New holozoans with cellular resolution from the early Ediacaran Weng’an Biota, SW China

Zongjun Yin1,2,3,4*, Weichen Sun1,2,5, Joachim Reitner6 and Maoyan Zhu1,2,4

1 State Key Laboratory of Palaeobiology and Stratigraphy, Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences, Nanjing 210008, China
2 Center for Excellence in Life and Paleoenvironment, Chinese Academy of Sciences, Nanjing 210008, China
3 Nanjing College, University of Chinese Academy of Sciences, Nanjing 210008, China
4 College of Earth and Planetary Sciences, University of Chinese Academy of Sciences, Beijing 100049, China
5 University of Science and Technology of China, Hefei 230026, China
6 Department of Geobiology, Geoscience Centre, University of Göttingen, Göttingen 37077, Germany

* Correspondence: zjyin@migpas.ac.cn

Abstract: Embryo-like fossils from the early Ediacaran Weng’an Biota (SW China, 609 myr ago), widely interpreted as holozoans, potentially provide insights into the early evolutionary development of metazoans and the rise of the animal kingdom. However, the biodiversity of the embryo-like fossil assemblage is largely underestimated and its more precise phylogenetic affinities within the holozoan tree are still under debate. We describe a new species of embryo-like fossil, Ostiosphaera rara n. gen. n. sp., from the Ediacaran Weng’an Biota. These 3D, phosphatized specimens exhibit a spherical morphology, a thick ornamented envelope with a circular opening and a membrane-bound, multicellular inner body. In terms of biological characteristics, O. rara shows similarities with a number of extant and fossil analogues, including testate amoebae, unicellular green algae, the cellular slime mould Fonticuclida and co-occurring Weng’an embryo-like fossils. Although the phylogenetic affinity of O. rara is difficult to constrain precisely based on the available evidence, it is reasonable to follow the holozoan interpretation for these fossils because they share the same grade complexity with co-occurring embryo-like fossils, such as Megaspheara and Helicoforaminina, in terms of the combination of biological features. These new holozoans resemble asexual reproductive gemmules of fossil and living demosponges in size, morphology, circular opening and cellular anatomy. If this similarity reflects biological affinity rather than convergent evolution, this discovery would force us to rethink the evolutionary history of Precambrian sponges.

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The Ediacaran Weng’an Biota (c. 609 myr ago, Doushantuo Formation, Weng’an, Guizhou Province, SW China) provides a taphonomic window during the Precambrian–Cambrian transitional time interval in which major animal clades evolved (Xiao et al. 2014b; Cunningham et al. 2017a, b). The phosphatized embryo-like fossils, including Megaspheara, Caveaspheara and Helicoforaminina (the MCH embryo-like fossil assemblage) from the Weng’an Biota, which were initially interpreted as animal embryos (Xiao et al. 1998; Xiao and Knoll 2000), have recently been more loosely constrained as holozoans (Yin et al. 2019, 2020), although their more precise phylogenetic affinities within the holozoan tree are still under debate (Y.J. Chen et al. 2006; L. Chen et al. 2014; Hultgren et al. 2011; Yin et al. 2016). The Weng’an MCH embryo-like fossil assemblage is thought to be significant for exploring the early evolution of holozoans 609 myr ago (Xiao et al. 2014b; Cunningham et al. 2017b), with great potential to shed light on the evolutionary origins of metazoans (Yin et al. 2019) because holozoans comprise all metazoans and their close unicellular relatives (Sebé-Pedrós et al. 2017; Ruiz-Trillo and de Mendoza 2020).

Regarding the origin of metazoans, there is a substantial gap between molecular clock estimates of the age of this event and estimates based on the fossil record (Cunningham et al. 2017a). For example, the results of molecular clock studies indicate that animals probably arose prior to the Cryogenian Period (720–635 Ma) and also that the major clades of bilaterians diversified during the subsequent Ediacaran Period (635–541 Ma) (Erwin et al. 2011; dos Reis et al. 2015). By contrast, the age of the earliest unequivocal animal fossils – namely, the celebrated megascopic Ediacaran biota – is late Ediacaran (575–541 Ma) (Zhu et al. 2008; Droser and Gehling 2015; Bobrovskiy et al. 2018; Chen et al. 2019; Dunn and Liu 2019). Some researchers have reported putative sponge biomarkers from the Cryogenian and Ediacaran systems (Love et al. 2009, 2020; Zumberge et al. 2018), but questions have been raised as to whether these organic molecules are unique to sponges (Antcliff et al. 2014; Nettersheim et al. 2019; Bobrovskiy et al. 2020). Furthermore, although body fossils interpreted as sponges have been discovered in Cryogenian and Ediacaran strata (Gehling and Rigby 1996; Maloof et al. 2010; Clites et al. 2012), reassessment of these fossils has demonstrated that none of them can be assigned to crown-group sponges with a high degree of confidence (Antcliff et al. 2014), even though some of them may have possessed a water canal system or spicules (Droser and Gehling 2015). Recently, some 890-myrr-old microstructures recovered from early Neoproterozoic microbial reefs were interpreted as possible body fossils of spicule-free demosponges (Turner 2021), but the age is much earlier than the molecular clock estimates of the age of animal origin.

Li et al. (1998) reported some putative sponges with spicule-like microstructures from the Weng’an Biota. However, later
observations suggested that those Weng’an sponges were more likely to be multicellular algal fossils preserved with discrete spines of large ornamented acritarchs, which are very common in the Weng’an Biota (Zhang et al. 1998). This challenge was then supported by in situ physical and chemical characterization of those spicule-like microstructures at the micro-to nanoscale (Muscente et al. 2015), which revealed that the spicule-like microstructures were rectangular rather than round in transverse section and carbonate in composition (Muscente et al. 2015). However, a more recent study of early Cambrian sponges with exceptionally preserved organic spicules suggested that the morphology and chemical composition of these Weng’an spicule-like microstructures are still compatible with their interpretation as sponges (Tang et al. 2019).

In 2015, Eocyathospunga, a sponge-grade body fossil preserving individual cells, was recovered from the early Ediacaran Weng’an Biota (Yin et al. 2015). However, this taxon lacks the diagnostic characters of crown-group sponges (Cunningham et al. 2017b) and is more likely to be either a stem-group sponge (Bottjer et al. 2020) or a ‘pre-sponge’ animal (i.e. a stem-group metazoan) (Cavalier-Smith 2017). At present, the oldest known microfossil specimens of crown-group sponges are spicules of early Cambrian age (c. 535 Ma) (Chang et al. 2017) and thus there is a c. 200 myr gap between the molecular clock estimates of the origin of sponges and estimates based on the fossil record (Sterling et al. 2010). However, the discovery of Weng’an spicule-like microstructures and Eocyathospunga suggests that there is great potential for the discovery of additional sponge-grade fossils in the taphonomic window of the Weng’an Biota if the molecular clock dating of the evolutionary origin of animals is just premature rather than completely wrong (dos Reis et al. 2015; Budd and Mann 2020; Beavan et al. 2021).

Given that the Ediacaran Weng’an Biota can preserve microfossils at the cellular and even subcellular level with relatively high fidelity (Xiao et al. 2014b; Yin et al. 2017; Cunningham et al. 2017b; Bottjer et al. 2020; Sun et al. 2020) and that sponges probably arose prior to the early Ediacaran in the light of molecular clock estimates, we focused on the unique window of the Weng’an Biota and investigated several spherical microfossils with a collar-like, circular opening using high-resolution X-ray tomographic microscopy. Our results illustrate that, in these fossils, a membrane-bound, multicellular inner body is enclosed by a thick ornamented envelope. Taken together, all the features (including size, morphology, envelope structure and multicellular internal body) of these new fossils suggest that they resemble co-occurring embryo-like fossils such as Megaspahaera, Caveasphaera and Helicoforaminina (Xiao et al. 2014a; Yin et al. 2019, 2020), but have about as much in common with them as they do with asexually reproductive sponge gemmules (Manconi and Pronzato 2015).

Materials and methods

The fossils presented here were collected from the upper grey facies of the Ediacaran Doushantuo Formation in Weng’an County, Guizhou Province, South China. Samples of dolomitic phosphorite were digested in c. 10% acetic acid and the insoluble microfossils were sorted manually under a stereomicroscope. The extant gemmules of the freshwater sponge Dralvia brownie were examined using a Zeiss Xradia 520 Versa CT scanner. The operating voltage and power of the X-ray tube were set at 50 kV and 5 W, respectively. For each scan, 3201 projections were collected over 360°, with the exposure time for each projection being either 12 or 15 s. To avoid beam-hardening artefacts, two thin filters (LE1 and LE2) were used. Unlike a conventional micro-CT, which relies solely on geometric magnification, the Zeiss Xradia 520 Versa uses a CCD-coupled 4× objective lens, producing a 0.65–0.84 μm voxel dimension for different specimens. The volume data were processed using Vgstudio Max (3.0 version). The specimens are repositioned at NIGPAS and the volume data are available on a 3D model database at NIGPAS (https://doi.org/10.12091/fossil-ontology.20220212).

Results

We describe a new, exceptionally well-preserved type of spherical microfossil, Ostiosphaera rara n. gen. n. sp., from the early Ediacaran Weng’an Biota. We used X-ray tomographic microscopy with sub-micron resolution to reconstruct their 3D architecture in great detail. Our results show that three available specimens, which measure c. 650 μm in diameter, possess a sculptured envelope with an external ornament (Fig. 1a–d), similar to that of co-occurring, embryo-like fossils, including Megaspahaera, Caveasphaera and Helicoforaminina (the MCH embryo-like fossils) (Xiao et al. 2014a), which have been widely accepted as holozoans (Huldgren et al. 2011; Chen et al. 2014; Yin et al. 2019, 2020). However, unlike the unperforated envelope of the Weng’an MCH embryo-like fossils, the envelope of the new microfossils has a single, circular, collar-like opening or aperture (Figs 1 and 2). The diameter of the aperture varies between specimens, ranging from c. 190 to 250 μm. Although the area surrounding the largest observed apertures is smooth (Fig. 1a), the area next to the smallest apertures is ornamented (Fig. 1c). This difference suggests that the original size of the aperture of some specimens may have been altered, with the largest apertures possibly being artefacts of post-mortem degradation, but we cannot rule out the possibility that the organism was capable of adjusting the size of the aperture. Evident soft deformation of the specimens suggests that the envelope was organic and pliable (Fig. 1c, d), just like that of Weng’an MCH embryo-like fossils, and therefore also that adjustment of the aperture size was possible.

Two of the new specimens preserve only the outer envelope, or consist of an inner cast in which no biological structure can be observed, only clotted or homogenised minerals (e.g. Fig. 1c, f). Fortunately, one specimen shows exceptional preservation of cellular structures (Fig. 1c, d, g–n). The outer envelope of this specimen ranges from 13 to 80 μm in thickness (Fig. 1g, h; Fig. 2a–e) and is thus much thicker than that of the Weng’an MCH embryo-like fossils, in which the envelope is generally no more than 10 μm (Yin and Zhu 2012; Yin et al. 2019, 2020). The observed variation in thickness is probably due to post-mortem degradation. A membrane-bound inner body is present immediately beneath the outer envelope (Fig. 1g, h). Despite the close mutual proximity of the outer envelope and membrane, the two structures are readily distinguishable (Fig. 1g, h, m, n). The envelope is thickest near the aperture, where it is formed into a hollow, collar-like opening (c. 90 μm in depth) that descends into the inner body (Figs 1g–i, 2b–e). The membrane-bound inner body consists of a diagenetic zone of void-filling minerals surrounding a shrunken central mass (Fig. 1g, h, m, n). Within this mass, minute minerals exhibiting a dark grey core and with high X-ray attenuation preserve the original biological structures, including 28 spherical vesicle-like structures measuring c. 40 μm in diameter (Figs 1g–i, 2a–d, f–h). Most of the vesicle-like structures are empty or filled with later diagenetic minerals and their outline is defined by a bright rim with high X-ray attenuation (Fig. 1i, j). Previous taphonomic studies have concluded that the stable rims defining the vesicle-like structures reflect a biological membrane from which the minerals grew (Sun et al. 2020, 2021). Nevertheless, several vesicle-like structures are solid and are...
outlined by a dark rim with low X-ray attenuation (Fig. 1i–l); this type of rim is similar to that of the well-preserved cell membrane of many of the MCH embryo-like fossils of the Weng’an Biota. Furthermore, the solid interior of the dark-rimmed vesicle-like structures (Fig. 1l) is similar in preservation to the cytoplasmic substance in the MCH embryo-like fossils, the material of which consists of dark, randomly oriented nanometre-scale crystals with relatively low X-ray attenuation. In addition to the 28 vesicle-like structures with clear boundaries, there are also numerous poorly preserved ‘ghost’ vesicle-like structures with unclear boundaries (Fig. 1j, k), suggesting that the inner body was originally fully filled with vesicle-like structures, only some of which have been preserved.

We noticed that the preservation mode of the vesicle-like structures is the same as that of cells of the co-occurring Sporosphaera (e.g. Landon et al. 2019, their fig. 1) and Megaclonophucus-stage Megasphaera (e.g. Xiao et al. 2014b, their fig. 10) because all the cells are membrane-bound and some are empty, whereas others are solid with void-filling cements or a permineralized cytoplasm-like matrix (Fig. 1g–i; Landon et al. 2019, their fig. 1). Given that the size, shape and membrane- and cytoplasm-like structures of the vesicle-like structures (Figs 1g–i, 2a–d, f–h) are consistent with that of permineralized cells of the co-occurring Sporosphaera (e.g. Landon et al. 2019, their fig. 1) and Megaclonophucus-stage Megasphaera (e.g. Xiao et al. 2014b, their fig. 10), we interpret the vesicle-like structures of Ostiosphaera as fossilized cells, rather than any other subcellular structures. The uniform size and shape of the cells further suggest a lack of cell differentiation (Fig. 2a–d, f–h).

Fig. 1. Phosphatized spherical microfossils from the Ediacaran Weng’an Biota. Surface renderings of (a) the top view and (b) the lateral view of specimen NIGP-WA-S01. Surface renderings of (c) the top view and (d) the lateral view of specimen NIGP-WA-S02. (e, f) Digital sections of parts (a) and (b), respectively, showing diagenetic internal structure. (g, i, k) Digital sections of specimen NIGP-WA-S02 showing the internal structures. (m, j, l) Enlargements of the framed areas in parts (g), (i) and (k), respectively, showing details of the vesicles. (h) Outlines of the boundaries of the envelope, inner body and the vesicles in part (g). The orange area represents the envelope, the light green line represents the membrane enclosing the inner body, the light green area represents the diagenetic zone and the blue area represents the shrunken mass of the inner body, where two vesicles can be observed. The blue arrow in part (j) indicates a ‘ghost’ vesicle with an enlargement displayed in the inset of part (k); note its hazy boundary. The blue arrow in part (l) indicates a well-preserved vesicle with membrane and internal structure. (m, a) Details of the boundaries of the envelope and the inner body. The red lines in part (a) represent the inner boundary of the envelope, whereas the green line represents the membrane enclosing the inner body. The two arrows in part (m) indicate the boundaries of the envelope and inner body.
Discussion

Several fossil and extant organisms and structures are similar to the Ostiosphaera from the Weng'an Biota in overall morphology and internal structure – for example, the early Ediacaran unicellular Sporosphaera (Landon et al. 2019), certain vase-shaped microfossils and testate amoebae (Fig. 3c) (Porter and Knoll 2000; Porter et al. 2003; Nikolaev et al. 2005; Lahr and Lopes 2009; Lahr et al. 2019), reproductive cysts of certain green algae (Fig. 3d), spore masses of the cellular slime mould Fonticulida (Brown et al. 2009), the co-occurring MCH embryo-like fossils (Yin and Zhu 2012; Xiao et al. 2014a; Cunningham et al. 2017b; Yin et al. 2020) or even sponge gemmules (Manconi and Pronzato 2015). To determine the affinity of Ostiosphaera, we compared it with the following potential analogues.

Comparison with Sporosphaera from the Ediacaran Weng'an Biota

Ediacaran Sporosphaera, a millimetre-scale encysted sphere that was also recovered from the Weng'an Biota, has been interpreted as...
a unicellular eukaryote with multicellular stages (Landon et al. 2019). They are slightly larger than the co-occurring Ostiosphaera, but they share features with the latter, including their overall morphology and internal cells. The internal cells (interpreted as endospores) of Sporosphaera vary from 25 to 125 μm in diameter (Landon et al. 2019), overlapping the cell size of the new microfossils. However, there are prominent differences beyond the superficial similarities between them. First, there is no circular aperture, but a simple slit-like excystment opening has been observed in each specimen of Sporosphaera. Second, there is, so far, no evidence to suggest that Sporosphaera developed an ornamented envelope akin to that of Ostiosphaera. Third, the multicellular mass of Ostiosphaera is membrane-bound, whereas no comparable membrane-like structure has been observed in any Sporosphaera specimen. Therefore, despite their superficial similarities, they cannot be attributed to the same taxon.

**Comparison with testate amoebae**

As a result of their high taxonomic diversity, the shells of vase-shaped microfossils (usually interpreted as extinct testate amoebae) and testate amoebae vary greatly in shape and chemical composition, but certain species possess a non-mineralized, organic spherical wall with a circular aperture (Fig. 3c) (Beyens and Meisterfeld 2001; Porter et al. 2003; Lahr et al. 2019), which closely resemble Ostiosphaera. Nevertheless, in general, the maximum length of most testate amoebae, both extant and fossil, is <200 μm (Porter and Knoll 2000; Beyens and Meisterfeld 2001; Porter et al. 2003) and the test of the amoebae is normally a single layer and only 2–5 μm thick (Porter and Knoll 2000; Beyens and Meisterfeld 2001; Porter et al. 2003). Hence, when compared with most testate amoebae, Ostiosphaera are larger in size and more complex in terms of envelope microstructure, although there are exceptions. For example, Gromia sphaerica, a deep-sea testate amoeba, produces large spherical tests up to 30 mm or more in diameter with a layered wall structure (Gooday et al. 2000). It is therefore difficult to distinguish the Weng’an Ostiosphaera from testate amoebae merely on the basis of size, envelope structure or overall morphology.

We argue that Ostiosphaera differs from testate amoebae in possessing multicellular developmental stages. Testate amoebae lack a multicellular stage in their life cycle, regardless of whether reproduction is asexual or (possibly) sexual (Beyens and Meisterfeld 2001; Lahr et al. 2011), which contradicts the multicellular structure observed in Ostiosphaera. It is worth considering the cytology of testate amoeba cells. Membrane-bound intracellular vesicles or vacuoles can be observed in certain testate amoebae (Anderson 1994; Lahr et al. 2019). These tiny intracellular vesicles or vacuoles indeed look like cells superficially if we only consider their shapes and omit the fact that they are only a few microns in diameter and therefore much smaller than the preserved cells of Ostiosphaera (Anderson 1994). In fact, the experimental taphonomy of giant sulfur bacteria (large bacteria with diameters up to 750 μm or even more) with a large membrane-bound vacuole and relatively small vesicles suggests that the vacuoles and vesicles collapse easily. They then become irregular in morphology, showing very low preservation potential (Cunningham et al. 2012). The consistencies in size and overall shape of the membrane-bound structures of Ostiosphaera are therefore not compatible with their interpretation as intracellular vesicles or vacuoles. On the contrary, they show strong similarities in terms of shape, size and preservation mode to the cells of the co-occurring Sporosphaera (e.g. Landon et al. 2019, their fig. 1) and Megaclononophycus-stage Megasphaera (e.g. Xiao et al. 2014b, their fig. 10). Given the very low probability that the vesicle-like structures of Ostiosphaera are fossilized intracellular vacuoles or vesicles, we therefore argue that Ostiosphaera is unlikely to be a testate amoeba, although this possibility cannot be ruled out completely.

**Comparison with unicellular green algae with multicellular developmental stages**

Certain single-celled green algae, such as members of Prasinophyceae and Chlorophyceae, produce ornamented (usually spiny) or smooth reproductive cysts and exhibit multiple internal cells when producing gametes (Taylor et al. 2009; Moczydłowska 2016; Hartman et al. 2018). These organic-walled cysts with gametes are normally smaller (~100 μm in diameter) and morphologically simpler than Ostiosphaera and the co-occurring MCH embryo-like fossils in terms of the thickness and histology of the cysts (Agić et al. 2016; Moczydłowska 2016; Hartman et al. 2018; Moczydłowska and Liu 2021). However, there are still exceptions. For example, reproductive cysts of prasinophyte, referred to as phycocymata, develop ornamented, bi-layered organic walls with diameters up to 100 μm (Taylor et al. 2009). Tasmanites algae (Tasmanaceae), interpreted as large prasinophyte phycocymata, even possess cysts measuring 100–600 μm in diameter (Taylor et al. 2009). Their cysts are usually composed of two layers and can be as thick as 80–100 μm (Vigran et al. 2016). These exceptions provide potential analogues for the new Weng’an microfossils in terms of the complexity of their large, thick and bi-layered cysts, although there are still differences between them. Tasmanites and all the other prasinophyte phycocymata lack a circular opening and they develop a simple slit to split the cysts when discharging daughter cells (Taylor et al. 2009; Vigran et al. 2016; Hartman et al. 2018). Moreover, unlike the membrane-bound cellular mass of Ostiosphaera, the algal daughter cells inside the cysts are not enclosed by a membrane.

Similarly, the desmidiean algal cysts (Chlorophyceae) are much smaller than Ostiosphaera and also develop a slit rather than a circular opening to allow the release of the internal daughter cells (Moczydłowska 2016; Moczydłowska and Liu 2021), whereas in the genus Acetabularia (the dasycladalean algae belong to Ulvophyceae), the reproductive cysts develop a small secondary, circular aperture with an operculum (Fig. 3d) (Agić et al. 2016). However, this is unlikely to be the case for Ostiosphaera because Acetabularia cysts are characterized by small sizes (around 100 μm in diameter) and thin walls (only a few microns in thickness) without a complex histology (Agić et al. 2016). Evidence of an operculum is absent in Ostiosphaera, although a taphonomic bias for this absence cannot yet be completely excluded. Another significant difference is that the daughter cells of Acetabularia are not enclosed by a membrane (Table 1).

**Comparison with the slime mould Fonticuida**

The sorus of the cellular slime mould Fonticuida, which is a tiny spherule made up of a large number of spores (i.e. a spore mass), superficially resembles Ostiosphaera. It always forms on top of a volcano-shaped stalk made of extracellular matrix and sorogenic cells (i.e. a fruiting body, also called a sorocarp) (Brown et al. 2009). However, the spherical spore mass is not enclosed by a cyst, but is more like the multicellular filastereans, in which a large number of cells are embedded within extracellular matrix materials (Brown et al. 2009; Ferrer-Bonet and Ruiz-Trillo 2017). Evidently, this is not the case for Ostiosphaera.

**Comparison with the co-occurring MCH embryo-like fossils**

Ostiosphaera closely resembles the co-occurring MCH embryo-like fossils in size and spherical gross morphology, as well as in having...
an ornamented envelope covering an internal cellular mass (Table 1) (Xiao et al. 2014a; Yin et al. 2019, 2020). The MCH embryo-like fossils were initially described as animal embryos (Xiao et al. 1998; Xiao and Knoll 2000) and this interpretation was supported by many later studies (Chen et al. 2006; Yin et al. 2007, 2013, 2016; Xiao et al. 2007a). However, it has been challenged by alternative hypotheses, including giant sulfur bacteria (Bailey et al. 2007), green algae (Butterfield 2011; Moczydłowska and Liu 2021), non-metazoan holozoans (mesomycetozoan-like protists) (Hultgren et al. 2011) and stem-group metazoans (Hagadorn et al. 2006; Chen et al. 2014). Although the bacterial and algal interpretations have been rejected in the light of experimental taphonomy and/or the complex developmental processes of the newly discovered embryo-like fossils (Yin et al. 2007, 2019, 2020; Xiao et al. 2007b; Cunningham et al. 2012; Chen et al. 2014), debates around the remaining hypotheses (non-metazoan holozoans, stem-group metazoans or crown-group metazoans) continue, in part due to the unknown diversity and incomplete development sequences of the MCH Weng’an embryo-like fossils (Xiao et al. 2014b; Cunningham et al. 2017b). Because non-metazoan holozoans (i.e. unicellular relatives of metazoans) plus total-group metazoans (i.e. extinct stem metazoans plus extinct and living crown metazoans) compose the holozoan tree (Sebé-Pedrós et al. 2017; Ruiz-Trillo and de Mendoza 2020) (Fig. 4), the MCH embryo-like fossils have currently been loosely constrained as holozoans with high confidence (Yin et al. 2019, 2020).

It is conceivable that the affinity of the new fossils is related to that of the MCH embryo-like fossils because their biological structures show the same level of complexity and great similarities.

Table 1. Comparison of features among new Weng’an fossils and their analogues

|                      | Diameter  | Morphology | Collar-like opening | Operculum | Envelope histology | Thickness of envelope (μm) | Multicellular inner mass |
|----------------------|-----------|------------|---------------------|-----------|--------------------|---------------------------|--------------------------|
| New Weng’an fossils  | 450–850 μm| Spherical  | No                  | No        | Single- or bi-layered | <20                       | Yes                      |
| Megasphaera          | 370–750 μm| Spherical  | No                  | No        | Bi-layered         | <20                       | Yes                      |
| Caveasphaera         | 500–900 μm| Spherical  | No                  | No        | Single-layered     | <20                       | No                       |
| Testate amoebae      | <300 μm;  | Spherical  | Yes                 | Yes       | Single-layered     | 3–80                      | Yes                      |
|                      | >1 mm     | vase-like  |                     |           |                    |                           |                          |
| Algal cysts          | 20–600 μm | Spherical  | No                  | Yes       | Single- or bo-layered | 10–100                    | Yes                      |
| Sponge gemmules      | 250–1250 μm| Spherical | Yes                 | No        | Bi- or multi-layered |                           |                          |

Therefore, we propose that, like the MCH embryo-like fossils, Ostiosphaera could be pinned to the holozoan tree (Fig. 4). Nevertheless, the new microfossils cannot be assigned to any existing genera of embryo-like fossils from the Weng’an Biota because there are still some considerable differences between them. First, the new specimens of Ostiosphaera differ from the MCH embryo-like fossils in having a much thicker envelope and a circular aperture with a deep collar in the thickest part of the envelope. Second, unlike Helicoforamina, the new specimens of Ostiosphaera do not develop spiral loops on the surface of envelopes (Xiao et al. 2014a; Yin et al. 2020). Third, the internal cellular mass of Ostiosphaera does not form branch-like structures like that of Caveasphaera (Xiao et al. 2014a; Yin et al. 2019). These differences in biological features suggest that Ostiosphaera does not belong to Megasphaera, Caveasphaera or Helicoforamina, but to an independent taxon.

Comparison with living unicellular relatives of metazoans

Despite our reasonable attribution of Ostiosphaera to the holozoans, it is noteworthy that their envelope ornament, histology and collar-like opening suggest that they are more complex in biology than living non-metazoan holozoans, including the choanoflagellates, filastereans and ichthyosporeans (Sebé-Pedrós et al. 2017; Ruiz-Trillo and de Mendoza 2020) (Fig. 4).

As the closest unicellular relatives of metazoans, choanoflagellates possess a spherical to ovoid cell body with a long flagellum surrounded by a microvilli collar (Leadbeater 2015). Their distinctive morphology distinguishes them from the new Weng’an microfossils. Some choanoflagellates evolved a multicellular stage in their life history, but the choanoflagellate colonies share nothing in common with Ostiosphaera in terms of morphology and anatomy (Leadbeater 2015). Some species with encystment show a few similarities with Ostiosphaera because they produce thickened cysts with multiple internal cells, but the cysts are relatively smaller and lack a circular opening (Leadbeater 2015).

Among the filastereans, only Capsaspora owczarzaki has been well investigated (Sebé-Pedrós et al. 2013; Suga and Ruiz-Trillo 2015; Ferrer-Bonet and Ruiz-Trillo 2017; Sebé-Pedrós et al. 2017). Capsaspora owczarzaki is a tiny (3–5 μm in diameter) unicellular amoeba with a complex life cycle, including an aggregate multicellular stage (Ferrer-Bonet and Ruiz-Trillo 2017; Sebé-Pedrós et al. 2017). In the aggregative stage, the amoebae gather together to form a multicellular mass embedded within an extracellular matrix rather than a thick cyst (Sebé-Pedrós et al. 2013; Ferrer-Bonet and Ruiz-Trillo 2017). Capsaspora owczarzaki is clearly much smaller in size and simpler in morphology and anatomy than Ostiosphaera.

Fig. 4. Simplified holozoan tree with potential positions (marked in blue) for the new Weng’an microfossils.
Ichthyosporeans (also known as mesomycetozoeans, including two groups, Dermocystida and Ichthyophonida) develop a large number of endospores (daughter cells) inside a cyst (the cell wall of the mother cell) during their life cycle (Mendoza et al. 2002; Suga and Ruiz-Trillo 2015). They look like Ostiosphaera to some extent, but there are also differences (Mendoza et al. 2002; Marshall et al. 2008; Marshall and Berbee 2011, 2013): (1) the cysts of Dermocystida are normally thin, whereas those of Ichthyophonida are thicker, but both lack complex ornaments; (2) like the other two groups of unicellular relatives of metazoans, the ichthyosporean cysts are significantly smaller than Ostiosphaera; and (3) ichthyosporeans release endospores from the mother cell wall via a slit-like structure rather than a circular opening.

Comparison with gemmules of demosponges

Within the holozoan tree, we note that – in terms of size, morphology, envelope histology and collar-like opening as well as internal cellular mass – these new microfossils also show considerable similarities to asexual reproductive gemmules of both fossil and extant sponges. As an asexual reproductive strategy, most freshwater sponges (Class Demospongiae, Order Spongillida; in this study, we follow the taxonomic system of Demospongiae adopted by the World Porifera Database (de Voogd et al. 2021)) (Manconi et al. 2013; Manconi and Pronzato 2015; Morrow and Cardenas 2015) and some marine sponges (Class Demospongiae, Order Clionaidae, Family Clionaidae; Class Demospongiae, Order Suberitida, Family Suberitidae; Class Demospongiae, Order Haplosclerida, Family Chalinidae) (Fell 1974; Simpson and Fell 1974; Jetton et al. 1987; Morrow and Cardenas 2015) produce gemmules to survive adverse conditions. Unlike testate amoebae, the tests of which show very high interspecific variation, demosponge gemmules are invariably spherical, ranging from 250 to 1250 μm in diameter (Simpson and Fell 1974; Gilbert and Simpson 1976; Williamson and Willianson 1979) (Table 1), which is compatible with our new microfossils.

Figure 5 shows high-resolution tomographic constructions of an extant sponge gemmule (D. brownie, belonging to the demosponge Order Haplosclerida, Family Metaniidae) (Volkmer-Ribeiro et al. 2017). As a result of dehydration and degradation, the envelope of the dry gemmule in Figure 5 has shrunk, resulting in an irregular spherical shape (Fig. 5a, c, d). In general, the multi-layered envelope of sponge gemmules ranges from 10 to >100 μm in thickness (Gilbert and Simpson 1976). For example, the bi-layered envelope of the figured gemmule is very thick and shows variations in thickness (40–120 μm) due to dehydration (Fig. 4c, d, g, h). A similar variation of envelope thickness also occurred in our new microfossils (Fig. 1). The envelope of gemmules usually encloses a membrane-bound solid mass of undifferentiated stem cells (archeocytes), which are
uniform in size and shape (Ilan et al. 1996; Manconi and Pronzato 2015), like the undifferentiated cell mass preserved in our fossils. These cells are ejected through a circular, collar-like aperture called the micropyle (Ilan et al. 1996; Schill et al. 2006; Manconi and Pronzato 2015) (Fig. 5a–g, i–l), to develop into new sponges when environmental conditions improve. The sponge micropyles show similarities with the circular, deep collar-like opening of our new Weng’an fossils (Figs 1 and 2). In the dry gemmule illustrated, only microbial filamentous material can be observed in the central of the envelope as a result of strong post-mortem degradation, not the cell mass (Fig. 4c, d, g, h, j), but the membrane-like structure and the collar-like micropyle are still complete (Fig. 4a, c, d, g, h, j, l). A similar membrane enclosing the inner body has also been observed in our microfossils (Figs 1 and 2).

Contrary to the figured gemmule in which plenty of reinforcing spicules developed, no spicule was observed in our fossils. The gemmules exhibit spicules in the majority of demosponges, but this is not the case for all demosponge gemmules. Some demosponges from both freshwater and marine environments produce gemmules without any spicules (Simpson and Fell 1974; Gilbert and Simpson 1976; Manconi and Pronzato 2007, 2015). In addition, examination of early Cambrian sponge fossils suggests that the earliest Precambrian sponges might be spicule-free (Tang et al. 2019). In short, the observable biological features of Ostiosphaera are compatible with the general biology of sponge gemmules.

**Does Ostiosphaera represent an organism dwelling in hermit shells?**

It is worth considering the possibility that the internal multicellular mass of Ostiosphaera may represent a separate organism that lives within an empty and abandoned cyst belonging to another organism. For example, some testate amoebae with endosymbiotic algae living within their cysts could produce more complex structures (e.g. Nikolaev et al. 2005, their fig. 1a). Despite concluding that Ostiosphaera are unlikely to be testate amoebae as a result of some significant differences between them, as argued here, the possibility that the new fossils represent one organism living inside another cannot be readily accepted or rejected based on current evidence. More specimens are needed to test this interpretation.

**Conclusions**

The new microfossils described here show the same level of biological complexity as the MCH embryo-like fossils from the Weng’an Biota (Table 1), but they belong to an independent new taxon of Ostiosphaera n. gen. n. sp. as a result of their distinct circular opening. Although the precise affinity of Ostiosphaera remains uncertain, detailed comparisons reject the interpretations of unicellular green algae or slime mould and we prefer to follow the holozoan interpretation (Fig. 4). Although the biological complexity of Ostiosphaera is higher than that of the living non-metazoan holozoans, at this point we still cannot completely rule out the possibility that it might represent a previously unknown extinct taxon of non-metazoan holozoans (Fig. 4).

Ostiosphaera also looks like certain testate amoebae with an organic test in terms of general size, morphology and a circular opening aperture (Table 1); however, the similarities in these characters cannot support a strong affinity interpretation because unlike testate amoebae, which are single-celled organisms without multicellular stages in their life cycles, Ostiosphaera possesses multicellular stages. If the vesicle-like structures of Ostiosphaera represent fossilized intracellular organelles rather than cells, then the possibility of Ostiosphaera as extinct testate amoebae cannot yet be completely excluded, although this seems unlikely in the light of the taphonomy of the Weng’an embryo-like fossils.

We noticed that Ostiosphaera also shows considerable similarities with sponge gemmules in terms of size, morphology, envelope histology and internal structure (Table 1). Gemmules are produced by fossil and extant demosponges from both freshwater and marine environments (Fell 1974; Simpson and Fell 1974; Manconi and Pronzato 2015; Morrow and Cardenas 2015; Pronzato et al. 2017). According to the current taxonomy and phylogeny of demosponges, these gemmule-forming species are distributed in multiple clades without a latest common ancestor (Morrow and Cardenas 2015 and references cited therein). We propose two scenarios to interpret this distribution pattern of gemmule formation in the sponge tree: (1) gemmule formation evolved independently multiple times in different demoseponge lineages; or (2) it reflects a symplesiomorphic trait descended from the ancestor of all demosponges and some demoseponge lineages later lost this trait. The latter scenario is more compatible with the parsimony of evolution. We therefore argue that gemmule formation might have evolved within the earliest crown-group or even stem-group demosponges.

Even though sponge body fossils from Precambrian deposits reported previously remain controversial (Antcliffe et al. 2014), it is believed that the demosponges, as early diverging clades of metazoaos, should have arisen long before the Cambrian. Both molecular estimates (Sterling et al. 2010; Erwin et al. 2011; Dohrmann and Worheide 2017) and biomarkers (Lowe et al. 2009, 2020; Gold et al. 2016; Zamberger et al. 2018) support this assumption (but see also Bobrovskiy et al. 2020). Recently, some possible non-spicular demosponge body fossils from 890 myr ago were recovered from early Neoproterozoic microbial reefs (Turner 2021). It would therefore be no surprise if total-group demosponges were found in early Ediacaran deposits. However, we hold back on concluding that Ostiosphaera are necessarily early Ediacaran demosponges because an ultimate test of the hypothesis that the new microfossils are sponges rather than an extinct group of non-metazoan holozoans requires further evidence, including more specimens and adult sponge body fossils.

At present, the earliest known, unambiguous crown-group sponge fossils are discrete spicules from the lower Cambrian, which means, again, that there is a 200 myr gap between molecular clock estimates of the time of origin of crown-group sponges and estimates of this date based on the fossil record (Sterling et al. 2010). If the biological similarities between Ostiosphaera and demoseponge gemmules reflect shared ancestral characters rather than convergent evolution, then the new discovery of Ostiosphaera has great potential to narrow this gap by c. 74 myr (by extending the known fossil record of crown-group sponges downwards) and to force us to rethink the Precambrian history of sponges.

**Systematic palaeontology**

Kingdom Eukaryota

Phylum Incertae sedis

Genus Ostiosphaera n. gen.

**Etymology:** ‘Ostin-’, Latin, ostium, in reference to the round opening structure of the fossils; ‘sphaera’, Latin, referring to the spherical morphology of the fossils.

**Type species:** Ostiosphaera rara n. gen. and sp.

**Species composition:** Only the type species Ostiosphaera rara is included in this genus.

**Diagnosis:** As for the type species.

**Occurrence:** ‘54’ phosphate mining Quarry in Weng’an County, Guizhou Province, SW China; Doushantuo Formation, Upper Phosphorite Member (Weng’an Phosphorite Member), early Ediacaran.

**Discussion:** This new genus differs from other spherical micro-fossils in the Weng’an Biota (Ediacaran Doushantuo Formation) described previously (including Megaphaera, Caveosphaera, Helicoformamia, Spiralicella and Sporosphaera) in its distinct opening.
Ostiosphaera rara new genus and species (Figs 1 and 2)

Etyymology: Latin, rara, referring to rarity of this taxon in the Weng’an Biota.

Holotype: The specimen displayed in Figures 1, c–e and 2 (NIGP170XX) is designated as the holotype. The holotype has been deposited in the collection of Nanjing Institute of Geology and Palaeontology (NIGP), Chinese Academy of Sciences.

Diagnosis: Spherical ornamented cyst (650 μm in diameter) with a circular aperture (190–250 μm), enveloping small, discrete spheroidal membrane-bound vesicle-like structures (40 μm in diameter).

Occurrence: The Upper Phosphorite Member of the early Ediacaran Doushantuo Formation, Weng’an phosphate mining area, Guizhou Province, SW China.

Material: Only three complete specimens have been recovered from macerations so far.

Description: A globular microfossil, c. 650 μm in diameter, developed ornamented cyst with a circular opening structure with diameter c. 190–250 μm. In general, the interior volume of the fossils is filled with later void-filling minerals without discernible biological structures. The well-preserved specimen contains dozens of small spheroidal membrane-bound vesicle-like structures measuring c. 40 μm in diameter.

Remarks: The newly discovered specimens bear some resemblance to co-occurring embryo-like fossils such as Sporosphaera, Megaspheara, Caveasphaera and Helicoformina in terms of general morphology and size. For example, they share a similar size, shape and ornamented envelope with Megaspheara, Caveasphaera and Helicoformina, but they differ from Megaspheara, Caveasphaera and Helicoformina in having a circular aperture with a deep collar in the thickest part of the envelope. They contain similar internal structures as Sporosphaera, Caveasphaera and Helicoformina in having a circular aperture and a deep collar in the thickest part of the envelope. They do not develop spiral loops on the surface of envelopes. Unlike Caveasphaera, the internal cellular mass of Ostiosphaera does not form branch-like structures.

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Author contributions ZY: conceptualization (lead), data curation (lead), formal analysis (lead), funding acquisition (lead), investigation (lead), methodology (lead), project administration (lead), resources (lead), visualization (lead), writing – original draft (lead), writing – review & editing (lead); WS: investigation (supporting), visualization (supporting); JR: investigation (supporting), resources (equal), writing – review & editing (equal); MF: formal analysis (supporting), funding acquisition (equal), writing – review & editing (equal).

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships which could have appeared to influence the work reported in this paper.

Data availability

The specimens are reposited at the NIGPAS, and the datasets generated during and/or analysed during the current study are available on the 3D model database of NIGPAS at https://doi.org/10.1209/1741-2560/30/020212.

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