mesoderm; light tan, endoderm.

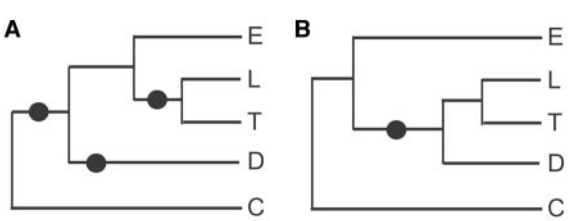

Fig. 4. Alternative bilaterian phylogenies and possible phylogenetic positions of *Vernanimalcula*. E, Ecdysozoa; L, lophophore-bearing lophotrochozoan protostomes; T, lophotrochozoans using trochophore-type larvae, i.e., spiralians; D, deuterostomes; C, cnidarian outgroup. L, T, and D in general display complete coelomic mesoderm within the body wall and surrounding the gut, from anterior to posterior end of the body plan (*25*). A large number of bilaterian groups are omitted from these diagrams, including the acoels, which in either of the phylogenies shown would occupy a basal position with respect to other Bilateria, including the coelomate *Vernanimalcula* (*26*–*28*). (**A**) Conventional phylogeny, supported in a majority of trials by molecular phylogeny (*6*, *29*, *30*). Here, the protostomes (represented by L+T+E) are a monophyletic clade, and the black circles represent three alternative stem group positions that *Vernanimalcula* might occupy. (**B**) Alternative phylogeny in which the protostomes are not monophyletic, but the bilaterian clade that shares the character of having a complete coelomic mesoderm (i.e., L+T+D) is monophyletic, and the resulting position that *Vernanimalcula* would occupy.

more, the organization of these fossils, taken together with their provenance, indicates that the genetic tool kit and pattern formation mechanisms required for bilaterian development had already evolved by Doushantuo times, long before the Cambrian. Therefore, the diversification of body plans in the Early Cambrian followed from the varied deployment of these mechanisms once conditions permitted, not from their sudden appearance at or just before the Cambrian boundary.

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The Bacterial Condensin MukBEF Compacts DNA into a Repetitive, Stable Structure

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Condensins are conserved proteins containing SMC (structural maintenance of chromosomes) moieties that organize and compact chromosomes in an unknown mechanism essential for faithful chromosome partitioning. We show that MukBEF, the condensin in *Escherichia coli*, cooperatively compacts a single DNA molecule into a filament with an ordered, repetitive structure in an adenosine triphosphate (ATP) binding–dependent manner. When stretched to a tension of ~17 piconewtons, the filament extended in a series of repetitive transitions in a broad distribution centered on 45 nanometers. A filament so extended and held at a lower force recondensed in steps of 35 nanometers or its multiples; this cycle was repeatable even in the absence of ATP and free MukBEF. Remarkably, the pattern of transitions displayed by a given filament during the initial extension was identical in every subsequent extension. Hence, after being deformed micrometers in length, each filament returned to its original compact structure without the addition of energy. Incubation with topoisomerase I increased the rate of recondensation and allowed the structure to extend and reform almost reversibly, indicating that supercoiled DNA is trapped in the condensed structure. We suggest a new model for how MukBEF organizes the bacterial chromosome in vivo.

Chromosomes must be organized into compact structures to be correctly segregated into daughter cells. Whereas the first levels of chromosome structure have been well described [e.g., folding of DNA into nucleosomes in eukaryotes (*1*)], higher orders of DNA compaction are still not understood. Proteins that play a critical role in the organization and higher order folding of mitotic chromosomes and bacterial nucleoids are members of the SMC family called condensins (*2*–*5*).

Condensins consist of an SMC dimer and non-SMC subunits. Each SMC protomer contains, in order, an N-terminal globular domain with a Walker-A consensus sequence, a long α helix, a hinge domain, a second long α helix, and a C-terminal globular region having both a Walker-B box and an ATP-binding cassette (ABC) signature motif. This polypeptide folds back onto itself to form an antiparallel coiled coil, with the hinge at one end and a globular head domain containing the DNA and ATP-binding regions at the other end (*6*). A partial structure of the SMC protein Rad50 shows that two globular heads come together in trans to form two ATP-binding pockets and a potential DNA binding groove (*7*). Folded SMC protomers dimerize through interactions of the hinges and then associate with two or three non-SMC factors. A complete condensin typically has a molecular mass of ~600 kD (*8*). This overall architecture is conserved among diverse organisms, which suggests that it is important for function in vivo.

Despite the multiple vital roles of SMC proteins in the cell, very little is known about the molecular mechanisms by which they act. One of the best characterized SMC proteins, 13*S* condensin from *Xenopus laevis*, localizes to a central axis along the length of in vitro–assembled *Xenopus* mitotic chromosomes (*9*). It is required for the initiation and maintenance of mitotic chromosome condensation (*10*), introduces global positive writhe into naked DNA in the presence of ATP (*11*), and is a weak DNA-stimulated ATPase (*12*). Condensins with similar functions and activities have also been described in yeast (*13*, *14*), worms (*15*), and bacteria (*16*, *17*).

The *E. coli* condensin, MukBEF, consists of MukB (the SMC subunit) and MukE and MukF (*8*, *17*). Mutational inactivation of any of the

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