Anatomy and plant affinity of *Chuaria*

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*Chuaria* is one of the few globally distributed macrofossil pioneers documented in the Precambrian. It is perhaps the most controversial fossil in term of its affinity despite more than one hundred years of study. Many mutually exclusive affinities have been suggested for this frequently encountered fossil. Although often treated as a multicellular alga, this interpretation remains inconclusive because the lacking unambiguous demonstration of cellular structures. In this paper the cellular details of *Chuaria* are clearly revealed for the first time. The cell walls in *Chuaria* suggest that it is a multicellular eukaryotic alga, in agreement with the latest biogeochemical analyses. Different thicknesses of cell walls suggest primary cellular differentiation in this organism. Membrane-like structures within the cells (the first to be reported in Precambrian fossils) imply a eukaryotic nature. This study partially resolves the century-long controversy over the affinity of *Chuaria*, and makes *Chuaria* one of the few recognized multicellular eukaryotes before the Neoproterozoic glaciation.

*Chuaria*, plant, cell wall, multicellular, eukaryote, membrane
Liulaobei Formation is composed of yellowish green shales that are interbedded with thin beds of quartz sandstone, calcareous siltstone, and argillaceous limestones, with the limestone interbeds becoming increasingly abundant toward the top of the section (Figure 1b). The formation yields abundant leiospheroid acritarchs [21] and macroscopic carbonaceous compression fossils such as *Chuaria*, *Tawuia*, *Ellipsophysa*, and *Sinossabellidites* [20,22,23]. Conformably overlying the Shouxian Formation, the Jiuliqiao Formation (~71 m thick) consists mainly of thin-bedded argillaceous limestone, and stromatolitic limestone, with calcareous siltstone intercalations. Macroscopic carbonaceous compression fossils, including *Chuaria*, *Ellipsophysa*, *Sinossabellidites*, *Pararenicola*, and *Protoarenicola*, are abundant in this formation [18,20,22,23]. The formation also contains a large quantity of leiospheroid acritarch [24].

Recent Sm-Nd dating of diagenetic chert from the Sidingshan Formation yielded an isochron age of 801±46 Ma [25], and SHRIMP zircon U-Pb measurements on dolerites that intrude the Huaiabei Neoproterozoic succession in the region provided ages of 976±24 Ma and 1038±26 Ma [26]. The acritarchs of the Liulaobei Formation are comparable to those of the late Riphean (Neoproterozoic) pre-Cryogenian assemblages well known from Europe and North America [21]. Taken together, these observations suggest that fossiliferous Liulaobei and Jiuliqiao Formations are pre-Cryogenian in age.

1 Materials and methods

The fossils were collected from the Jiuliqiao and Liulaobei Formations, Shouxian, Anhui Province, China (Figure 1a,b). The specimens were first observed and photographed with an Aigo digital viewer. Replicas were made on some of the specimens [27], demineralized with HCl-HF-HCl, coated with gold, and observed using a Leo 1530 VP SEM at the Nanjing Institute of Geology and Palaeontology, Nanjing, China (NIGP). For comparison, a sample collected from a Triassic cycad leaf was processed and observed in the same way. EDXMA (energy dispersive X-ray microanalysis) on both *Chuaria* and its sediment matrix was carried out using the same SEM. A total of 14 specimens were observed using the SEM. Afterwards the replica was embedded in SPI-Pon812 resin, ultrathin-sectioned, stained with lead citrate, and observed using a Hitachi-7650 TEM at Nanjing Normal University, Nanjing, China. All *Chuaria* specimens are deposited in NIGP, while the fossil cycad is deposited in Shenzhen Fairy Lake Botanical Garden, Shenzhen, China (SFLBG). All micrographs were saved in TIFF format, and prepared for publication using Adobe Photoshop 7.0.

2 Results

The specimens of *Chuaria* were embedded in the yellowish slightly calcareous siltstone of the Liulaobei (Figure 2a–h)
and Jiuliqiao Formations (Figure 3a–g) in Shouxian, Anhui Province, China. They were circular, 1.47–1.95 mm in diameter, or oval, up to 4.5 mm × 6.5 mm, preserved as dark organic compressions (Figures 2a,b; 3a,f). Replicas made on the specimens were cleaned with HCl-HF-HCl, coated with gold and observed using SEM (Figures 2c–h; 3b,g). The organic materials appear dark brown or black in color under the light microscope (Figures 2a–b; 3a,f). Organic material in all fourteen of the specimens observed by SEM consistently revealed preserved cellular details (Figures 2c–h; 3b–e,g). The cells were polygonal in profile, 10–32 μm long and 3.6–18 μm wide (Figures 2d–h; 3b,g). The cells were separated by cell walls (Figures 2e,h; 3b,g), which varied in thicknesses (Figures 2e, h; 3b,g). The cells with thin walls were either slightly flattened, isodiametric polygons, or highly flattened, more or less elongate polygons (Figures 2e; 3b,g). The thin cell walls appear as a thin sheet about 100 nm thick, either sandwiched between cells or, where the adjacent cell had detached, the cell wall was perforated with holes 350 nm to 1 μm in diameter in surface view (Figures 2e–h; 3b,g). The thick cell walls, frequently seen at the margin of the specimen, were up to 2 μm thick.

![Figure 2](image-url)
Adjacent thick cell walls were not completely fused but were separated by a fissure (Figure 2h). Sometimes membrane-like structures were observed in the cells (Figure 3c–e). These membrane-like structures appeared different in sections at different levels of the same region in the same cell, suggesting a three-dimensional configuration (Figure 3d,e). Energy dispersive X-ray microanalyses performed on *Chuaria* and its sediment matrix indicated that the fossils were composed of organic material rich in carbon and distinct from the sediment matrix (Figure 1c). The above mentioned cells and cell walls of *Chuaria* were similar to those seen in a Triassic cycad leaf (Figure 3h,i), in which the cells appeared as blocks of organic material sandwiching thin two-dimensional cell walls (Figure 3i). There were also pits on the cell walls when the cells were removed (lower-left corner of Figure 3i).

**Figure 3** Specimens of *Chuaria* with cellular details and ultrastructures, and their comparison with those of a fossil cycad. a–e (PB21279) and f–g (PB21278), deposited in NIGP; h–i (LNFC-2007-001), deposited in SFLBG. All are SEM except Figure 3a, f, h (LM) and Figure c–e (TEM). Figure 3b, g and i is of replicas. a, A circular specimen with dark organic material. Bar = 1 mm. b, Cells with cell walls (white arrow) sandwiched between them. Bar = 5 μm. c, Several layers of membrane-like structures in the cells. Bar = 100 nm. d, e, The same membrane-like structures in the same cell. Note the subtle difference between the corresponding structures (arrows) at different levels. Bar = 100 nm. f, A specimen of dark organic material. Note its circular shape and the dark organic material. Bar = 1 mm. g, A broken surface showing cells (C), cell walls in cross- (black arrow) and surface-views (at the right), and pits on the cell walls (white arrows). The inset shows a detailed view of the pits, with a 1 μm bar. Bar = 5 μm. h, A Triassic cycad (*Holozamites hongtaoi* [28]) leaf from western Liaoning, China. Note the dark drop of the liquid that latter condenses into the replica showed in Figure 3i. i, Cells with preserved organic material in the cycad leaf. Note the cell walls (black arrow) either sandwiched between cells (C) or freely standing where the cells have detached (white arrows), and cell wall with pits in surface view (lower-left corner).
3 Discussion

Chuaria is a circular, discoidal fossil of millimeter dimensions, consisting of carbonaceous compressions. It is also one of the oldest known macroscopic body fossils [7]. Despite its long history of study, the biological affinity of Chuaria has been problematic for a variety of reasons. Well-preserved coalified specimens of Chuaria were studied here using the replica technique to reveal cellular details. This technique makes a faithful impression of cellular structures, which also may bring some organic material with intact cellular features to be studied and demonstrated clearly using SEM and TEM (Figures 2c–h; 3b–e.g). The thin cell walls appeared as sheets of organic material sandwiched between cells (Figures 2e; 3b,g). This was confirmed by observation on original samples that had cleaned with HCl and thus, eliminated the possibility of artifacts introduced by the replica technique. The space between the cells and their cell walls may reflect the original difference in organization and differentiated shrinkage in the fossils, and this space remained unfilled despite subsequent compression. This space is distinct from the previously reported cracks in the “amorphous” envelope of Chuaria (see Plate 4, Figure 2 in Steiner [10]), which lacked a cell wall between the adjacent blocks. Disturbance during demineralion channels (pits and plasmodesma) between these cells. An inorganic infilling, if present, would have been dissolved to some degree during the demineralizing process. The congruence between observations before and after demineralization suggests that, in this case, the cell wall was composed of organic material derived from the organisms themselves. Most of the thin cell walls appeared as sheets of organic material about 80–150 nm thick, and their thickness was independent of the gap between cells. An inorganic in-filling, if present, would be expected to have a thickness closely correlated with the gap dimension; (4) pits were frequently observed on the cell walls (Figures 2e; 3g). They appeared as irregularly scattered circular holes on the cell wall, quite similar to those seen in another cycad fossil plant (lower-left corner in Figure 3i). Their existence suggests possible communication channels (pits and plasmodesmata) between these cells, which are a feature of eukaryotes. Their presence is hard to explain by inorganic infilling; (5) the thick cell walls (Figure 3h–i) framed concave lumina, just like those of living plants; (6) some of the thick cell walls were composed of two layers (Figure 2h) apparently representing two adjacent cell walls. This phenomenon cannot be attributed to inorganic infilling between organic materials but compares well with cell walls in living plants; and (7) membrane-like structures within the cells of Chuaria (Figure 3c–e) were very similar to the ER or other membranes seen in other much younger fossil plants [29–36]. These membrane-like structures suggest strongly a eukaryotic nature of Chuaria. In short, the constant and coherent spatial relationships among the cells, cell walls, pits on the cell walls, and membrane-like structures in the cells of Chuaria are similar to those in extant and fossil plants and suggest that cell walls are an authentic feature of Chuaria. These cellular details indicate that Chuaria belongs to the plant kingdom.

There are many features that distinguish plants from animals, e.g. the presence of chlorophyll and chloroplasts, vacuoles, and cell wall. Among these, cell wall is by far the most idiosyncratic anatomical feature that can be used to distinguish plants from animals. The presence of cell walls in Chuaria eliminates the possibility that it is an animal. Its multicellularity, pits on cell walls, and membrane-like structures eliminate the possibility of it being a prokaryote, as suggested previously [11,12]. The cells with thick cell walls were observed only at the margins of the organism, suggesting a possible role in retaining the form of the organism (as in living plants) (Figure 2h). This observation is in accord with Amand’s study [8] on Chuaria although his conclusion concerning its affinity is different from ours. The latest geochemical studies indicate that Chuaria contains aliphatic biopolymer, suggesting an algal origin, which is in agreement with our conclusion [17,19]. Although the three-dimensional distribution of the cell types in the organism is still unclear, the presence of different types of cells implies that there is a primary cellular differentiation in Chuaria.

Many fossils of similar age and morphology have been regarded as Chuaria. Many of them differ from the present observations, especially in their dimensions. Therefore it is likely that not all fossils named as Chuaria belong to the same biological taxon. However, the consistent SEM observations on all fourteen specimens of Chuaria studied here certainly suggest that all of these macroscopic Chuaria belong to the plant kingdom. It is logical to assume that Chuaria has an important role as a primary producer in the Proterozoic ecosystem.

There are relatively few well-recognized multicellular macroscopic eukaryotes recorded for the period before the Neoproterozoic glaciation [37–40]. Our study indicates that these discoid Chuaria were important eukaryotic elements during the Proterozoic, and that they represent the evolutionary level of the multicellular eukaryotes at that time.

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