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## **Tubular microfossils from the Ediacaran Weng'an Biota (Doushantuo Formation, South China) are not early animals**

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### **Abstracts**

The early Ediacaran Weng'an Biota (ca. 609 Ma) of the Doushantuo Formation (Guizhou Province, China) encompasses an abundant and exquisitely preserved assemblage of phosphatic microfossils that have provided unique insight into the origin and early evolution of multicellular eukaryotes. However, the affinities of these early organisms are far from certain, including the tubular microfossils *Crassitubulus*, *Quadratitubus*, *Ramitubulus*, and *Sinocyclocylcicus*. These taxa have been widely accepted as stem-cnidarians or, alternatively, interpreted as filamentous cyanobacteria, or multicellular algae. We use high-resolution X-ray tomographic microscopy to analyse the structure and development of the four taxa. Our data and analysis allow us to conclude that these four taxa were not biomineralized.

*Crassitubulus*, *Quadratitubus*, and *Sinocyclocylcicus*, may be grouped on the basis

that they exhibit alternating complete and incomplete cross walls, and bipolar growth; which makes them favourably comparable to filamentous cyanobacteria. In contrast, *Ramitubulus* exhibits only complete cross walls, unipolar growth and dichotomous branching. These features are difficult to reconcile with a cyanobacterial interpretation. They are, instead, more indicative of multicellular algae-like Cambrian *Epiphyton*. Thus, the Weng'an tubular microfossils constitute a disparate assemblage of cyanobacteria and algae, but none represents early Ediacaran animals.

**Keywords:** Ediacaran; Doushantuo Formation; Weng'an Biota; tubular microfossils; microtomography

## 1. Introduction

The early Ediacaran Weng'an Biota of the Doushantuo Formation in Guizhou, Southwest China (ca. 609 Ma) (Zhou et al., 2017) is a unique Lagerstätte. With fossils preserved to a cellular or sub-cellular level of resolution (Hagadorn et al., 2006; Xiao et al., 2014a, 2014b; Cunningham et al., 2017; Yin et al., 2017). The Lagerstätte provides an important window to study the origin and early evolution of multicellular eukaryotes. The majority of the fossil remains are microscopic in scale and have been interpreted to represent early developmental stages. Their phylogenetic affinity and evolutionary significance, however, have been difficult to constrain because of the lack of adult remains (Xiao et al., 2014a; Cunningham et al., 2017). Because the age of the Biota falls in the time interval when early animals, based on molecular clocks, may have evolved (Erwin et al., 2011; dos Reis et al., 2015; Cunningham et al., 2016); many previous surveys attempted to identify adult metazoans from the Biota (Xiao et al., 2000; Chen et al., 2002, 2004; Liu et al., 2008; Yin et al., 2015). Among these, the tubular microfossils *Ramitubus increscens*, *Ramitubus decrescens*, *Sinocyclocyclicus guizhouensis*, *Quadratitubus orbigniatius*, and *Crassitubus costatus* have been regarded as the most credible candidates. They have been widely interpreted as cnidarians and, therefore, the oldest record of eumetazoans (Xiao et al., 2000, 2014a; Chen et al., 2002; Liu et al., 2008). However, apart from suggestions of

a close relationship with crinoids (Xue et al., 1992) or some unknown early biomineralized animals (Li et al., 1997), Liu et al. (2010) alternatively interpreted *Crassitubus*, *Sinocyclocyclicus* and *Quadratitubus* as filamentous cyanobacteria, and Cunningham et al. (2015) categorized all four genera as unspecified algae. Given the potential evolutionary significance of these tubular microfossils, we use high-resolution X-ray tomographic microscopy to characterize the three-dimensional structure of representative specimens, to reevaluate their diagnostic characteristics, and to test existing interpretations of their affinities. On this basis, we draw a distinction between *Crassitubulus*, *Quadratitubulus*, and *Sinocyclocyclicus*, which are comparable to filamentous cyanobacteria, and *Ramitubulus*, which exhibits strong similarity to multicellular algae-like *Epiphyton*. We find no evidence to support the interpretation of any of the Weng'an tubular microfossils as animals.

## 2. Material and methods

The fossils in this study were collected from the Upper Phosphate Member (or Weng'an Phosphate Member) of the Doushantuo Formation in the Weng'an phosphate mining area in Guizhou Province, Southwest China (Zhu et al., 2007; Chen et al., 2009; Cunningham et al., 2017). The Upper Phosphate Member is composed of the upper grey dolomitic phosphorite layer and the underlying lower black phosphorite layer. The fossils were recovered from the samples out of the grey interval through acetic acid digestion (7% to 10% with water). After recovery by manually picking from the acid insoluble residue, some well-preserved specimens were scanned at the Micro-CT Laboratory of Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences (NIGPAS), using a Carl Zeiss Xradia 520 Versa X-ray tomographic Microscope. The operating voltage and power settings for the X-ray tube were 55Kv and 4.5W. X-ray projections were obtained using a CCD-based 4X objective, resulting in voxel dimensions of 0.4583 to 0.5229  $\mu\text{m}$ . As most of the tubular fossils were too long for the maximum sizes of the field views under submicron resolution set up, we scanned these specimens part-by-part with the help of the "stitching" model in the "Scout-and-Scan" software of the Xradia 520

Versa (Wu et al., 2018). All the specimens figured in this paper have been deposited in NIGPAS or the Institute of Geology, Chinese Academy of Geological Sciences. Following best practice for digital morphology (Davies et al., 2017), the tomographic data arising from our study are available from Geobiodiversity Database (<http://www.geobiodiversity.com>).

### 3. Results

#### 3.1. *Ramitubus*

*Ramitubus* is the only one among the four tubular genera to display dichotomous branching, with up to four hierarchies of dichotomy in any one of the known specimens, all of which are incomplete, and the longest is 4 mm in length (Liu et al., 2008). The divergent angle between bifurcating branches ranges from 20° to 37°, with mean angle of 30° (n = 9, Figs. 1, 2) (Liu et al., 2008). *Ramitubus* occurs in clusters and always exhibits a circular cross section (Figs. 1, 2). All of the 13 specimens we studied expand in diameter in the direction of branching, with the diameter of any single branch varying from 95 to 200 µm (Liu et al., 2008). Constrictions are commonly observed on both the exterior and interior of the tube, and they can invariably be traced between sister branches of the same individual (Figs. 1C, 2A-D, arrowed). Generally, the tube diameters decrease slightly where the constrictions occur, and then the tubes grow thicker to regain the original diameter (Fig. 2A-D, F; also refer to pls. 1 and 2 in Liu et al., 2008). None of the specimens discovered so far has an enveloping sheath. Complete cross walls, flat or slightly warped, were observed in well-preserved specimens (Figs. 1E, I, 2F-H; Movie S1 in the supplementary data), arranged regularly with an average spacing of ~12.4 µm along the long axis of the tube (Liu et al., 2008). However, cross walls are commonly incompletely preserved as opposed to incompletely developed (Fig. 1J-L) (Cunningham et al., 2015). The taphonomic basis of these incompletely preserved cross walls is evidenced by the fact that they define vugs that are occupied by void-filling cement (Fig. 1J, L) and do not exhibit the patterning of alternating complete

and incomplete cross walls seen in the other tubular taxa of the Weng'an Biota (Cunningham et al., 2015). For example, one specimen exhibits secondary cavities with irregular margins and void-filling cement (Fig. 1J); cross walls are visible at the margins of the tube but they are truncated in the middle. In two other specimens, the cross walls are all-but absent, preserving just the base of the cross walls in places, the remaining space occupied by phosphatic filaments or diagenetic linings (Fig. 1K, L).

### 3.2. *Sinocyclocyclicus*

*Sinocyclocyclicus* has a straight, non-branching form with a circular cross section (Fig. 3A, B). Some specimens preserve blunt terminations (Fig. 3C). The outer sheath is smooth, and the inner cross walls can be observed when the smooth external sheath is lost or broken (Fig. 3A, E). Tube diameter ranges from 132 to 272  $\mu\text{m}$  and the maximum reported length is 1186  $\mu\text{m}$  (Liu et al., 2008). Cross walls are regularly spaced and approximately planar, except where they curve gently to meet the wall of the tube (Fig. 3D, F). Cross walls alternate between complete and incomplete, the latter occurring in two ranks, the first of which extends to approximately half the radius, while the second-rank walls find their limit between those of the first-rank incomplete cross walls (Fig. 3D, F). Brittle deformation of cross walls is apparent in some specimens (e.g., fig. 1b and e in Cunningham et al., 2015).

### 3.3. *Quadratitubus*

*Quadratitubus* has a straight, non-branching form (Fig. 3G, H), with an approximately square cross section with rounded corners (Fig. 3J). The diameter (i.e., length of each side in cross section) varies from 160 to 250  $\mu\text{m}$  (Liu et al., 2008). The tube surface is smooth when an enveloping sheath with thickness around 3–5  $\mu\text{m}$  is preserved (Liu et al., 2008), or annulated when the sheath is absent (Fig. 3H). Cross walls are regularly spaced and alternate between complete and incomplete (Fig. 3I, K). Brittle deformation of the cross walls is not uncommon (e.g., pl. 7, fig. 10 in Liu et al., 2008; fig. 1d and f in Cunningham et al., 2015), but ductile deformation is rarely observed (refer to fig. 6C in Liu et al., 2008). In some specimens, sparse

constrictions can be observed from exterior of the tube when the outer sheath is not preserved (Fig. 3H, I, K, arrows), but the tube diameter does not change around the constricted area; which is different from *Ramitubus*.

#### 3.4. *Crassitubus*

*Crassitubus* is non-branching and has a circular cross section as well as an enveloping sheath, but it differs from *Sinocyclocyclicus* in having a curved, twisted, or even a knotted configuration (Fig. 3L-N). Tube diameter ranges from 140 to 220  $\mu\text{m}$  and most specimens are less than 1mm in length (Liu et al., 2008). The enveloping sheath is thicker than in *Sinocyclocyclicus* and *Quadratitubus*, and a multi-laminated structure can be observed in well-preserved specimens (Fig. 3O-Q). In some but not all the specimens, one or two longitudinal ridges can be observed on the outer surface of inner tube when the enveloping sheath is absent (Fig. 3M, N; refer to pl. 6, figs. 3, 7 in Liu et al., 2008; fig. 3e and f in Cunningham et al., 2015). Like *Sinocyclocyclicus* and *Quadratitubus*, *Crassitubus* exhibits regularly spaced and alternating complete and incomplete cross walls (Fig. 3Q). Constrictions also occur in this taxon, and like *Quadratitubus*, the tube diameter does not change around the constriction (Fig. 3Q). At high resolution, the tube appears to have divided into two parts at the point of constriction, and the dividing end of the tube turned to blunt (Fig. 3Q), similar to the blunt end of *Sinocyclocyclicus* (Fig. 3C).

#### 4. Discussion

The phylogenetic affinities of the Weng'an tubular microfossils are far from resolved. They were originally interpreted as crinoid stems and arms, or components of the variably enigmatic Cambrian small shelly fauna. They have also been considered stem- or crown-cnidarians, filamentous cyanobacteria, or other alga (Xue et al., 1992; Li et al., 1997; Xiao et al., 2000; Chen et al., 2002; Liu et al., 2008, 2010; Cunningham et al., 2015).

Xiao et al. (2000) compared these fossils with Ordovician tabulates and interpreted them as possible stem-cnidarians based principally on their tubular

morphology and the presence of cross walls. They highlighted the gregarious habit of the tubes and suggested that the fossils were benthic colonial organisms; they viewed the branches of *Ramitubulus* as a result of asexual reproduction by budding, and compared the cross walls observed in all of the tubular taxa to the tabulae of tabulate corals (Xiao et al., 2000). However, *Crassitubulus*, *Quadratitubus*, *Ramitubulus*, and *Sinocyclocyclicus*, do not appear to have been biomineralized in life, as evidenced by ductile deformation (Liu et al., 2008; Cunningham et al., 2015) and the incomplete preservation through secondary phosphatization of cross walls in all of the taxa. The presence of brittle fracturing in some specimens, which inspired Cunningham et al. (2015) to conclude that at least some of the taxa were biomineralized, can be dismissed on the observation that cellular and subcellular soft tissue remains in the Weng'an Biota also sometimes exhibit brittle deformation, which indicates a post mortem, post-fossilization phenomenon (Liu et al., 2008). The absence of biomineralization in *Ramitubus*, *Sinocyclocyclicus*, *Quadratitubus*, and *Crassitubus*, is therefore significant, since it is inconsistent with the condition in tabulate corals. Finally, Xiao et al. (2000) supported their cnidarian interpretation for these tubular taxa on the presence of a large terminal chamber in one specimen (refer to fig. 2D in Xiao et al., 2000), which they compared to tabulate calices. However, this specimen is broken and this end of the fossil clearly does not represent the biological termination of the living organism. Void spaces occur in various areas of the Weng'an tubes (Fig. 11-L), but their irregular truncation of cross walls demonstrates that they result from decay and incomplete fossilization, making the cnidarian interpretation ever more unlikely.

While most previous researchers have considered all of the Weng'an tubular fossils as comprising an essentially homogenous group, Liu et al. (2010) established new taxa and drew a distinction between *Ramitubus* versus *Sinocyclocyclicus*, *Quadratitubus* and *Crassitubus* which they compared to filamentous cyanobacteria. *Sinocyclocyclicus*, *Quadratitubus* and *Crassitubus* were distinguished by (1) a blunt apical termination which is particularly clear in *Sinocyclocyclicus* and *Quadratitubus*; (2) a smooth enveloping sheath (especially thick and laminated in *Crassitubus*); (3)



alternately arranged complete and incomplete cross walls; and (4) unique constrictions suggesting tube division in *Quadratitubus* and *Crassitubus*. All of these features are compatible with modern filamentous cyanobacteria, supporting a cyanobacterial affinity of the three genera. Nonetheless, the cnidarian interpretation of *Ramitubus* remained (Liu et al., 2010), following Xiao et al. (2000), Chen et al. (2002), and Liu et al. (2008).

Cunningham et al. (2015) interpreted *Crassitubus*, *Quadratitubus*, *Ramitubus* and *Sinocyclocyclicus* as a largely homogeneous group, though they considered the tubes of *Quadratitubus* and *Sinocyclocyclicus* to have been more rigid than those of *Crassitubus* and *Ramitubus*. Our evidence suggests that this distinction is a taphonomic artefact of post mortem, post-fossilization compaction. Cunningham et al. (2015) observed so-called ‘cell clusters’ at terminal positions in tubes of *Crassitubus*, and polygonal granule-like structures between the cross walls of *Sinocyclocyclicus*, drawing comparison to red algae, such as the coralline alga *Amphiroa*, and green algae *Spirogyra*.

The competing hypotheses of affinity for the tubular fossils are distinguished by their interpretation of the cross walls and whether or not the four genera are closely related. The shared presence of cross-walls is critical to constraining their phylogenetic assignment. The cross walls with diameters ranging from 130 to 270  $\mu\text{m}$  are too small to bear comparison to the tabulae of tabulate corals. The arrangement of alternating complete and incomplete cross walls is also inconsistent with the tabulae of tabulates. Furthermore, tabulate corals exhibit  $\text{CaCO}_3$  biomineralization, while our analysis indicates that the tubes were unmineralised *in vivo*. Even if we were to assume that tabulates (perhaps anthozoans in general) had a non-biomineralizing Neoproterozoic history, there is no credible evidence for the polyp chamber that inspired the cnidarian interpretation in the first instance. The remaining interpretation of the cross-walls is that they represent cell boundaries (Liu et al., 2010; Cunningham et al., 2015).

There is no material evidence to support a common affinity for all four of the tubular taxa other than their tubular gestalt and co-occurrence. Our results reveal that

*Crassitubus*, *Quadratitubus*, and *Sinocyclocliticus* are similar in that they are linear, have enveloping sheaths and blunt terminations, and exhibit regularly spaced complete and incomplete cross walls, whereas in *Ramitubus* the cross walls are always complete, and exhibit polarized dichotomous branching (Table 1). The blunt apical tube ends of *Sinocyclocliticus*, *Quadratitubus* and *Crassitubus* indicate they were not attached to a surface, whereas the unipolar growth of *Ramitubus* indicates that it was an epibenthic organism. The branching pattern and variation in stem diameter suggest that in *Ramitubus*, the tube grew by terminal addition. In contrast, the alternating complete and incomplete cross walls of *Crassitubus*, *Quadratitubus*, and *Sinocyclocliticus* are compatible with a pattern of intercalary growth in which the incomplete cross walls reflect incipient development of complete cross walls.

Liu et al. (2010) compared *Crassitubus*, *Quadratitubus*, and *Sinocyclocliticus*, with filamentous cyanobacteria, based on their non-branching configuration, thick multilaminated enveloping sheath, uniform diameter, constriction-like narrow gaps between hormogonia-like tubes and blunt apical ends. All of these features are compatible with living cyanobacteria such as *Oscillatoria kawamurae*. Liu et al. (2010) interpreted the complete cross walls as cell boundaries, and the incomplete cross walls as reflecting the mechanism of cell division that is common in cyanobacteria (Carr and Whitton, 1973). In particular, the narrow gap seen in the tubes of *Quadratitubus* (Fig. 3K) and *Crassitubus* (Fig. 3Q) can be compared with the separation disk between two hormogonia of living *Oscillatoria* cyanobacteria, in which a short trichome slides off from a parental filament to form an independent daughter filament. This reflects an asexual mode of reproduction in cyanobacteria.

The thin ridges observed on the surface of *Crassitubus* (Fig. 3M, N) cannot readily be interpreted under a cyanobacterial model, if they were biological in origin. They could be diagenetic artefacts, reflecting cements or sediment deposited along fractures of the thick enveloping sheaths that were developed secondarily along the long axis of the tubes. This may explain why the ridges can only be observed in some, but not all of the specimens, and the number of the ridges varies among specimens. The non-branching tubes are thicker than extant cyanobacteria, since the diameter of

living filamentous cyanobacteria is no more than 80  $\mu\text{m}$  or so (Demoulin and Janssen, 1981; Schulz and Jorgensen, 2001; Bengtson et al., 2017), while the diameters of *Crassitubus*, *Quadratitubus* and *Sinocyclocyclicus* range between 130 to 270  $\mu\text{m}$  (Liu et al., 2008). However, the limited size difference between them is not a barrier to uniting them in affinity, because the diameters of these tubular fossils are still within the size range of living bacteria. For example, individual cells of the living filamentous sulfur bacteria *Thiomargarita namibiensis* achieve very large size, typically ranging from 100 to 300  $\mu\text{m}$  in diameter, and can be up to 750  $\mu\text{m}$  in diameter (Schulz et al., 1999). Furthermore, giant filamentous cyanobacteria with diameters ranging from 113 to 614  $\mu\text{m}$ , have been reported from the Neoproterozoic (Tonian) Liulaobei Formation (Pang et al., 2018).

The affinity of *Ramitubus* must be distinct. The patterns of polarized terminal growth and dichotomous branching exclude it from a cyanobacterial affinity, and we have already presented evidence precluding a cnidarian interpretation. As a matter of fact, the branching morphology and growth mode of *Ramitubus* strongly resembles branching Cambrian *Epiphyton*, an enigmatic group of benthic, colonial reef building organisms that have not only been compared to the living red alga *Corallina* (Riding and Toomey, 1972; Luchinina and Terleev, 2008), but also to cyanobacterial calcimicrobes (Pratt, 1984; Zhang et al., 2019). The angle between sister branches of *Epiphyton* ranges 20° to 37°, similar to that of *Ramitubus*. *Epiphyton* also resembles *Ramitubus* in its circular cross section and the degree of diameter variation along the length of a single colony (38 to 98  $\mu\text{m}$ ) (Figs. 1, 2). Moreover, the horizontal to slightly concave cross walls in *Epiphyton* are closely comparable to the complete cross walls in *Ramitubus* (Fig. 2H, I), as is the mode of branching in which large cells are succeeded by paired smaller cells at the point of ramification. Despite a modest size difference, these features strongly support an interpretation of *Ramitubus* as an early Ediacaran relative of Cambrian *Epiphyton*. Although the systematic position of *Epiphyton* remains controversial, the available evidence suggests that the affinity of *Ramitubus* lies with multicellular algae rather than with animals.

## 5. Conclusions

There is no credible evidence to support a metazoan affinity for the Weng'an tubular microfossils *Crassitubus*, *Quadratitubus*, *Ramitubus*, and *Sinocyclocyclicus*. The tabulae identified in previous studies are, rather, cell boundaries, and the putative polyp chamber is an artefact of decay and incomplete fossilization. We attribute these taxa to two principal groups: non-branching *Sinocyclocyclicus*, *Quadratitubus* and *Crassitubus*, and dichotomously branching *Ramitubus*. The non-branching group exhibits evidence of bipolar growth through binary cell division, manifest as incomplete cross walls that alternate with complete cross walls; we interpret them as cyanobacteria and they would have propagated asexually by forming hormogonia. Branching *Ramitubus* was benthic and colonial, growing by terminal addition; it compares closely to Cambrian *Epiphyton* and likely represents a multicellular alga.

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## Figure captions

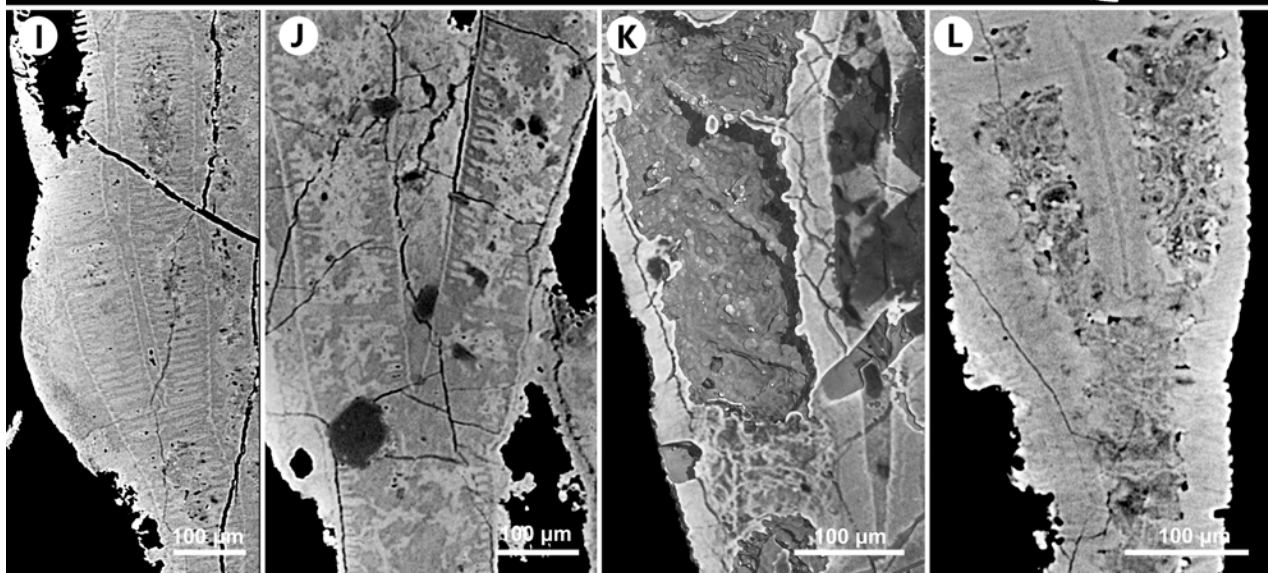
Fig. 1. Images of the Ediacaran tubular microfossil *Ramitubus increescens*. (A-D) Surface renderings of four specimens; the arrows in (C) indicate constrictions. (E-H) Digital sections of the specimens shown in (A-D), displaying internal structures. (I-L) Close-up views of the areas marked by frames in (E-H), respectively, showing more detail of the internal structures.

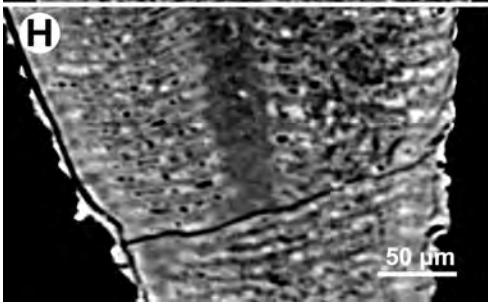
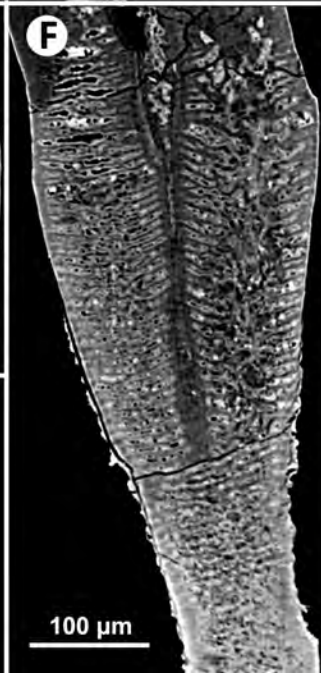
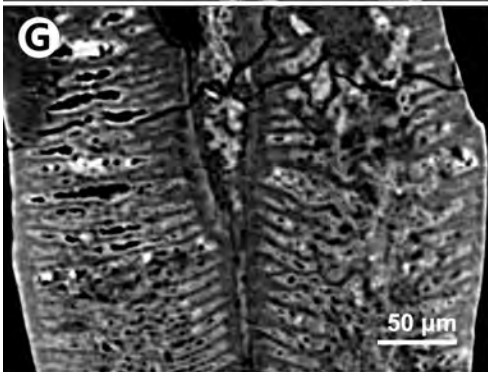
Fig. 2. Ediacaran tubular microfossil *Ramitubus* and Cambrian tubular microfossil *Epiphyton*. (A-H) Images of Ediacaran *Ramitubus*; (A-C) surface renderings from



different views; (D) a digital slice of (C); the arrows in (A-D) indicate constrictions; (E) a virtual 3D section through (C), showing internal structures; (F) a close-up view of framed area in (D); (G, H) enlarged views of the branching area in (F), showing more detail of internal structures. (I) An image of *Epiphyton* cited from Luchinina and Terleev (2008, pl. 1, fig. 2a), showing cellular structures.

Fig. 3. Ediacaran non-branching tubular microfossils from the Weng'an biota. (A-F) *Sinocyclocyclicus guizhouensis*; (A, B) SEM images; (C, D) transmitted optical micrographs of thin sections, showing regular spaced cross walls; (E) a close-up view of the framed area in (A), showing the thin outer wall; (F) a close-up view of the framed area in (D), the lines indicate the alternating complete and incomplete cross walls. (G-K) *Quadratitubus orbigniatius*; (G, H) SEM images of two specimens; (I) thin section of the specimen in (H), the arrows in (H, I) indicate constrictions; (J) cross section of the specimen in (G); (K) a close up view of the framed area in (I), showing detail of the constriction structure. (L-Q) *Crassitubus costatus*; (L, M) SEM images of two specimens; (N) a synchrotron radiation X-ray tomographic microscopic image of *Crassitubus costatus* cited from Cunningham et al., 2015 (fig. 3e in Cunningham et al., 2015); the arrow heads in (M, N) indicate the thin ridges; (O, P) transmitted optical photomicrographs of two specimens; (Q) a close-up view of the framed area in (O), showing the enveloping sheath and the constriction structure (arrows).







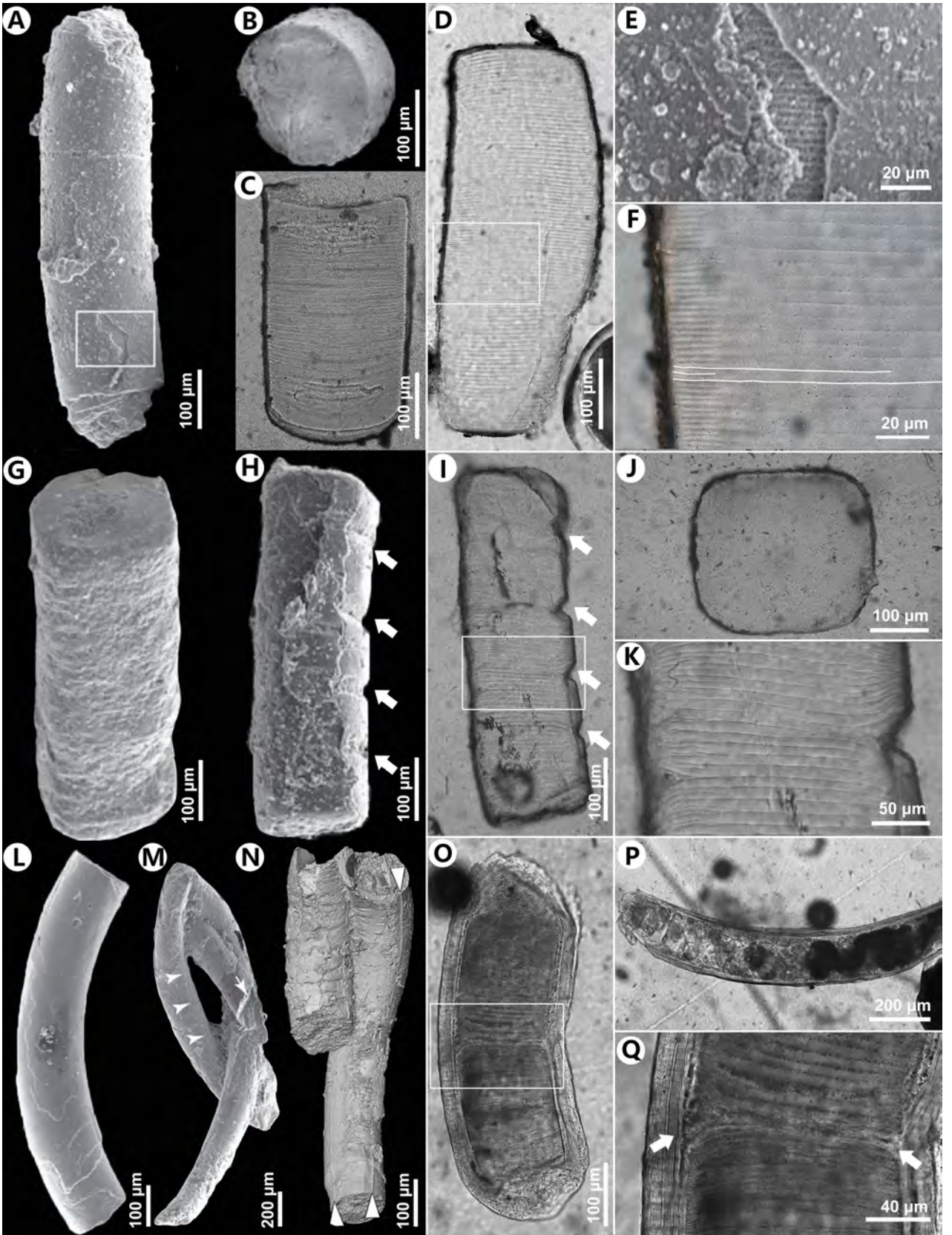


Table 1. Biological features of Ediacaran tubular microfossils from the Weng'an Biota, Cambrian fossil *Eipiphyton* and extant cyanobacteria.

Notes: ● means “Yes”, ○ means “No”.

Taxa		Shape of cross section	Diameter (μm)	Branching form	Enveloping sheath	Complete cross wall	Incomplete cross wall	Unipolar growth	Bipolar growth
Tubular fossils	<i>Ramitubus</i>	circle	95-200	●	○	●	○	●	○
	<i>Sinocyclocyclicus</i>	circle	132-272	○	●	●	●	○	●
	<i>Quadratitubus</i>	quadrangular	160-250	○	●	●	●	○	●
	<i>Crassitubus</i>	circle	140-220	○	●	●	●	○	●
Cambrian <i>Eipiphyton</i>		circle	56-121	●	○	●	○	●	○
Extant cyanobacteria		circle	<80	○	●	●	●	○	●