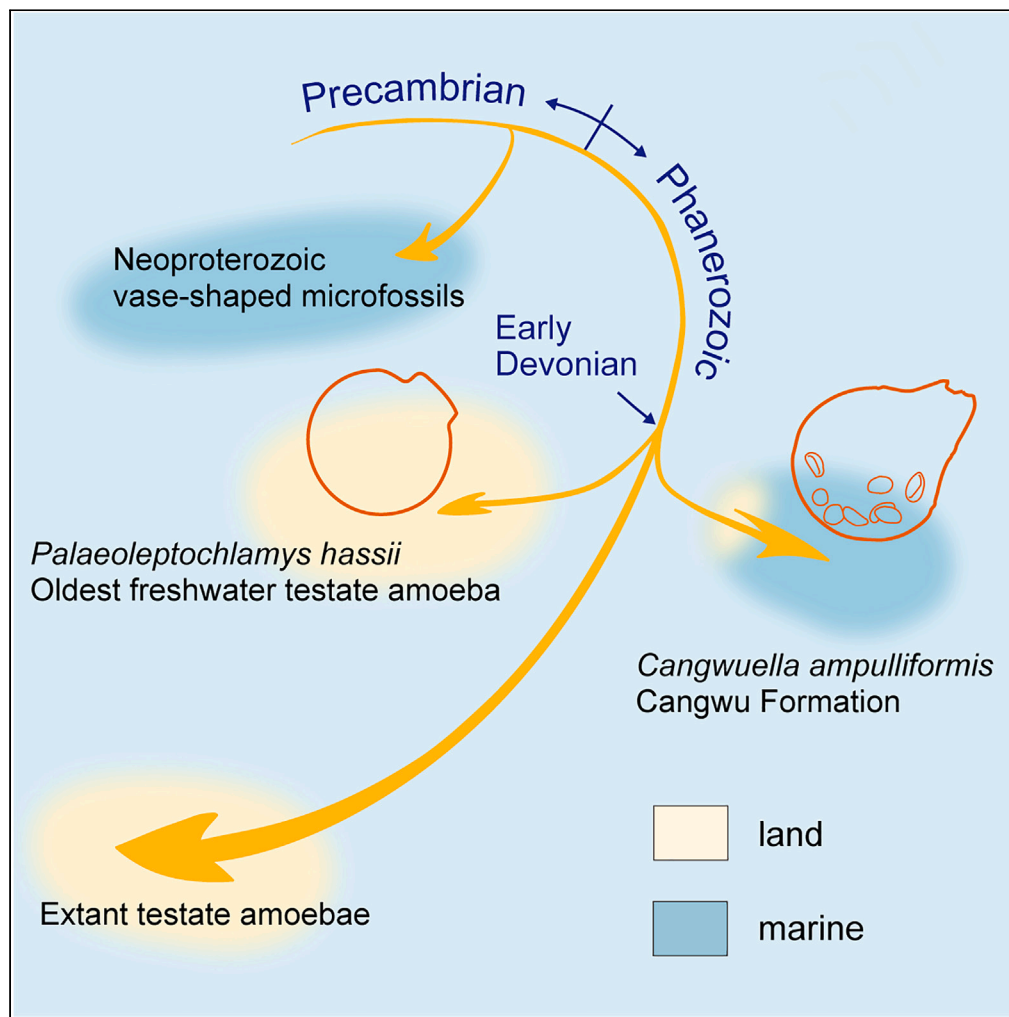


## Article

## Shallow-marine testate amoebae with internal structures from the Lower Devonian of China



Kai Wang, Hong-He Xu, Bing-Cai Liu, ..., Peng Tang, Jian-Feng Lu, Yi Wang

hhxu@nigpas.ac.cn

**Highlights**

A fossil species of Arcellinida is described from the Lower Devonian of China

Smooth-walled acetabuliform structures are shown in the shell of this testate amoeba

Shallow-marine arcellinid testate amoebae occurred during the Early Devonian

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## Article

## Shallow-marine testate amoebae with internal structures from the Lower Devonian of China

Kai Wang,<sup>1,2</sup> Hong-He Xu,<sup>1,4,\*</sup> Bing-Cai Liu,<sup>1,2</sup> Jiao Bai,<sup>1,2</sup> Yao Wang,<sup>3</sup> Peng Tang,<sup>1</sup> Jian-Feng Lu,<sup>1</sup> and Yi Wang<sup>1</sup>

## SUMMARY

Testate amoebae, a polyphyletic protist group inhabiting a wide variety of extant ecosystems, have evolved as far back as early Neoproterozoic. However, their fossil record is discontinuous and biased toward empty shells. Here, we report an arcellinid testate amoeba species, *Cangwuella ampulliformis* gen. nov., sp. nov., from a shallow-marine community in the Early Devonian of Guangxi, southwestern China. With the aid of scanning electron microscopy and X-ray micro-tomography, we find that the shell of our testate amoeba contains some acetabuliform structures. Although such configuration does not match exactly with the known internal structures in extant testate amoebae, our fossils highlight the potential of exploring the ecological relationships between fossil testate amoebae and their associated organisms, and increase our knowledge on the diversity of testate amoebae in Early Devonian environments.

## INTRODUCTION

Protists, a widespread form of microcosmic life, play a crucial part in earth's biosphere.<sup>1</sup> Among these microbial eukaryotes, testate amoebae stand out with their unique test (shell), usually 20–150 μm in size, and in the tree of eukaryotic life, are known as a polyphyletic group that consists of three unrelated taxonomic lineages, including Amoebozoa, Cercozoa and Stramenopiles,<sup>2,3</sup> within which the species of Arcellinida in Amoebozoa account for the vast majority of testate amoeba population. As free-living protists, extant testate amoebae are distributed in most kinds of freshwater or terrestrial habitats, e.g., river, pond, peatland and soil, but as well as in brackish and marine waters,<sup>4</sup> and are of great significance to the flow of matter and energy in ecosystems.<sup>5,6</sup>

It has been generally considered that the evolutionary history of testate amoebae could be traced back to the late Tonian (early Neoproterozoic, ca. 800–720 Ma), as evidenced by the extensive records of marine vase-shaped microfossils in this period.<sup>7–12</sup> However, not much is known about the Paleozoic testate amoeba fossils, as of now. The oldest unambiguous occurrence of Paleozoic testate amoebae is *Palaeoleptochlamys hassii*, a freshwater arcellinid species from the Lower Devonian Rhynie chert,<sup>13</sup> followed by scattered reports from the upper Carboniferous and Permian.<sup>14–17</sup> There is also dearth of knowledge about the internal structures of fossil testate amoebae. Except few possible reproductive cells or resting cysts in phosphatized or amber-preserved specimens,<sup>18,19</sup> no other bio-related structures inside the shells of fossil testate amoebae have been observed. Here, we report some exquisitely preserved vase-shaped microfossils from the Lower Devonian Cangwu Formation in Guangxi, southwestern China (Figures 1 and S1), and based on morphological characters, interpret them as a new fossil species of arcellinid testate amoebae. Additionally, we show distinct acetabuliform structures preserved in the shell of our testate amoeba, infer possible sources of these internal structures, and discuss implications of our fossils for the evolution of testate amoebae.

## RESULTS AND DISCUSSION

## Systematic paleontology

## Order

Arcellinida Kent 1880.

## Family

Incertae sedis.

<sup>1</sup>State Key Laboratory of Palaeobiology and Stratigraphy, Nanjing Institute of Geology and Palaeontology and Center for Excellence in Life and Palaeoenvironment, Chinese Academy of Sciences, Nanjing 210008, China

<sup>2</sup>University of Chinese Academy of Sciences, Beijing 100049, China

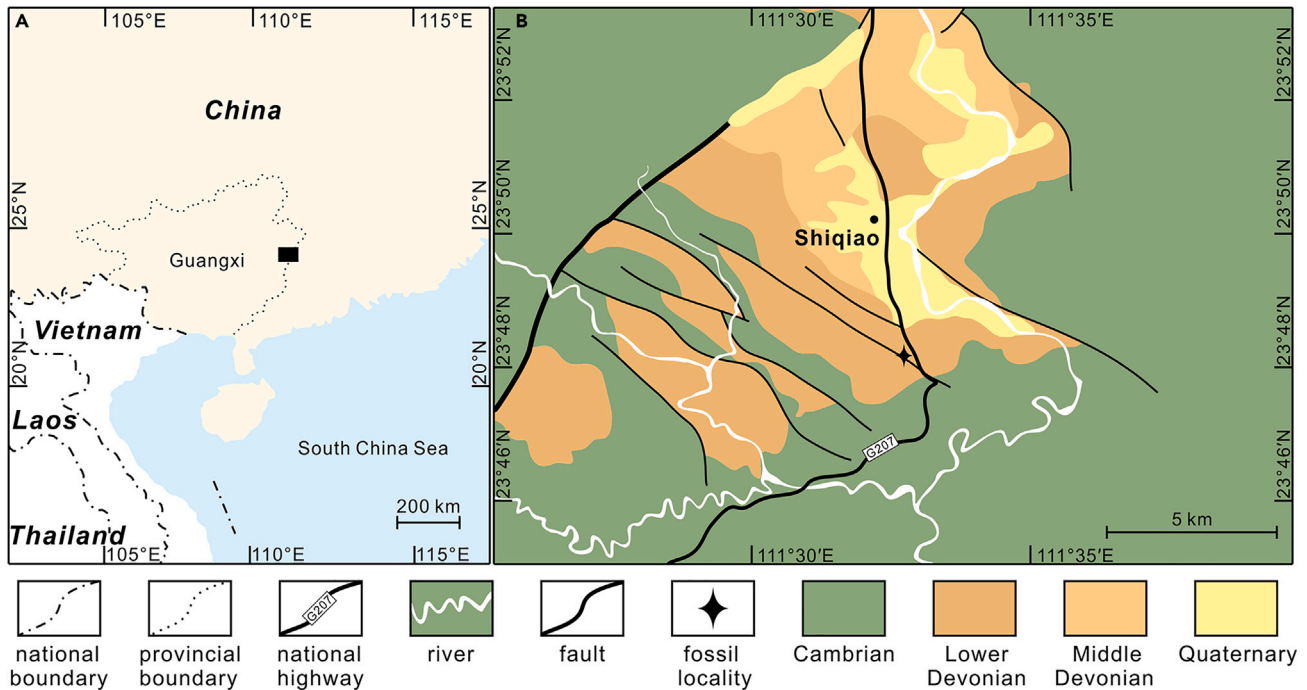
<sup>3</sup>School of Geography and Tourism, Qufu Normal University, Rizhao 276826, China

<sup>4</sup>Lead contact

\*Correspondence: hhxu@nigpas.ac.cn

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**Figure 1. Geographic and geologic map of the fossil locality**

(A) Geographic map of Guangxi Zhuang Autonomous Region, southwestern China. Black rectangle shows the general location of the studied area. (B) Geologic map of the southern part of the Shiqiao Town, showing the outcrops of Lower Devonian and the sampling locality.

### Genus

*Cangwuella* gen. nov.

### LSID (Life Science Identifier)

urn:lsid:zoobank.org:act:05F5D18F-E38F-4C01-BC86-B08D275799F2.

### Type species

*Cangwuella ampulliformis* sp. nov.

### Etymology

Named for its occurrence in the Cangwu Formation.

### Diagnosis

Vase-shaped shells consist of a bulbous chamber and a noticeably protruding neck. The length and width of chamber are almost equal. Shell wall shows honeycomb structure in the cross section, and is rigid, organic, never or very slightly compressed in the aperture view, without the appearance of scales, granules or other ornaments. Neck gradually tapers toward the top. Aperture is circular to oval, with a slightly undulated margin.

### Species

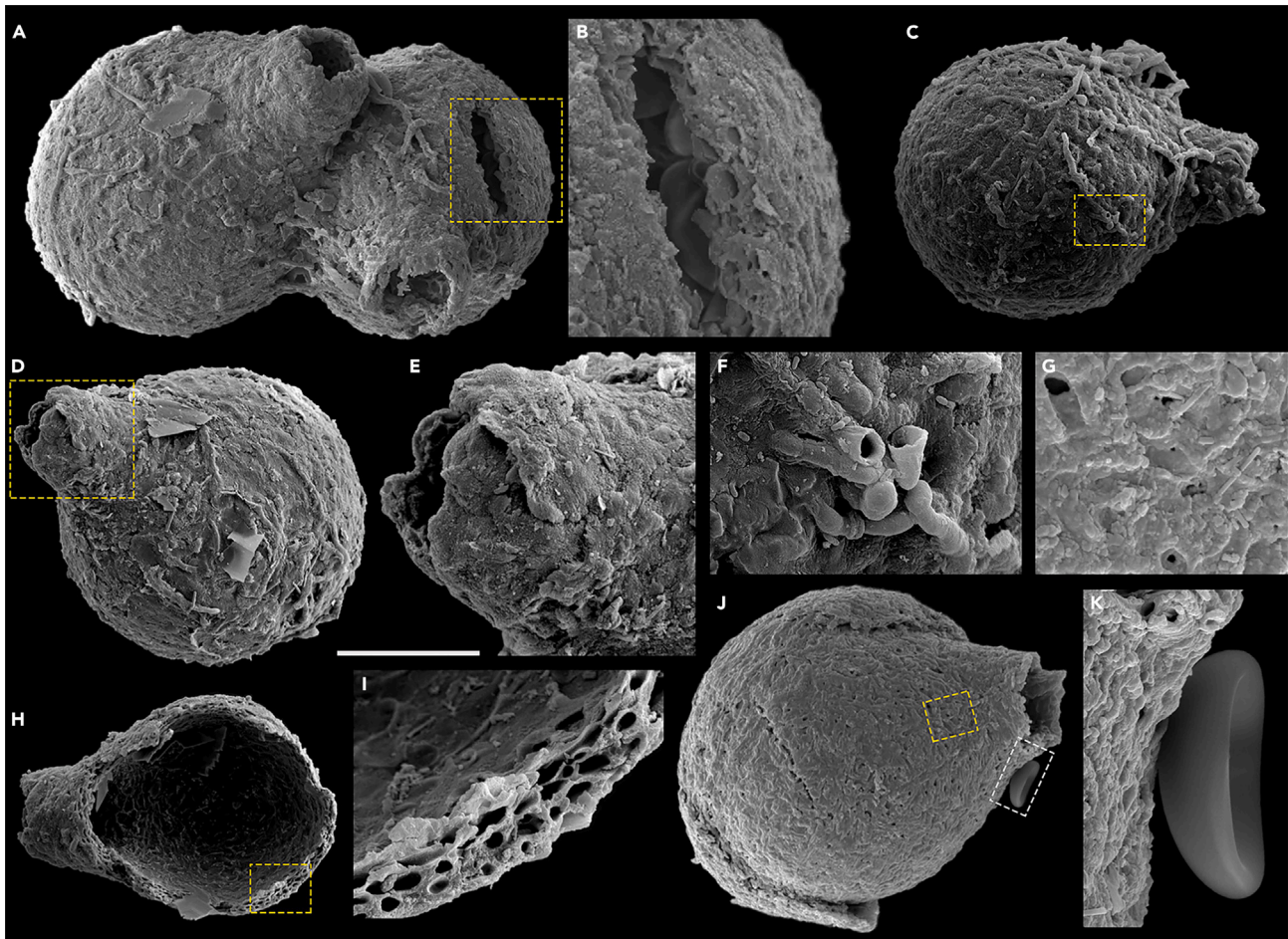
*Cangwuella ampulliformis* sp. nov.

### LSID

urn:lsid:zoobank.org:act:665ED98A-C122-4845-A16F-7A3EB7018548.

### Holotype

NIGP 179657 (Figure 2D).



**Figure 2. Scanning electron microscopy photographs of *Cangwuella ampulliformis* gen. nov., sp. nov. from the Cangwu Formation in Guangxi, southwestern China**

- (A) Two attached tests with a laceration on the shell of the right one, NIGP 179653.  
 (B) Close-up of the yellow in Figure 2A, showing the acetabuliform structures preserved inside the shell.  
 (C) A test covered with filaments, NIGP 179648.  
 (D) A test with a well-preserved aperture, holotype, NIGP 179657.  
 (E) Close-up of the yellow box in Figure 2D, highlighting the undulating margin of the aperture.  
 (F) Close-up of the yellow box in Figure 2C, showing that the filaments are hollow.  
 (G) Close-up of the yellow box in Figure 2J, showing the 'pores' on the shell wall surface.  
 (H) A test with a broken chamber, paratype, NIGP 179644.  
 (I) Close-up of the yellow box in Figure 2H, showing the honeycomb structure, which is composed of three layers.  
 (J) A test with an individual acetabuliform structure falling out of the chamber, near the aperture, NIGP 179658.  
 (K) Close-up of the white box in Figure 2J, showing the acetabuliform structure. Scale bar is 50  $\mu\text{m}$  for Figures 2A, 2C, 2D, 2H and 2J; 20  $\mu\text{m}$  for Figures 2B and 2E; 10  $\mu\text{m}$  for Figures 2F, 2G, 2I and 2K.

### Paratypes

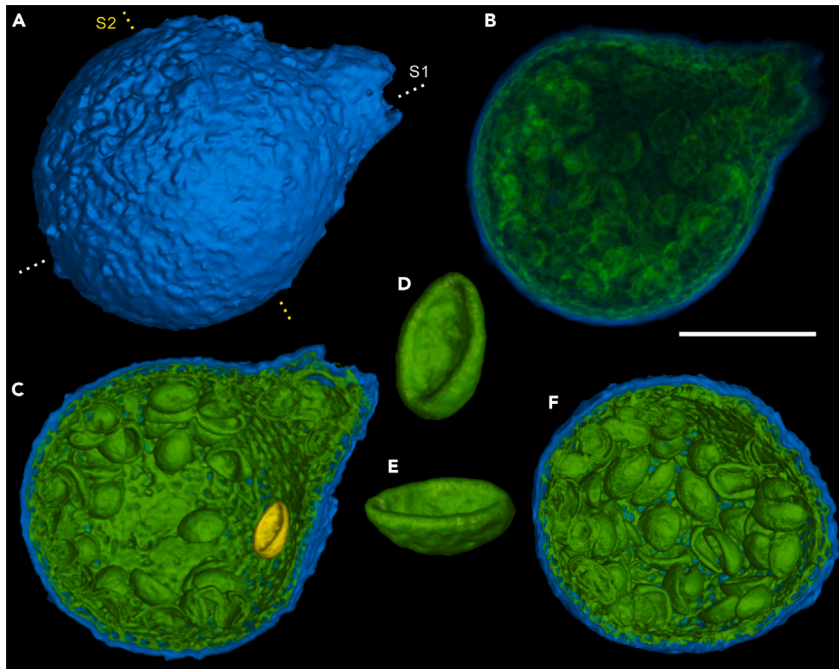
NIGP 179644 (Figure 2H) and NIGP 179659 (Figures 3A and S2A).

### Repository

Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences, with serial numbers and prefix NIGP.

### Etymology

From the Latin *ampulliformis*, referring to the vase-shaped shells.



**Figure 3. Three-dimensional reconstruction of *Cangwuella ampulliformis* gen. nov., sp. nov., based on synchrotron radiation X-ray tomography**

(A) Lateral view, with S1 and S2 marking the location of slice views in Figures 3C and 3F respectively, paratype, NIGP 179659.

(B) Transparent view of Figure 3A, showing the acetabuliform structures preserved inside the shell.

(C) View from S1 in Figure 3A.

(D) Proximal (vertical) view of the yellow structure in Figure 3C, showing the oval shape.

(E) Equatorial (lateral) view of the yellow structure in Figure 3C, showing the round-bottomed acetabuliform shape.

(F) View from S2 in Figure 3A. Scale bar is 50  $\mu\text{m}$  for Figures 3A, 3B, 3C and 3F; 20  $\mu\text{m}$  for Figures 3D and 3E.

#### Locality and horizon

Cangwu County, Guangxi Zhuang Autonomous Region, China; the Shiqiaofeng Member, Cangwu Formation (?Pragian-Emsian, Lower Devonian).

#### Diagnosis

As for the genus.

#### Description

Test is vase-shaped, consisting of a basal bulbous chamber and an upper neck (Figures 2, 3, and S2). Test is 100–147  $\mu\text{m}$  long and 64–112  $\mu\text{m}$  wide, slightly compressed in lateral view and bilaterally symmetrical. Test outer surface is rough and always covered with filaments (Figures 2A, 2C, S2C, and S2G). The filaments are intertwined and attached to the test. Fracture of the filaments shows that they are hollow tubes (Figure 2F). Some "pores", 0.4–1.3  $\mu\text{m}$  in diameter ( $n = 10$ ), can be recognized on the test (Figure 2G), but with no evidence of directly penetrating the test walls. No embedded scales, particles spines and other typical ornaments are seen on test. Neck is hollow, extends from chamber with a wide base (the connection between the neck and the chamber) and communicates with the chamber, showing a gradually tapering shape from the basal portion to the top. Aperture is the only natural opening of the whole test and located at the end of the neck. A more completely preserved specimen shows that the aperture possesses undulating margin without collar (Figures 2D and 2E). The wall of the test chamber, seen from a broken specimen (Figure 2H), is ca. 5  $\mu\text{m}$  thick with a reticular inner surface and three-layered honeycomb structure (Figure 2I). Small smooth-walled structures are seen inside the test chamber (Figures 2B, 3, and S3–S5; Video S1) or near the aperture (Figures S2E and S2H). The individual form is 17–23  $\times$  10–13  $\mu\text{m}$  in size ( $n = 17$ ), oval in proximal (vertical) view (Figure 3D), and round-bottomed acetabuliform in equatorial (lateral) view (Figure 3E). Detailed morphometric data of new taxon is shown in Table 1.

**Table 1. Morphometric data of *Cangwuella ampulliformis* from the Cangwu Formation (Pragian to Emsian, Early Devonian) in Guangxi, southwestern China (measurements in  $\mu\text{m}$ )**

Character	Min	$\bar{x}$	Max	M	SD	SE	CV	n
shell length	100	123.1	147	118	15.17	4.80	12.33	10
shell (chamber) width	64	94.4	112	95.4	13.58	3.63	14.39	14
chamber length	71	90.0	107	91	10.49	3.32	11.66	10
neck length	20	34.1	55	30.3	9.80	2.96	28.78	11
neck width	34	43.8	51	44.3	6.46	1.95	14.75	11
aperture width	18	27.1	38	27.2	5.85	1.76	21.60	11
shell width/shell length	0.63	0.72	0.80	0.73	0.05	0.02	7.12	10
shell width/chamber length	0.89	0.98	1.08	0.99	0.06	0.02	6.15	10
neck length/shell length	0.18	0.27	0.39	0.27	0.06	0.02	21.01	10
neck width/neck length	0.91	1.34	1.72	1.41	0.22	0.07	16.75	11
neck width/shell width	0.40	0.49	0.64	0.48	0.07	0.02	13.64	11
aperture width/neck width	0.37	0.54	0.63	0.58	0.09	0.03	16.61	8

Where the morphometric data were measured on specimens is shown in [Figure S1C](#).

Notes: Min, minimum;  $\bar{x}$ , arithmetic mean; Max, maximum; M, median; SD, standard deviation; SE, standard error of the arithmetic mean; CV, coefficient of variation, %; n, the number of measured specimens.

### Comparisons

As of now, the coeval testate amoeba fossils are only known from the Lower Devonian Rhynie chert, in which several vase-shaped specimens were found and described as *Palaeolectochlamys hassii*—the earliest freshwater testate amoeba.<sup>13</sup> The Rhynie material was observed in a thin section of permineralized silica, whereas our specimens were found in the organic remains extracted from clastic rocks using palynological technique. *P. hassii* is similar to our new taxon in chamber shape, which is bulbous or spheroidal, and never or very slightly compressed in apertural view. The wall of both species is rough, rigid and organic, without any evidence of exogenous scales or particles. It is reported that in light microscopy, the Rhynie testate amoebae are brighter in color and more translucent than sporopollenin-coated plant spores<sup>13</sup>; our fossils, in turn, appear opaque under transmitted light. This indicates that either the chemical composition (though unknown) or thickness of the organic shell wall might be different between the Rhynie material and our fossils. One thing to point out is that although specimens of *P. hassii* are found among the remains of cyanobacterial filaments and banded tubes of nematophyte (possibly fungus-derived), they show no filamentous attachments on their shell<sup>13</sup>; however, in some cases, there are many filaments attached to our shells.

More obvious contrasts between these two arcellinid amoeba taxa lie in size, neck, aperture and internal structure of shell. *Palaeolectochlamys*, generally smaller ( $54 \times 50 \mu\text{m}$  in dimension) than *Cangwuella*, has a very short neck (no more than 1/14 of its shell length) and a distinct aperture with an internally reinforced collar, which is clearly absent in our specimens. Furthermore, no biogenic remains are discerned inside the shell of *Palaeolectochlamys*, whereas our fossils contain many acetabuliform structures.

### Justification for the affinity of new vase-shaped microfossils

The vase-like shape of our specimens is reminiscent of chitinozoans, a widely occurring Ordovician to Devonian group of organic-walled microfossils with a hollow vesicle consisting of a distinct chamber and neck. Chitinozoans have a series of diagnostic surface appendages on the flank and the chamber margin. The opening of chitinozoans is sealed with an operculum or blocked from the chamber by an internal plug (prosome), and some distinctive structures, e.g., callus, mucron, and peduncle at the opposite side of the aperture.<sup>20</sup> None of these typical characteristics of chitinozoans is verified in our specimens. Nonetheless, some vesicles of chitinozoans, e.g., the genus *Sphaerochitina*<sup>21,22</sup> possess glabrous round-bottomed chambers somewhat resembling our tests. Such chitinozoans have a noticeably longer neck with trumpet-like aperture, whereas the neck of our tests is always slightly constricting toward the aperture. The walls of those chitinozoans, in some cases, are wrinkled, although such feature is completely unexpressed in *Cangwuella*, indicating that the wall of our specimens is more robust. Another important difference is that the wall structure of *Cangwuella* shows honeycomb structure ([Figure 2I](#)), which has not been observed in chitinozoans. Therefore, our vase-shaped shells do not belong to chitinozoans.

Several testate protistan groups, in particular those of Rhizaria, Cercozoa and Amoebozoa, produce analogues to the *Cangwuella* specimens in terms of their gross morphology. Soft body parts of these protists, e.g., pseudopodium, usually provide informative characters for identification. However, they are unlikely to survive fossilization, thereby leaving fossil groups an empty external shell only. Hence, the following comparisons between *Cangwuella* and these protists are based on the shell morphology.

Among the protists belonging to foraminifera (Rhizaria), there are many single-chambered (monothalamous) forms, i.e., monothalamids,<sup>23</sup> that possess organic-walled or agglutinated shells with terminal aperture. These monothalamous foraminifera have a very wide range of size from less than 100  $\mu\text{m}$  to more than 5000  $\mu\text{m}$ ,<sup>24</sup> obviously overlapping the size of our specimens, and they are diverse in shape,<sup>25</sup> including cylindrical, lenticular, ovate and spherical, as well as pyriform to flask-shaped shells. Therefore, the present vase-shaped microfossils, if only considering overall morphological characters, seem quite similar to monothalamous foraminifera. However, there are actually differences between them. The undulating margin of aperture shown in our specimens are unknown in monothalamous foraminifera. The aperture of the latter is generally more like a simple opening, without undulating lip, and not associated with a conspicuously elongate neck. Also, monothalamous foraminifera are not as fairly robust as the vase-shaped microfossils in shell wall. In fact, the shell wall of many monothalamous foraminifer species is soft and flexible<sup>26–29</sup> and always collapsed when dehydrated. Comparable morphological distortions are not seen in our specimens. Some monothalamous foraminifers, allogromiids and saccamminids, show that there could be many stercomata (waste pellets) occupying much of the shell interior.<sup>30</sup> The stercomata are oval, 10–20  $\mu\text{m}$  in dimension, and thus morphologically similar to the internal structures in the shell of *Cangwuella*. However, detailed studies using scanning and transmission electron microscopies show that these stercomata, not always uniform in size and shape, are subangular grains composed of plate-like particles and bound together by fibers.<sup>31</sup> This is clearly not the case for our fossils.

Of morphologically highly diverse cercozoan amoebae,<sup>32</sup> taxa with broadly vase-shaped shells can be found in at least four families, including Chlamydrophyidae, Pseudodifflugiidae, Euglyphidae, and Paulinellidae. Three genera of Chlamydrophyidae, *Chlamydrophyrs*, *Diaphoropodon* and *Lecythium*, and one genus of Pseudodifflugiidae, *Pseudodifflugia*, have been observed, though not commonly, to possess pyriform shell that has a protruding aperture and then superficially resembles our fossils. However, they are usually 10–70  $\mu\text{m}$  or even smaller in dimension,<sup>33,34</sup> with only very few examples (e.g., *L. mutabilis*) over 100  $\mu\text{m}$ <sup>34</sup> and matching the size of *Cangwuella*. Unlike these chlamydrophyids and pseudodifflugiids, neither large aggregate forms<sup>34,35</sup> nor distinct characters such as rods attached to shell<sup>33</sup> are seen in our specimens. The euglyphid testate amoebae can be easily distinguished from *Cangwuella* on the basis of shell wall. Most of these euglyphid amoebae are characterized by siliceous scales with different shape, size and arrangement,<sup>36–39</sup> whereas such structure is not shown in any of our specimens. Shells without scales indeed occur in Euglyphida, as represented by the genera *Ovulinata* and *Micropyxidiella* of Paulinellidae,<sup>40,41</sup> but they are much smaller (normally less than 20  $\mu\text{m}$  in diameter) than *Cangwuella*. For the above reasons, the fossils described herein are unlikely to be of a cercozoan affinity.

Counterparts of the new microfossils are also from arcellinid amoebae (Amoebozoa), in particular, from the members of Hyalospheniidae and Difflugiidae. Some hyalosphenid testate amoebae, e.g., *Apodera*, *Hyalosphenia*, *Nebela* and *Padaungiella*,<sup>37,42,43</sup> have close similarities to our fossils both in size and shape (in lateral view). The shell of most hyalospheniids, however, is laterally compressed in apertural view, which is clearly not the case for our specimens. Few exceptions, e.g., *Nebela golemanskyi*,<sup>44</sup> are almost uncompressed; however, their wall is composed of characteristic scales that are not observed in the fossils here. The shells completely composed of organic matter, without agglutinated exogenous material, are seen in *Hyalosphenia*, but again, they obviously show lateral compression. The family Difflugiidae provides good alternatives for the morphological comparisons of our fossils to the extant protists, of which potential analogues lie within the genus *Difflugia* (Arcellinida, Longithecina). This genus, one of the most species diverse groups of testate amoebae, includes many taxa that possess pyriform shells and are laterally uncompressed.<sup>37</sup> For example, *Difflugia bryophila*, *D. capreolata*, *D. gassowskii*, *D. oblonga*, *D. parva*, *D. petricola* and *D. pyriformis*, are all composed of a bulbous chamber and a single distinct neck; accordingly, they bear a strong resemblance to our specimens in shape. Previous taxonomic review of the pyriform species of *Difflugia*<sup>45</sup> shows that these taxa can be clustered into at least seven species complexes, in terms of size and shape. The dimensions of our fossils well conform with the *D. petricola*-complex, which is 96–151  $\mu\text{m}$  in length, 58–99  $\mu\text{m}$  in width and 20–36  $\mu\text{m}$  aperture diameter.<sup>45</sup> However, there is still an obvious difference between *Difflugia* and our fossils, although they share an almost identical shape. *Difflugia* has a

clearly agglutinated shell, which is characterized by exogenous material (xenosomes) like diatom frustules or mineral grains<sup>37</sup> from ambient environment, whereas the wall of our specimens basically consists of organic matrix, with little or no exogenous particles and scales.

The attribution of our fossils to testate amoebae is in accord with the evidence for fossilization potential of the latter. For extant testate amoebae, purportedly there are four main shell types, i.e., agglutinate, calcareous, siliceous and proteinaceous,<sup>37,46</sup> all of which appear to be non-resistant. However, there are indeed precedents that testate amoeba fossils occur in palynological preparations of the Late Paleozoic-Mesozoic samples: *Arcella arenaria*, *A. artocrea*, *A. gibbose*, *Amphitrema flavum*, *Centropyxis arcelloides*, *C. hirsuta*, *Diffflugia gramen*, *D. oblonga*, *D. pyriformis*, etc., from the Manjir Formation (Early Permian) of India<sup>15,47</sup>; *A. arenaria*, *A. vulgaris*, *Centropyxis aculeata*, *C. arcelloides*, *Trigonopyxis arcua*, etc., from the Raniganj Formation (Late Permian) of India<sup>14</sup>; *A. flavum*, *C. aculeata* and cf. *D. oblonga* from the Biyadh Formation (Barremian, Cretaceous) of Saudi Arabia.<sup>48</sup> These remains suggest that at least some fossil testate amoebae are acid-resistant, although the exact chemical composition of their shell wall is unknown.

By morphological comparisons, the most reasonable attribution of our vase-shaped microfossils falls within Arcellinida. *Diffflugia*-like arcellinid testate amoebae might be the closest candidate, although lower taxonomic identification levels for them are uncertain. Such an affinity for *Cangwuella* is compatible with the morphological reconstruction for possible fossil shells of ancient arcellinid testate amoebae.<sup>49–51</sup> Our vase-shaped fossils show a close similarity of general shape to the ancestral Longithecina, although there is difference between them with respect to shell type. However, it should be borne in mind that 1) what the shell type (organic or agglutinated) of the most recent common ancestor of Arcellinida is remains an open question<sup>51</sup>; 2) the reconstructed ancestor of Hyalosphenidae has an agglutinated shell, but there are indeed completely organic-shelled groups occurring in the extant hyalosphenids, e.g., *Hyalosphenia papilio*.

### Possible sources of the acetabuliform structures inside *Cangwuella*

Organically connected fossils show a snapshot of ancient organisms and play key roles in solving biological affinity of different groups. The hard shells, acting as those of testate amoebae, provide natural room for photosynthetic symbionts to live, food to be stored and/or digested, some soft-bodied small zooplanktons to parasitize, and the like. In the shell of *Cangwuella*, we see that a number of small, smooth-walled, acetabuliform structures are clustered into mass and inserted or closely attached to the inner shell wall, instead of being distributed in the center of shell or equidistantly arranged within the shell (Figures 3, S3, and S4). It is estimated that the *Cangwuella* shell might accommodate up to 160 complete acetabuliform structures, if the chamber is filled with them. Judging from their configuration, the shell and acetabuliform structures are not likely to represent just an incidental co-occurrence, but indicate certain relationship between them.

Testate amoebae can produce the resting cyst in the shell when encountering harsh environment. As the cytoplasm shrinks, cyst of extant testate amoebae is formed and enveloped by a membrane. There is normally only one resting cyst in a single shell of extant testate amoebae, and the cyst is over 60% of the whole shell size (estimated from Figure 9 sample of the ref.<sup>46</sup>). This is obviously not the case for our specimens: the acetabuliform structures described herein far outnumber the resting cysts of testate amoebae. Therefore, we consider that the acetabuliform structures inside *Cangwuella* do not represent resting cysts.

Some rotifers are observed to parasitize testate amoeba shells for dwelling and egg laying, although relevant studies are scarce. Hitherto such relationship to testate amoebae has been recorded in at least four rotifer species, *Asciaporrecta arcillicola*, *A. diffflugicola*, *Dicranophorus diffflugiarum*, and *Habrotrocha angusticollis*, that inhabit the shells of arcellinid testate amoebae.<sup>52</sup> These rotifers lay smooth and ellipsoidal eggs inside testate amoeba shells, and show a superficial similarity to the internal structures of *Cangwuella*. The single acetabuliform structure found herein is not too far off from the rotifer egg size (40–60 × 24–26 μm<sup>52</sup>). However, the number of rotifer eggs per testate amoeba shell is usually 1–4,<sup>52</sup> much less than that of the acetabuliform structures in our specimens. Although our single acetabuliform structure is in a reasonable size to be considered an animal egg, to our knowledge, there are no other known animals that inhabit and lay eggs in the shell of testate amoebae as rotifers do. Even if there were exceptions, it is still puzzling that what the parasitic soft-bodied animals were and how they laid so many eggs per testate amoeba shell.

An alternative is that the acetabuliform structures of *Cangwuella* might be some body plates derived from another testate amoeba, and used for shell building. This phenomenon, termed kleptosquamy,<sup>53</sup> is



possibly an ancestral character in the hyalosphenids. It actually occurs in many extant hyalosphenids, and some groups not closely related to hyalosphenids, e.g., *Argynnia dentistoma*. These arcellinid testate amoebae acquire body plates by capturing euglyphids and use these euglyphid plates to construct their own shell. However, they show strong differences when compared with the *Cangwuella* specimens. We do not consider that our fossils have to do with kleptosquamy for the following reasons. Firstly, the whole shell of euglyphid is just a few dozen micrometers in size, and hence its body plates are much smaller than our structures; Secondly, these plates is composed of silica, which is non-resistant in the hydrofluoric acid maceration of palynological preparation; Thirdly, kleptosquamy is present in the testate amoebae with a siliceous or agglutinated shell, instead of those organic-walled forms like our fossils; Lastly, in the testate amoebae with kleptosquamy, e.g., *Apodera vas*, the stolen body plates are evident on their external wall surface,<sup>53</sup> but this is completely absent in our specimens.

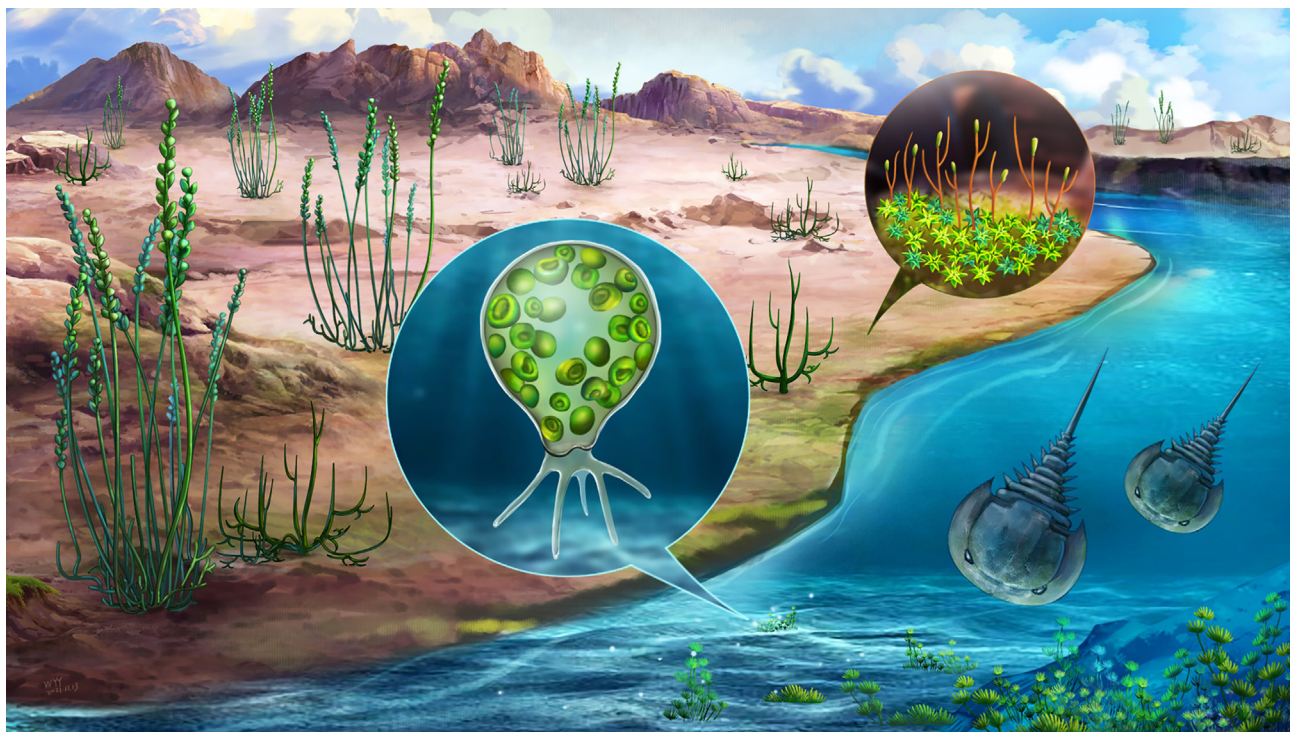
Testate amoebae are mainly phagotrophic, and feed on a very wide variety of organisms, including bacteria, algae, fungi, protists and even some small metazoans.<sup>46</sup> The acetabuliform structures described herein certainly could be considered as some undigested food remains, but a more significant issue is that whether they associate with the testate amoeba via endosymbiosis, a relationship that has been widely established within a variety of microbial eukaryotes<sup>54</sup> and evolved across the major lineages of testate amoebae,<sup>55</sup> including Arcellinida of Amoebozoa, Euglyphida of Cercozoa and Amphitremidae of Stramenopiles. Among the extant arcellinids, some mixotrophic species live as the host of their endosymbionts (trebouxiophytes), and show a comparable configuration to our fossils. It has been argued that the photosynthetic symbionts inside these arcellinid testate amoebae, of which *Hyalophenia papilio* and *Heleopera sphagni* are the most common groups,<sup>56</sup> turn out to be of unexpectedly low diversity and closely related to the green microalga *Chlorella* (Trebouxiophyceae, Chlorellaceae).<sup>55</sup> Recent examination of the relationship between *Hyalosphenia papilio* and its intracellular algae indicates that such endosymbiosis might be not stable, and instead, they only transiently perform photosynthesis (function as endosymbionts) before being digested as a food source.<sup>57</sup> We note that the presence of sporopollenin (highly resistant material) has been reported in the cell wall of several *Chlorella* species.<sup>58–62</sup> On this basis, although occurring within an easily degradable cytoplasm of testate amoebae, the endosymbiotic green algae do have the potential for being fossilized and remaining in the testate amoeba shell. However, the extant examples differ from our fossils in two ways: (1) The endosymbiont algae are usually no more than 4 μm in diameter, with only a few up to 10 μm (as measured from the light microscopy micrographs of mixotrophic testate amoebae in ref.<sup>55</sup> and<sup>56</sup> obviously smaller than our acetabuliform structure; (2) Such host-symbiont relationship is known from the freshwater or terrestrial testate amoebae rather than those living in shallow-marine habitats.

Although the morphological evidence of our shell strongly points toward an arcellinid affinity, all the known kinds of bio-related structures inside the shell of extant testate amoebae are poorly matched to our fossils. It does not necessarily mean, however, that all these possible sources are entirely excluded from future considerations, because of lacking well-studied examples of internal structures in extant testate amoebae.

### Implications for the evolution of testate amoebae

Although it is considered that the fossil record of testate amoebae starts from the Neoproterozoic, there is a huge gap between the late Neoproterozoic and Devonian.<sup>63,64</sup> Vase-shaped microfossils (VSMs) are diverse and widespread in the Tonian Period, exemplified by the occurrences in the Chuar Group of Grand Canyon (USA),<sup>11,12</sup> the Callison Lake Formation of Canada,<sup>9,10</sup> and the Urucum Formation of Brazil.<sup>8</sup> In contrast, there is only one previously reported fossil record of the Devonian testate amoebae,<sup>13</sup> which is more or less coeval with our fossils. The discovery of VSMs from the Cangwu Formation provides additional information on the distribution of testate amoebae in the Early Devonian, thereby improving understanding of the potential diversity of testate amoebae during this period or even earlier.

If it is true that the ancient VSMs belong to Arcellinida, a salient issue arising from this view would be the time of origin of freshwater arcellinid lineages. This is complicated not only by the gap in the known fossil record, but also the obvious distinction of habitat between fossil and extant arcellinids. All the Neoproterozoic VSMs are discovered in marine deposits,<sup>65</sup> but according to current knowledge, extant arcellinids are mostly associated with terrestrial environments such as humid soils, peatlands and mosses.<sup>66</sup> Molecular dating suggests that terrestrial arcellinids likely diversified along with the radiation of early land plants.<sup>66</sup> This estimation is supported by the freshwater testate amoebae from the Rhynie chert.<sup>13</sup> Our testate amoeba co-occurs with several types of plant remains and more recently reported *Houia* (Euchelicerata)



**Figure 4. Palaeoecosystem reconstruction of the Lower Devonian Cangwu Formation in southwestern China**

This figure shows the paleoecosystem with a coast setting. Testate amoeba *Cangwuella ampulliformis* gen. nov., sp. nov. lived in a nearshore environment with algae and the euclerid *Houia*. Tall to diminutive plants on land belong to Zosterophylloids and rhyniophytoids respectively.

(STAR Methods), the latter of which has been considered to live in marine habitats.<sup>67–69</sup> It can be suggested that the fossil-bearing horizon represents a shallow-water marine environment (Figure 4). Our fossils, then, would play a significant role in extending the record of marine VSMs into the Early Devonian, during which the earliest freshwater testate amoebae occurred.<sup>13</sup> Recent advances in molecular phylogeny and microbial paleobiology of testate amoebae raise a scenario that, within Arcellinida lineages, the marine-freshwater transition probably occurred multiple times and only during the Phanerozoic.<sup>49</sup> That arcellinids occurred in both the freshwater and shallow-marine environments in the Early Devonian concurs with the very possibility proposed by Lahr,<sup>49</sup> although more fossil evidence is required to provide details and testify for such evolutionary history of Arcellinida.

### Limitations of the study

Deeper significance of the new vase-shaped fossils undoubtedly rests on the relationship between the acetabuliform structures and shell. However, it is rather difficult to address this issue because of limitations of fossil preservation. For example, in order to demonstrate a host-symbiosis relationship, it is required to show that two organisms lived together at the same time, but the cytoplasmic membrane in testate amoebae is almost impossible to be preserved together within the rigid shell. Therefore, we refrain from concluding that the Cangwu material is the direct proof for a predator-prey or host-symbiosis relationship between testate amoebae and other microbial eukaryotes, but regardless, these vase-shaped microfossils would draw attention to less concerned fossil testate amoebae in Early Paleozoic-Devonian rocks.

### Conclusions

Testate amoeba, *Cangwuella ampulliformis* gen. nov., sp. nov., (Order Arcellinida, probably Infraorder Longithecina) is described from the Lower Devonian Cangwu Formation in Cangwu County, Guangxi, southwestern China. It is flask-shaped with a bulbous chamber (length/width ratio close to 1) and a gradually constricted neck, morphological characters of which demonstrate great similarities to extant members of Longithecina. The acetabuliform structures inside *Cangwuella*, though do not perfectly correlated with known analogues

from extant testate amoebae, open up the possibility of revealing ancestral state for some certain kind of ecological relationship, e.g., predation, parasitism, or commensalism, that occur in extant protists. Our fossils, in combination with the Rhynie chert material, show that Arcellinida inhabited both the shallow-marine and freshwater environments in the Early Devonian, strengthen the interpretation that the transition of Arcellinida from marine to freshwater environment occurred in the Phanerozoic, and highlight the prospect of calibrating the evolutionary path of Arcellinida by bridging the gaps in fossil records.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2023.106678>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, H.X.; Visualization, K.W., B.L., and J.B.; Investigation, K.W. and H.X.; Writing – Original Draft, K.W. and H.X.; Writing – Review and Editing, K.W. and H.X.; Funding Acquisition, H.X.; Resources, H.X., P.T., J.L., Y.W. (Yao Wang), and Y.W. (Yi Wang); Supervision, H.X.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Biological samples</b>		
Microfossils from the Lower Devonian Cangwu Formation in Guangxi, southwestern China	Repository at the Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences	NIGP179644 - NIGP179659
<b>Deposited data</b>		
X-ray micro-tomography raw data	<a href="https://www.zenodo.org/">https://www.zenodo.org/</a>	Target URL: <a href="https://doi.org/10.5281/zenodo.5740371">https://doi.org/10.5281/zenodo.5740371</a>
<i>Cangwuella</i> Wang et al. 2023, gen. nov.	<a href="http://www.zoobank.org">http://www.zoobank.org</a>	urn:lsid:zoobank.org:act:05F5D18F-E38F-4C01-BC86-B08D275799F2
<i>Cangwuella ampulliformis</i> Wang et al. 2023, sp. nov.	<a href="http://www.zoobank.org">http://www.zoobank.org</a>	urn:lsid:zoobank.org:act:665ED98A-C122-4845-A16F-7A3EB7018548.
<b>Software and algorithms</b>		
Three-dimensional reconstruction of the microfossils	<a href="https://www.volumegraphics.com/en/products/vgstudio-max.html">https://www.volumegraphics.com/en/products/vgstudio-max.html</a>	VGStudio Max 3.0

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Hong-He Xu ([hwxu@nigpas.ac.cn](mailto:hwxu@nigpas.ac.cn)).

#### Materials availability

- Specimens illustrated in this paper are deposited in Nanjing Institute of Geology and Palaeontology (NIGP), Chinese Academy of Sciences (CAS), with serial numbers and prefix NIGP.
- This study did not generate new unique reagents.

#### Data and code availability

- Raw X-ray micro-tomography data have been deposited at Zenodo and are publicly available as of the date of publication. DOI is listed in the [key resources table](#).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

Our samples were collected from one of well-exposed outcrops of the Cangwu Formation, which is located ca. 4 km south of Shiqiao Town, near national highway 207, in Cangwu County, Guangxi Zhuang Autonomous Region, southwestern China (Figures 1 and S1; GPS locality: 23°48'21.82"N, 111°32'52.82"E). In studied area, the Cangwu Formation overlies the Cambrian metamorphic rocks and underlies the Hanzhen Formation respectively. Lithologically, the Cangwu Formation can be divided into two members: the lower Shangwan Member (sandstone) and the upper Shiqiaofeng Member (siltstone) (Figure S1A). Microfossil specimens are preserved in the latter lithologic member, as the same layer as the previously reported Zosterophylloids with rooting system.<sup>70,71</sup> Previously discovered fossils in the Cangwu Formation also include the plant macrofossils *Changwuia schweitzeri*, *Drepanophycus* sp., *Taeniocrada decheniana*,<sup>72</sup> *Demersatheca contigua*<sup>73</sup> and rhyniophytoids.<sup>74</sup> Recently, the euchelicerate *Houia* was found in the Shiqiaofeng Member of the Cangwu Formation.<sup>67</sup> In the overlying Hanzhen Formation, more animal fossils have been reported, including bivalves *Glossites* sp., *Leptodesma schiqiaoensis*,

*Modiomorpha* sp. and *Praecardium* sp., and a species of ostracod—*Paramoelleritia xiangzhouensis*<sup>75,76</sup> that is regarded to indicate an age from late Emsian to Eifelian.<sup>77</sup> Based on biostratigraphy, fossiliferous horizon is tentatively considered to be of an age from Pragian to Emsian (410.8 – 393.3 Ma, Lower Devonian).

## METHOD DETAILS

Samples were prepared using palynological technique<sup>78</sup> to extract specimens from rocks. After hydrochloric-hydrofluoric (HCL-HF) acid maceration and water rinsing to neutralization, organic residues were sieved with nylon mesh (10 $\mu$ m mesh openings) to remove small impurities. Specimens were examined under a binocular stereo microscope for further microfossil analyses. A total of 16 specimens with typically vase-shaped forms were obtained and then photographed with the Tescan MAIA3 scanning electron microscope. Six specimens were observed to have acetabuliform structures inside their shells. In order to observe intact acetabuliform structures and their distribution inside the shell, we picked one specimen (shown in [Figure 3](#)) to take X-ray micro-tomography by using the Zeiss Xradia 520 Versa tomographic microscope. Image data were processed by the VGStudio Max 3.0. All of the above processes were carried out in the NIGP, CAS.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Morphometric data of our fossil specimens were measured with the software ImageJ 1.48v. Further statistical analyses were conducted using the software Microsoft Excel 2021 to determine minimum, arithmetic mean, maximum, median, standard deviation, standard error of the arithmetic mean, and coefficient of variation. Details of the results can be found in [Table 1](#).