Diversity and evolutionary growth of biomineralized columns in early Cambrian phosphatic-shelled brachiopods

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Abstract Biologically controlled mineral crystals producing organic-inorganic composites (hard skeletons) by metazoan biomineralizers has been an evolutionary innovation for more than half billion years on Earth. Among them, linguliform brachiopods are the only invertebrates that secrete phosphate to build their skeletons. One of the most distinct shell structures is the organic-phosphatic columns applied exclusively by phosphatic-shelled brachiopods. However, the complexity, diversity and biomineralization process of these microscopic columns are far from clear in brachiopod ancestors. Here, exquisitely well preserved columnar structures are discovered for the first time in the earliest eoobolids. The hierarchical shell architectures, epithelial cell moulds, and the shape and size of cylindrical columns are scrutinised in *Latusobolus xiaoyangbaensis* gen. et sp. nov. and *Eoobolus acutulus* sp. nov from the Cambrian Series 2 Shuijingtuo Formation of South China. The secretion and construction of the stacked sandwich model of columnar shell, which played a significant role in the evolution of linguliforms, is highly biologically controlled and organic-matrix mediated. Furthermore, a continuous transformation of anatomic features resulting

from the growth of columns is revealed between Eoobolidae, Lingulellotretidae and Acrotretida, shedding new light on the evolutionary growth and adaptive innovation of stacked sandwich columns among early phosphatic-shelled brachiopods during the Cambrian explosion.

Introduction

The early Cambrian witnessed a great burst of animal body plans and biomineralized shell architectures around half billion years ago (Briggs, 2015; Erwin, 2015, 2020; Budd and Jackson, 2016; Murdock, 2020; Zhang and Shu, 2021; Yun et al., 2021; Z.L. Zhang et al., 2021). The novel biomineralizations, biologically controlled mineral crystals producing organic-inorganic composites (hard skeletons), in these animals have played a vital role in the survival and fitness of early species (Balthasar and Cusack, 2015; Cuif et al., 2010; Li et al., 2022; Skovsted et al., 2008; Yun et al., 2022), and in turn build the fundamental bricks of complex marine ecosystems (Bicknell and Paterson, 2018; Buatois et al., 2020; Chen et al., 2022; Zhang et al., 2010; Z. Zhang et al., 2020). Since the early Cambrian to the present day, this adaptive evolution has been excellently demonstrated and continuously preserved in brachiopods, one of the key members of Cambrian Evolutionary Fauna (Carlson, 2016; Harper et al., 2017a, 2021; Sepkoski, 1984). Among them, the phosphatic-shelled brachiopods are some of the most common animals in early Cambrian faunas. With high fidelity preservation and high abundance of biomineralized shells in the fossil record, their morphological disparity, diversity of shell structures and growth patterns, and ecology complexity are preserved in great detail (Chen et al., 2021; Claybourn et al., 2020; Zhang, 2018; Z. L. Zhang et al., 2020a, 2021b).

Studying the processes by which organisms form biomineral materials has been the focuse at the interface between earth and life sciences (Leadbeater and Riding, 1986;

Simonet Roda, 2021). Brachiopods are very unique animals in having the ability to secrete two different minerals, calcium phosphate and calcium carbonate, making them the ideal group to further explore the processes of biomineralization (Simonet Roda et al., 2021a; Williams, 1977). Hard tissues composed of calcium phosphate with an organic matrix are also largely present in vertebrates, which have remarkably shaped the evolutionary trajectory of life on Earth (Ruben and Bennett, 1987; Wood and Zhuravlev, 2012). The origin of phosphate biomineralization in the evolutionary distant invertebrate brachiopods and vertebrates is still a big mystery in animal evolution (Luo et al., 2015; Neary et al., 2011; Simonet Roda, 2021). Thus, more research is needed in order to understand how to relate hierarchical structure to function in the very early examples of calcium phosphate-based biomineralization process (Kallaste et al., 2004; Weiner, 2008). It is noteworthy, that South China has been considered as one of centres for the origination and early dispersal of phosphatic-shelled brachiopods (Z. L. Zhang et al., 2021a). It provides a great opportunity to explore the unique biomineralization process and consequent adaptive evolution of their earliest representatives during the Cambrian radiation of life.

The shell forming process of brachiopods have long represented a conundrum, which is crucial to unravel their poorly resolved phylogeny and early evolution (Carlson, 2016; Cusack and Williams, 2007; L. Holmer et al., 2008; Murdock, 2020; Streng et al., 2007; Temereva, 2022; Williams et al., 2004; Williams and Cusack, 1999). Extensive studies have been conducted on living and fossil shells, but most of them are focused on articulated or carbonate-shelled representatives (Cusack et al., 2010; Griesshaber et al., 2007; Simonet Roda et al., 2021b; Simonet Roda, 2021; Ye et al., 2021). By contrast, the linguliform brachiopods, which are composed of an organic matrix and apatite minerals that show extremely intricate architectures and permit exquisite preservation, are less well understood, and their shell structural complexity and diversity, especially of their fossil representatives require further studies (Cusack et al., 1999; Streng et al., 2007; Williams and Holmer, 1992; Zhang et al., 2017). The building of shells by microscopic cylindrical columns is a unique

feature, which is restricted to the phosphatic-shelled brachiopods and their assumed ancestors (L. Holmer et al., 2008; Holmer, 1989; Holmer et al., 2002; Skovsted and Holmer, 2003; Williams and Holmer, 2002a). These type of columns were previously believed to be exclusively restricted to micromorphic acrotretide brachiopods, a group which demonstrate more complex hierarchical architectures and graded structures compared to simple lamella shell structure in older lingulides (Holmer, 1989; Williams and Holmer, 1992). As a result of further studies, diverse columnar shell structures have been recognised in more brachiopod groups, including stem group taxa *Mickwitzia* and *Micrina* (Skovsted and Holmer, 2003), lingulellotretid *Lingulellotreta* (L. Holmer et al., 2008), eoobolid *Eoobolus* (Streng et al., 2007; Z. L. Zhang et al., 2021a), and the possible paterinide *Bistramia* (L. Holmer et al., 2008). However, the columnar shells among the oldest linguliforms and their evolutionary variations have not been scrutinised.

For the first time, exquisitely well preserved columnar shell structures are described from the oldest known eoobolid brachiopods. *Latusobolus xiaoyangbaensis* gen. et sp. nov., and *Eoobolus acutulus* sp. nov. are described here, based on new specimens from the Cambrian Series 2 Shuijingtuo Formation of southern Shaanxi and western Hubei in South China. In this study, the shell architectures, epithelial cell moulds, and the shape and size of cylindrical columns are minutely examined, shedding new light for understanding the architecture intricacy, biomineralization process and evolutionary fitness of early phosphatic-shelled brachiopods.

Results

Systematic palaeontology

Brachiopoda Duméril, 1806

Linguliformea Williams, Carlson, Brunton, Holmer and Popov, 1996

Lingulata Gorjansky and Popov, 1985

Lingulida Waagen, 1885

Linguloidea, Menke, 1828

Eoobolidae, Holmer, Popov and Wrona, 1996

Genus ***Latusobolus*** Zhang, Zhang and Holmer gen. nov.

Type species. *Latusobolus xiaoyangbaensis* sp. nov., here designated.

Etymology. From the Latin '*latus*' (wide) with the ending '*obolus*', to indicate the transversely oval outline of both ventral and dorsal valves, morphologically similar to *Obolus*. The gender is masculine.

Diagnosis. For a full description and discussion of *Latusobolus* gen. nov., refer to Appendix 1.

Latusobolus xiaoyangbaensis Zhang, Zhang and Holmer sp. nov.

Figure 1 and ***Appendix 2—figures 1-4***, ***Appendix 3—table 1***.

Etymology. After the occurrence at the Xiaoyangba section in southern Shaanxi, China.

Type material. Holotypes, ELI-XYB S5-1 BR09 (***Appendix 2—figure 1M–P***), ventral valve and ELI-XYB S4-2 BO11 (***Appendix 2—figure 2M–P***), dorsal valve, from Cambrian Series 2 Shuijingtuo Formation at the Xiaoyangba section (Z. L. Zhang et al., 2021a) near Xiaoyang Village in Zhenba County, southern Shaanxi Province, China.

Diagnosis. As for the genus.

Description. For a full description and discussion of *Latusobolus xiaoyangbaensis* gen. et sp. nov., refer to Appendix 1.

Genus ***Eoobolus*** Matthew, 1902

Type species. *Obolus* (*Eoobolus*) *triparilis* Matthew, 1902 (selected by Rowell, 1965)

Diagnosis. See Holmer et al. (p. 41) (Holmer et al., 1996).

Eoobolus acutulus Zhang, Zhang and Holmer sp. nov.

Figure 2 and ***Appendix 2—figures 5–7***, ***Appendix 3—table 2***.

Etymology. From the Latin '*acutulus*' (somewhat pointed), to indicate the slightly acuminate ventral valve with an acute apical angle. The gender is masculine.

Type material. Holotypes, ELI-AJH S05 BT11 (***Appendix 2—figure 5E–H***), ventral valve, and ELI-AJH S05 1-5-07 (***Appendix 2—figure 5M***), dorsal valve, from Cambrian Series 2 Shuijingtuo Formation at the Aijiahe section (Z. L. Zhang et al., 2016) near Aijiahe Village in Zigui County, north-western Hubei Province, China.

Diagnosis. For a full description and discussion of *Eoobolus acutulus* sp. nov., refer to Appendix 1.

Discussion

Complex linguliform brachiopod shells

Although the supposed living fossil *Lingula* has long been considered to virtually lack morphological evolutionary changes (Schopf, 1984), more recent studies have shown that it actually has experienced dramatic modifications in many aspects (Liang et al., 2023), including internal organs (Zhang et al., 2008a), life mode (Topper et al., 2015), shell structures (Cusack et al., 1999), and even genome (Goto et al., 2022; Luo et al., 2015). The complexity and diversity of linguliform shell architecture was increasingly recognised in the

pioneering study of Cusack, Williams and Holmer (Cusack et al., 1999; Holmer, 1989; Williams and Cusack, 1999; Williams and Holmer, 1992). What's more, such complex architectures have a wide distribution in closely related brachiopod groups at the beginning of Cambrian, when they made their first appearance. In connection with an ongoing comprehensive scrutiny of well-preserved linguliform shell architectures from the early Cambrian limestones of South China, their complexity and diversity hidden in the conservative oval shape is becoming more and more intriguing. However, compared to their living representatives, the shell structures are relatively simple, revealing profound modifications during the long evolutionary history (Cusack et al., 1999; L. Holmer et al., 2008; Williams, 1977; Williams and Cusack, 1999).

In general, the shells of organo-phosphatic brachiopods are stratiform, composed of an outer periostracum and inner rhythmically-disposed succession of biomineralized lamellae or laminae (Holmer, 1989; Williams, 1977). The organic periostracum, serving as a rheological coat to the underneath shell, is scarcely fossilized. However, its wrinkling and vesicular features have largely been preserved as superficial imprints (pits, pustules, fila, grooves, ridges, rugellae, drapes, reticulate networks, spines) on the surface of the primary layer, and are important characters in understanding brachiopod phylogeny (Holmer 1989; Holmer *et al.* 1996; Cusack *et al.* 1999). By contrast, the structures preserved in the secondary layer have been characterised in fossil and living groups by three ancient fabrics –columnar, baculate, laminated – all of which persists in living shells except for the columnar fabric (Cusack et al., 1999).

The primary layer commonly consists of one heavily biomineralized compact lamina composed of apatite granules, with a thickness from 2 μm to 20 μm (***Figures 1B**, **C*** and ***2G***). Usually, the concentric growth lines are regularly distributed on primary layer surface of post-metamorphic shell. By contrast, the surface ornamentation tends to be less ordered in distribution, demonstrating a strong phylogenetic differentiation. Superficial pustules are one of the most distinct patterns and are readily recognised in one of the oldest brachiopod

group, the Eoobolidae (Holmer et al., 1996). The pustules are roughly round in outline, composed of apatite aggregates, and range from 2 μm to 20 μm in diameter (***Figure 1G***). Such pustules are also found on early obolid, zhanatellid and acrotheloid shells with relatively wide size variations from 5 μm to 30 μm. Although different in size, the similar pattern may indicate a same secretion regime that originated as vesicles during the very early stages in periostracum secretion (Cusack et al., 1999). The thickness of the underneath secondary layer varies greatly in different brachiopod groups, depending on shell component and fabric type. Columnar, baculate and laminated fabrics are incorporated into the basic lamination component to form the diverse stratiform successions of secondary shell layer.

The fossil record reveals that the columnar shell structure is generally preserved in most early linguliforms (L. Holmer et al., 2008; Streng et al., 2007; Williams, 1977; Zhang, 2018; Z. L. Zhang et al., 2020a, 2021a). It is a multi-stacked sandwich architecture (multi columnar laminae are stacked in a vertical direction), which is initially developed in the earliest linguliform *Eoobolus* as an early stage (Z. L. Zhang et al., 2021a). One to three stacked sandwich units with short columns can also be found in *Latusobolus xiaoyangbaensis* gen. et sp. nov. (***Figure 1***) and *E. incipiens* (***Figure 4A***), while the number of stacked sandwich unit increased in later eoobolids (***Figure 2***), obolids and lingulellotretids (***Figure 4B–D***). Eventually, the acrotretides developed a more complex columnar layer with multiple stacked sandwich units (***Figure 4F–J***). Moreover, the columnar shell structures were also found in stem group brachiopods, e.g., *Mickwitzia*, *Setatella* and *Micrina*, but with different column size and numbers of lamination (L. Holmer et al., 2008; Holmer et al., 2002; Skovsted et al., 2010; Williams and Holmer, 2002a). It is assumed here that the columnar layer maybe plesiomorphic in linguliform brachiopods, and was inherited from stem group brachiopods.

Biomineralization process of organo-phosphatic columnar shell

Metazoans are known for secretion of very different types of biominerals through the biological mineralization process, which linked the soft organic tissues of life with minerals of solid earth in processes that would revolutionised the Earth's fossil record (Addadi and Weiner, 2014; Lowenstam and Weiner, 1989; Simonet Roda, 2021; Wood and Zhuravlev, 2012). Because of the fine nature of organo-phosphatic biomineralization in linguliforms (Cusack et al., 1999; Williams and Cusack, 1999), they have an exquisitely finely preserved shell architectures (***Figures 1-2***, ***3A***, ***B*** and ***4***), including the epithelium cell moulds (***Figure 3C*** and ***F–I)*** among the earliest eoobolids, lingulellotretids and acrotretids. This permits us to reconstruct the biomineralization process of their apatitic cylindrical columns and address key questions how these hierarchical structures relate to mechanical functions. Although the biomineralization process of living brachiopods at the cellular level is not well known yet, many biochemical experiments (Cusack et al., 1999, 1992; Lévêque et al., 2004; Williams and Cusack, 1999) reveal the high possibility of the biologically controlled and organic matrix mediated extracellular mineralization during brachiopod shell secretion, which can be compared to the hard tissue forming process of mollusc shells and vertebrate teeth (Golub, 2011; Neary et al., 2011; Simonet Roda, 2021).

Many generally polygonal structures(***Figure 3C*** and ***F***), preserved on the internal surface of successively alternating laminae (***Figure 3G–I***), have generally been considered to represent the moulds of epithelial cells (McClean, 1988). The average size of epithelial cells is 20 µm, ranging from 5 to 30 µm. The rheological vesicle environment may cause the shape variation of epithelia, while different secreting rate could result in different sizes; usually smaller epithelial cells had high secretion rates (Z. L. Zhang et al., 2016). As the active secretion activity of outer epithelial cells, they can be easily embedded in the newly formed inner and outer bounding surfaces of shell laminae (McClean, 1988; Winrow and Sutton, 2012), which left shallow grooves between epithelial margins as intercellular boundaries *(**Figure 3F***). Epithelial cells are preserved as moulds – presumably by the

phosphatization of smooth organic sheets (***Figure 4G*** and ***H***) – that had been secreted relatively slow by the outer plasmalemma of the outer mantle (Cusack et al., 1999).

These active cells are responsible for the secretion of linguliform periostracum with rhemorphic wrinkling features (Cusack et al., 1999), which left typical microtopography on the external surface of primary layer beneath (***Figure 3A*** and ***B***). On the other hand, they also secreted linguliform biomineral in a rheological extracellular environment, inferred from living *Lingula* as apatite growing from an amorphous calcium phosphate precursor, which forms the basic crystals of apatite around 5 nm in diameter (Lévêque et al., 2004; Williams and Cusack, 1999). These nanoscale crystals were packaged into apatite granules with an average size of 100 nm (***Figures 1I*** and ***2K***), which acted as the fundamental component building up the hierarchical stratiform columnar shells, including the primary layer and secondary layer (***Figure 5***). The apatite granules were probably coated or saturated with organic compounds to form granule aggregations or clusters (spherular mosaics) as irregular spherules or rods of about 500 nm (***Figures 1I*** and ***2H****;* ***Appendix 2—figure 7J*** and ***K***), usually leaving gaps between the aggregation boundaries in fossils after the degradation of organic counterparts (***Figure 2G****;* ***Appendix 2—figure 4K***). These apatite spherules are aggregated in the planar orientation as a compact thin lamella less than 4 µm. Several thin lamellae are closely compacted as the primary layer (***Figure 1B*** and ***C***). Some less obvious gaps were preserved, and they may indicate the existence of organic matrix intercalated randomly (***Figures 1D***, ***I*** and ***2F***; ***Appendix 2—figures 4I***, ***J*** and ***7J***). On the other hand, similar nanometre scale networks of spherules are aggregated to be linearly organised as an orthogonal column perpendicular to the layer surface. Each columnar composite unit consists of numerous columns disposed orthogonally between a pair of compact lamella (Cusack et al., 1999; Holmer, 1989). Multi columnar laminae are stacked in a vertical direction from exterior to interior to form the secondary layer, applying a stacked sandwich model (***Figure 5***), which differs from the layer cake model. A very thin gap, commonly less than 1 µm, between each pair of stacked column lamina is very obvious in well preserved

specimens (***Figures 1C***, ***D***, ***I***, ***2C***, ***F***, and ***4C**; **Appendix 2—figures 4I*** and ***7J***), probably indicating one organic membrane acts as an extracellular matrix with functions of a template guiding mineral nucleation (Addadi and Weiner, 2014; Cusack et al., 1999; Lévêque et al., 2004). This is also supported by the symmetrical nature of the stacked columnar architecture, revealing a homogeneous organic substrate responsible for the succeeding rhythmic sequence. Although newly secreted columnar unit may succeed the older one unconformably with overlap (***Figure 2C***) and be involved in lateral facies changes (***Figure 2K*** and ***4C***), it is supposed to reflect intracellular deviation with the same secretory cycle with the outer mantle as a whole as in living lingulids (Cusack et al., 1999; Williams et al., 1992). The secreting and building process of the early Cambrian phosphatic-shelled brachiopod columnar shell, secreted by the underlying outer epithelium cells of the mantle lobe, is demonstrated in ***Figure 5***.

It is worth noting that nanoscale openings are permeated on the terminal end of the columns (***Figures 1C**, **4E*** and ***H***) and surface of columnar laminae (***Figures 1H**, **2H***, ***3E*** and ***4G***) on well preserved specimens. The openings are round in shape with a mean size of 600 nm. Observation from some natural fractures of shells, some canals can be traced continuously through several columnar sequences (***Figures 2G*** and ***4E***), while in most other poorly preserved fossils, they are filled with secondarily phosphatised spherules or mosaics (***Figures 1I*** and ***2G***), occasionally leaving random gaps (***Figures 1J***, ***2F*** and ***4I***). These canals are very likely empty space of degraded organic material, which is confirmed by a different taphonomic process that preserves canals in contemporaneous Cambrian siliciclastic rocks (Duan et al., 2021). The regular arrangement of the canal systems and closely related columns revealed that the process is organic matrix-mediated, and moreover the even disposition of the organic matrix in columns indicate the rheological and center areas, on which biologically controlled biomineralization took place that the nucleation, growth and aggregation of the deposited amorphous calcium phosphate is directed by the same group of epithelial cells (Cusack et al., 1999; Pérez-Huerta et al., 2018; Simonet Roda,

2021; Weiner and Dove, 2003). In the stacked columnar units, the chambers between each columns delineated by a pair of compact lamellae (***Figure 4F*** and ***J***) would be originally filled with glycosaminoglycans (GAGs) as in living *Lingula* (Cusack et al., 1999; Williams et al., 1994). The chambers are often filled with coarse spherular mosaics when being secondarily phosphatised, resulting in being indistinguishable between columns, paired compact lamellae and organic membrane (***Figures 1D*** and ***2F***). At the margin of mature shells, especially the ventral pseudointerarea where the vertical component of growth vector becomes increasingly important, the short columns are replaced by relatively higher columns, resulting in the transformation from two stacked columnar units into one unit during growing anteriorly (***Figures 2C***, ***K***, ***3I*** and ***4C***), which may demonstrate allometric growth of shell (Cusack et al., 1999).

The most intriguing and enigmatic phenomenon of skeletal biomineralization is the evolutionary selection of calcium carbonate and calcium phosphate in invertebrates and vertebrates, respectively (Lévêque et al., 2004; Luo et al., 2015). However, the Brachiopoda is a very unique phylum that utilises both. For the subphylum Linguliformea, the appearance of apatite as a shell biomineral dates back as far as the early Cambrian (Topper et al., 2013; Ushatinskaya, 2002) and persists to the present (Carlson, 2016). Calcium phosphate can build a relatively less soluble skeletal component as compared with calcium carbonate shells, but with the disadvantage of a greater energetical and physiological cost (Wood and Zhuravlev, 2012). The acquisition of this specific biomineral in phosphatic-shelled brachiopods has been considered an ecological consequence of the globally elevated phosphorous levels during phosphogenic event in the calcite seas with a low Mg:Ca ratio and/or high CO_2 pressure (Balthasar and Cusack, 2015; Brasier, 1990; Cook and Shergold, 1984; Wood and Zhuravlev, 2012). In such situations, linguliforms were likely to utilise the sufficient phosphorous in ambient waters by chance from a brachiopod ancestor with an unmineralized shell coated with detrital grains, like *Yuganotheca* found in the Chengjiang Lagerstätte (Zhang et al., 2014), possibly as an evolutionary response of prey to an

escalation in predation pressure during the Cambrian explosion of metazoans (Cook and Shergold, 1984; Wood and Zhuravlev, 2012). Consequently, organic-rich biomineral composites of linguliform brachiopod shells possessed innovative mechanical functions, providing competitive superiority and adaptation on Cambrian soft substrate as well as reducing susceptibility to predation (Wood and Zhuravlev, 2012). Thus, even with the physiological cost of biomineralization in demanding metabolism keeps increasing, linguliforms keep their phosphate shells during dramatically oscillations of sea water chemistry and temperature over 520 million years.

Given the long history of this subphylum, the possession of a phosphatic shell must have had numerous advantages. The innovative columnar fabric can economically increase the thickness and strength of the shell by the presence of numerous, stacked thinner laminae, comparable with the laminated fabric seen in obolids (Cusack et al., 1999; Z.-F. Zhang et al., 2016). Furthermore, the sandwich architecture also increases the toughness, flexibility and the ability to resist crack propagation by filling the space between the compact lamellae with organic material, comparable with the baculate fabric (Lévêque et al., 2004; Merkel et al., 2009). Thus, the stacked sandwich model of the columnar shell possesses a greater advantage of mechanical functions and adaptation with a superior combination of strength, toughness and flexibility, resembling the colonnaded and reinforced concrete constructions of our buildings. This type of more efficient and economic shell may also have been responsible for the early diversity of linguliform brachiopods during the Cambrian explosion, resulting in this group becoming a significant component of the Cambrian Evolutionary Fauna (Bassett et al., 1999; Sepkoski, 1984; Zhang et al., 2008b; Z. Zhang et al., 2020; Z. L. Zhang et al., 2021b). New data from nuclear magnetic resonance spectroscopy and X-ray diffraction reveals that apatite in brachiopod shells is highly ordered and thermodynamically stable crystalline that is more robust in the extremes of moisture, ambient osmotic potential and temperature, unlike the poorly ordered crystal of vertebrate bone (Neary et al., 2011).

Evolution of stacked sandwich columns in early brachiopod clades

Evolutionary transformations have repeatedly modified the organo-phosphatic architecture consisting of various aggregates of spherular apatite, held together by a scaffolding of glycosaminoglycan complexes, fibrous proteinaceous struts and chitinous platforms, in linguliform brachiopod shells since the early Cambrian (Cusack et al., 1999). As one of the oldest type of brachiopod shell architectures, the columnar shell has previously been regarded as an unique character of acrotretide brachiopods (Cusack et al., 1999; Holmer, 1989), however, more recent discoveries of columnar shell in a diversity of early Cambrian stem group brachiopods have revealed that the same biomineralization strategy is also utilised in a wider range of early brachiopod and brachiopod-like groups (L. Holmer et al., 2008; Holmer et al., 1996; Skovsted et al., 2010; Streng et al., 2007; Ushatinskaya and Korovnikov, 2014; Zhang, 2018; Z. L. Zhang et al., 2021a). This increases the need for a better understanding of the origin and adaptive modification of stacked sandwich columns in the early lophophorate evolution.

The Eoobolidae is presently considered to be the oldest known linguliform brachiopods (Z. L. Zhang et al., 2021a). The biomineralized columns in *Eoobolus incipiens* from Cambrian Stage 3 probably represents an early and simple shell structure type with a poorly developed secondary columnar layer (***Figure 4A***). The columns are relatively small with a mean diameter of 1.8 µm ranging from 0.6 to 3.0 µm, and a mean height of 4.1 µm (***Appendix 3—table 3***). Furthermore, the complete secondary layer is just composed of two to three stacked columnar units, resulting in a shell thickness of about 30 µm. Such simple shell structure is also developed in slightly younger *Latusobolus xiaoyangbaensis* gen. et sp. nov. (***Figure 1***), but with a slightly higher column of about 6.2 µm. From Cambrian Age 4, the unit number of layers increases rapidly in the Eoobolidae, growing as many as 10 stacked columnar units in *Eoobolus acutulus* sp. nov. (***Figure 2***), and as many as 10 in *Eoobolus*? aff. *priscus*, and the shell thickness increases to 50 µm. However, the size of individual columns keeps within a very stable range, around 4 µm in height and 2 µm in

diameter. Compared with Eoobolidae, the Lingulellotretidae, demonstrates a more developed columnar shell, which has a relatively larger number of columnar units up to 20, effectively increasing the shell thickness to 70 µm. The column size is very similar in both *Lingulellotreta malongensis* and *L. ergalievi* (***Figure 4B*** and ***D***), and matches that of contemporaneous *Eoobolus*. But the slightly younger *L. ergalievi* has more columnar units, resulting in a thicker shell than the former.

Among all early Cambrian linguliforms with columnar shells, the acrotretides have developed the most complex shell structure (***Figure 4F–J***). Furthermore, the successive fossil record in Cambrian unveiled an obvious pattern of the increasing growth of stacked columns in acrotretides from a very simple type in *Palaeotreta shannanensis* like *E. incipiens* to slightly developed *Palaeotreta zhujiahensis* like *L. malongensis*, and to the most developed *Eohadrotreta zhenbaensis* onward (***Figure 6***). The diameter of column increases about two times in acrotretides from linguliforms, whereas the height of column increases to 10 µm in *Eohadrotreta zhenbaensis* and 29 µm in *Hadrotreta primaeva*, which is about 10 times as high as in *Eoobolus variabilis*. The number of columnar units has also increased to 30, which collectively increased the shell thickness to a maximal value of more than 300 µm.

The homology and continuity of the columnar shell in early linguliforms outlines a clear picture, most likely representing an evolutionarily continuous transformation between Eoobolidae, Lingulellotretidae and Acrotretida, which represent the major components of early Cambrian benthic communities (Chen et al., 2021; Claybourn et al., 2020; Topper et al., 2015; Zhang et al., 2008a; Zhang, 2018; Z. L. Zhang et al., 2020a). However, the possible occurrence of this shell fabric within the family Obolidae cannot be discussed here, as detailed information of the possible columnar shell structures in *Kyrshabaktella* and *Experilingula* are too poorly known (Cusack et al., 1999; Streng et al., 2007; Williams, 1977). The small size and simple patterns of stacked columnar shells remain very stable in the Eoobolidae since its origin, which likely limits both the shell thickness and overall body size, with ventral and dorsal valves of the family below 6 mm until Miaolingian (middle Cambrian).

Stacked sandwich columns are a character of the *Lingulellotreta* shell structure as well; however more columnar units are developed to slightly increase the shell thickness and species reach body size twice that of Eoobolidae (Zhang et al., 2007). A continuous transformation of anatomic features can be deduced from the growth of columns between the two clades. Firstly, the stacked columns are markedly developed at the pseudointerarea area of ventral valve, which causes the great elevation of the pseudointerarea above the shell floor (***Figure 2A***). It leaves a large amount of space for the posterior extension of the digestive system, which are well protected by the covering mineralized ventral pseudointerarea. This is evidenced by the discovery of a curved gut under the pseudointerarea in the soft tissue preserved Chengjiang Lagerstätte (Zhang et al., 2007). Secondly, with the continuous growth of the ventral pseudointerarea from both lateral sides, the opening obolide-like pedicle groove is sealed off, resulting in a unique pedicle opening exclusively observed in the lingulellotretid brachiopods. Thus, the pedicle protruding opening between the ventral and dorsal valves of Lingulida is transformed to a new body plan where the pedicle opening is restricted in ventral valve. It supports the scenario that the columnar shell structure is monophyletic in at least Linguloidea, and that the younger lingulellotretid columnar shell was derived from an *Eoobolus*-like ancestor during late Cambrian Age 3.

In another evolutionary direction, acrotretide brachiopods fully utilise the columnar fabric including the derived camerate fabric as shell structure in the whole clade (Streng and Holmer, 2006). The similarity and gradually evolutionary transformations of columns from simple forms in lingulides to complex types in acrotretides suggests the stacked sandwich columns did not evolve independently in actrotretides. In terms of the derived camerate fabric, more mineralized material is utilised compared to its precursor, the columnar fabric (Streng et al., 2007). The column size, including height and diameter, and the number of stacked columnar units are constantly increasing to about 10 to 20 times greater in acrotretides than in *E. incipiens and L. xiaoyangbaensis*, since late Cambrian Age 3 (***Figure 6***). Despite the increase in size and number, the whole body size of acrotretides is restricted

in millimetre size (Holmer, 1989; Williams et al., 2000). A continuous transformation of anatomic features and shell structure functions can be deduced from the evolutionary growth of columns in early acrotretides. Firstly, the stacked columns were markedly developed at the pseudointerarea area of the ventral valve, which resulted in a greater elevation of the pseudointerarea above the valve floor, compared to that of *Lingulellotreta*. Two transformations subsequently changed an obolid-like flat ventral valve (*Palaeotreta shannanensis*) to a cap shape vale (*P. zhujiahensis*) (Z. L. Zhang et al., 2020b), and a conical shape (*Eohadrotreta zhenbaensis*) (Zhang et al., 2018), and eventually to a tubular shape (*Acrotreta*) (Holmer and Popov, 1994). During this time, dorsal valves constantly kept a flat shape. The obolid-like ventral pseudointerarea changed from orthocline to catacline and eventually to procline with strongly reduced propareas, while ventral muscular system moved to the elevated posterior floor, resulting in forming the new apical process (Popov, 1992; Zhang et al., 2018). With the increasing growth of the individual columns, the thick organo-phosphatic shell with increased strength might provide more mechanical support to the conical or tubular valve in a fluid environment.

Based on the similar ontogenetic transformation with the increasing growth of the shell in Lingulellotretidae and Acrotretida, the forming of the ventral pedicle foramen is very likely homologous, modified from a lingulide-like pedicle groove between the two valves. Furthermore, the similarity and continuity in increasing the number and size of the stacked columns suggested that columnar shell structure is synapomorphy in Linguloidea and Acrotretida. However, the phylogenetic puzzle of whether the columnar fabric is paraphyletic with the baculate fabric in Linguliformea, or even in Lingulata hangs on two pieces of important fossil evidence. The shell structure of the earliest linguliform brachiopods needs to be discovered on more well-preserved fossils globally. Moreover, the shell structure in widely distributed early Cambrian stem group brachiopods needs to be scrutinized comprehensively. The complex shell in stem group taxa *Micrina* and *Mickwitzia*, demonstrating advanced columnar features in specimens yonger than *Eoobolus incipiens*, might reveal the

plesiomorphy of the columnar shell in Linguliformea (L. E. Holmer et al., 2008; Skovsted and Holmer, 2003; Williams and Holmer, 2002b). But this assumption depends on future work and whether the columnar shell is preserved in older stem groups. In another scenario, baculate, laminated and columnar fabric might have originated independently from an unmineralized ancestor like the agglutinated *Yuganotheca* during the early Cambrian (Cusack et al., 1999; Zhang et al., 2014). No matter what scenario is true in nature, the origin of the innovative columnar shell with a stacked sandwich model has played a significant role in the evolution of linguliform brachiopods. The evolutionary growth of stacked columns matches the increasing diversity of phosphatic-shelled brachiopods during the Cambrian explosion. Among them, the micromorphic acrotretides demonstrated the superb application of columnar shell combined with its innovative conical shape and possible exploitation of secondary tiering niches (Topper et al., 2015; Wang et al., 2012; Zhang et al., 2008a, 2018; Z. L. Zhang et al., 2021b), having flourished during the Great Ordovician Biodiversification Event, thriving and playing an important role in marine benthic communities for more than 100 million years (Bassett et al., 1999; Brunton et al., 2001; Congreve et al., 2021; Harper et al., 2017b, 2015; Z. L. Zhang et al., 2020b). The fitness of the diminutive body size of acrotretides is likely a trade-off between the increasing metabolic demand of phosphate biomineralization after the Cambrian phosphogenic event and the increased chance of evolutionary survival and adaptation of producing a high mechanical protect skeleton for protection in the shallow water environment (Cook and Shergold, 1984; Garbelli et al., 2017; Lévêque et al., 2004; Neary et al., 2011; Simonet Roda, 2021; Wood and Zhuravlev, 2012).Their relatively large surface/volume ratio mechanically requires a strong support with the composition of stacked sandwich columns and a relatively lower density of the shell by organic biomineralized material for the secondary tiering life. Such adaptive innovations may account for the flourish of phosphatic-shelled acrotretides in the latter half of the Cambrian, continuing to the Great Ordovician Biodiversification Event, thriving and playing an important role in marine benthic communities for more than 100 million years

Material and Methods

The brachiopod material studied here was collected from the Cambrian Series 2 Xihaoping Member of the Dengying Formation and the Shuijingtuo Formation at the Xiaoyangba section of southern Shaanxi (Z. L. Zhang et al., 2021a), and the Shuijingtuo Formation at the Aijiahe section and Wangjiaping section of western Hubei (Z. L. Zhang et al., 2016). All specimens are recovered through maceration of limestones by acetic acid (~10%) in lab, and deposited in the Early Life Institute (ELI), Northwest University, China. Selected specimens were coated and studied further using Fei Quanta 400-FEG SEM at Northwest University, Zeiss Supra 35 VP field emission at Uppsala University and JEOL JSM 7100F-FESEM at Macquarie University. Measurements of length, width and angle of different parts of *Latusobolus xiaoyangbaensis* gen. et sp. nov. and *Eoobolus acutulus* sp. nov. are performed on SEM images of well-preserved specimens by TpsDig2 v. 2.16.

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Zhiliang Zhang, Conceptualization, Investigation, Methodology, Resources, Visualization, Validation, Funding acquisition, Writing – original draft, Writing – review and editing; Zhifei Zhang, Investigation, Resources, Funding acquisition, Writing – review and editing; Lars Holmer, Investigation, Writing – review and editing; Timothy Topper, Investigation, Writing – review and editing; Bing Pan, Writing – review and editing; Guoxiang Li, Investigation, Writing – review and editing.

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【Figure captions】

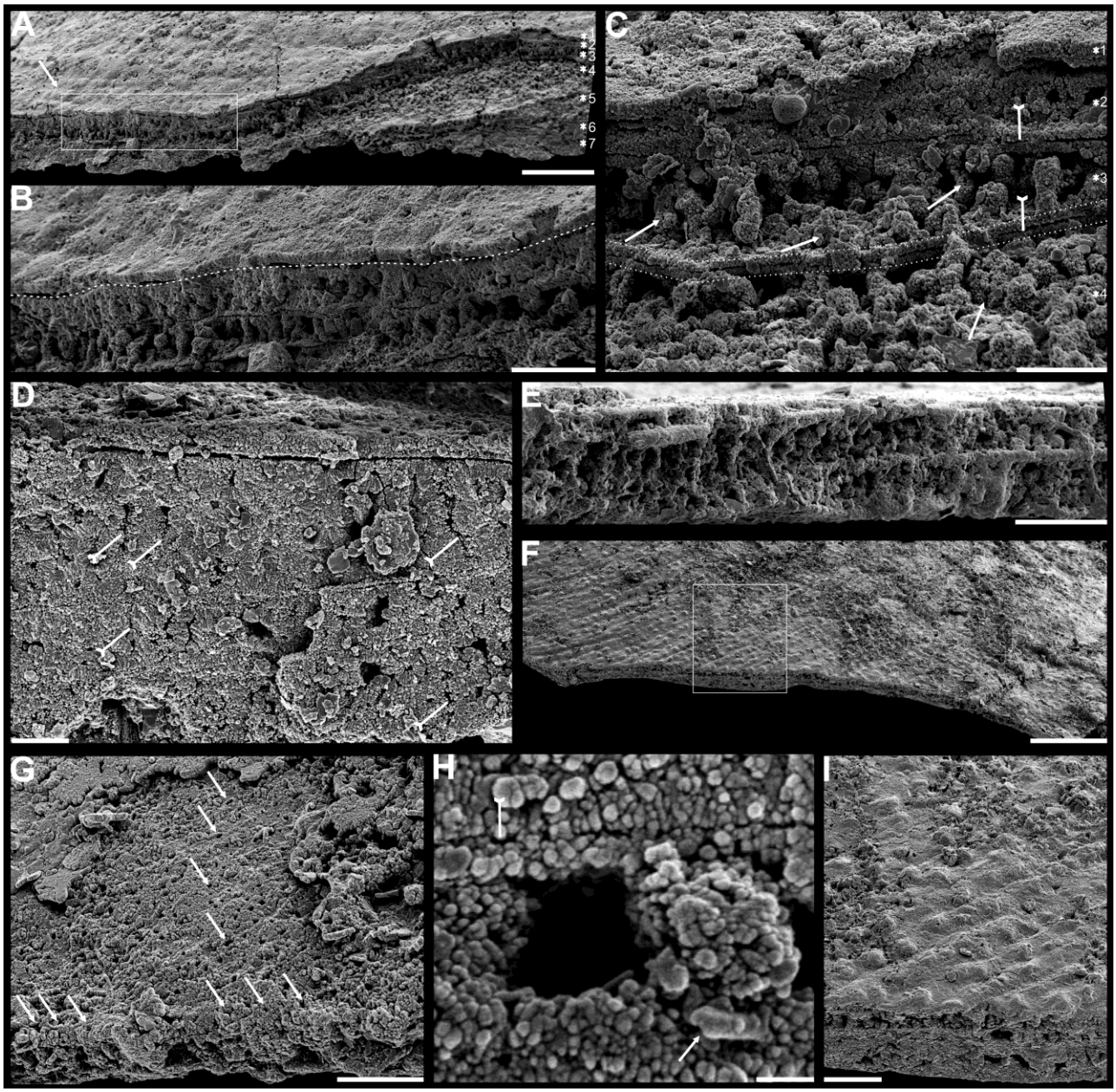

Figure 1. Shell ultrastructure of *Latusobolus xiaoyangbaensis* gen. et sp. nov. from the Cambrian Series 2 Shuijingtuo formation in southern Shaanxi. **A-C**, ELI-XYB S5-1 BS01. **A**, cross section of a ventral lateral margin, note post-metamorphosis pustules by arrow and shell layers 1-7, box indicates area in **B**. **B**, enlarged view of **A**, showing primary-secondary layer boundary by dashed line. **C,** enlargement of shell layers 1-4 of **A**, note organic canals of columns (arrows), organic layer (tailed

arrows) between two stratiform lamellae by dashed lines. **D**, poorly phosphatised columns of ventral valve, note organic canals by tailed arrows, ELI-XYB S5-1 BR06. **E**, columns of dorsal valve, ELI-XYB S5-1 BS17. **F**, cross section of a ventral lateral margin, show developed post-metamorphosis pustules, box indicates the area in **G**, ELI-XYB S4-2 BO06. **G**, enlarged primary layer pustules and secondary layer columns underneath. **H**, one layer of dorsal columnar shell with the exfoliation of above primary layer, noting column canals on the layer surface by arrows, ELI-XYB S4-2 BO08. **I**, apatite spherules of granule aggregations of ventral columnar shell, note granule rods by arrow and organic membrane by tailed arrow, ELI-XYB S4-2 BO06. Scale bars: **A**, 50 µm; **B**, **E**, **G**, 20 µm; **C**, **H**, 10 µm; **D**, 5 µm; **F**, 100 µm; **I**, 1 µm.

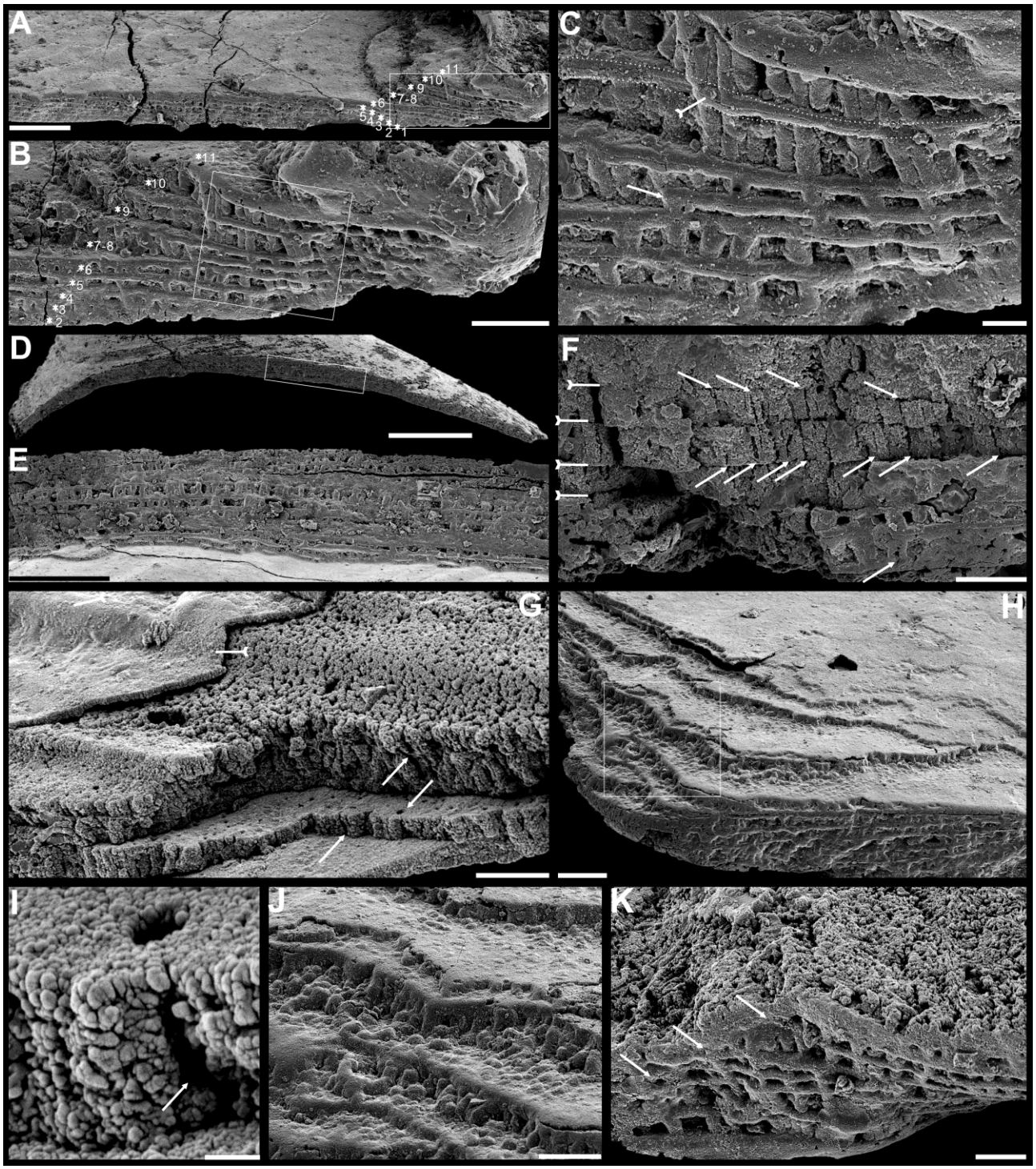

Figure 2. Ultrastructure of ventral *Eoobolus acutulus* sp. nov. from the Cambrian Series 2 Shuijingtuo formation in Three Gorges areas. **A-C**, ELI-AJH S05 BT12. **A**, cross section of a ventral lateral margin, note stacked columnar units 2-11, box indicates area in **B**. **B**, enlarged view of **A**, showing shell layers 2-11. **C**, enlarged view of **B**, show thin organic layer (tailed arrow) between two stratiform lamellae by

dashed lines, the fusion of two columnar layers by arrow. **D-F**, ELI-AJH 8-2-3 BT02. **D**, cross section of shell margin, box indicates area in **E**. **E**, different preservation condition of columns. **F**, poorly phosphatised columns, note the opening of organic canals along the organic lamellae by arrows, and space between two stratiform laminae by tailed arrows. **G-H**, ELI-AJH 8-2-3 BT03. **G**, note organic canals on the cross section and surface of columnar layer by arrows, and partly exfoliated primary layer by tailed arrow. **H**, magnified columns composed of granule spherules with canal in **G** by arrow. **I**, cross section of shell margin, box indicates area in (**J**), ELI-AJH 8-2-3 BT04. **J**, enlarged short columns. **K**, imbricated columnar layers (arrows), ELI-AJH S05 BT12. Scale bars: **A**, **E**, 50 µm; **B**, **I**, 20 µm; **C**, 5 µm; **D**, 200 µm; **F**, **G**, **J**, **K**, 10 µm; **H**, 1 µm.

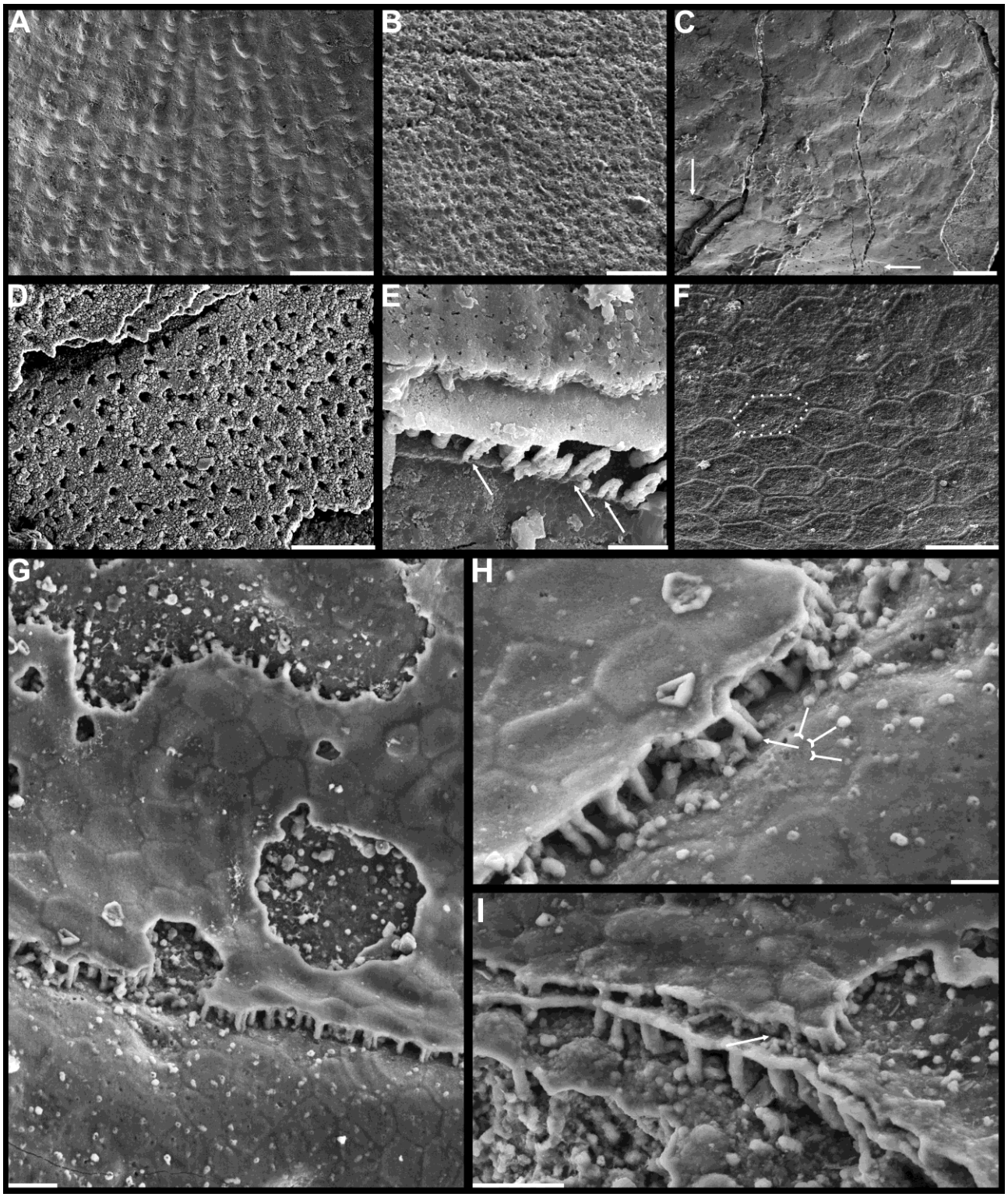

Figure 3. Shell ornamentation, ultrastructure and epithelial cell moulds of Cambrian Series 2 brachiopods. **A**, post-metamorphic pustules of *Latusobolus xiaoyangbaensis* gen. et sp. nov., ELI-XYB S4-3 AU11. **B-D**, *Eoobolus acutulus* sp. nov. **B**, metamorphic hemispherical pits, ELI-AJH 8-2-2 Lin01. **C**, epithelial cell

moulds, note column openings on layer surface beneath by arrow, ELI-AJH S05 N31. **D**, enlarged column openings on layer surface, ELI-WJP 7 CE05. **E-I**, *Eohadrotreta zhenbaensis*. **E**, partly broken columns, note organic canals by arrows, ELI-AJH F36. **F**, polygonal epithelial cell moulds on valve floor, dash lines indicate margin of one epithelial cell, ELI-WJP 6 R79. **G-I**, ELI-AJH Acro 053. **G**, epithelial cell moulds on median septum with columns between. **H**, enlarged view of **G**, note rudiment of columns by tailed arrows and one column on epithelial cell margin by arrow. **I,** three layers of epithelial cell moulds on cardinal muscles with columns between (arrow). Scale bars: **A**, **D**, 50 µm; **B**, **E**, **F**, **H**, 10 µm; **C**, **G**, **I**, 20 µm.

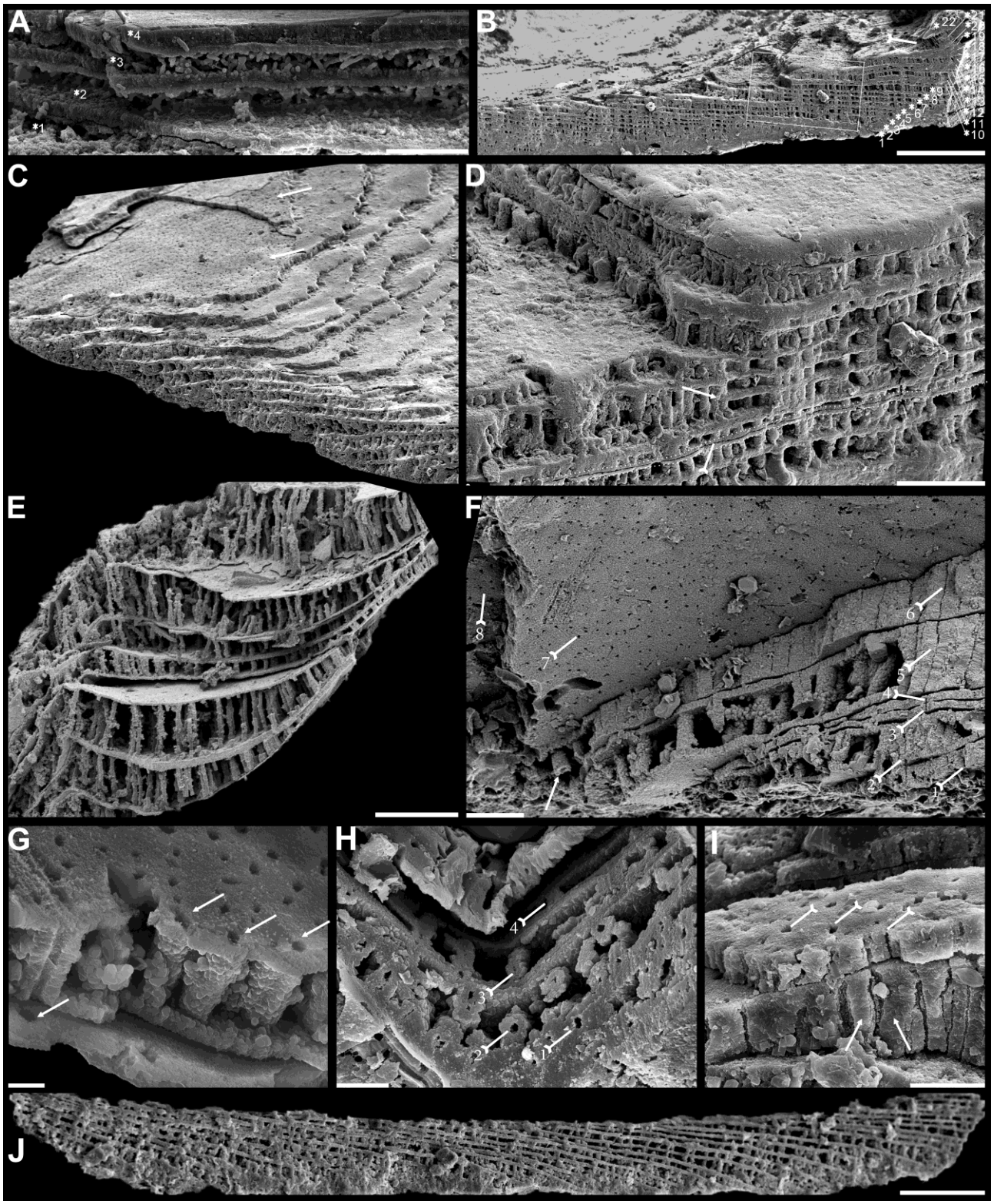

Figure 4. Columnar layers of Cambrian Series 2 brachiopods. **A**, *Eoobolus incipiens*, AJXM-267.5 DT12. **B-D**, *Lingulellotreta ergalievi*, ELI-AJH 8-2-3 CI11. **B**, cross section of shell margin, box indicates area in **C**, note stacked columnar units 2-22,

and raised pseudointerarea (tailed arrow). **C**, enlarged view of thin organic layer (tailed arrow) between two stratiform lamellae by dashed lines, the fusion of two columnar units into one by arrow. **D**, imbricated growth pattern of columnar layers. **E**, *Palaeotreta zhujiahensis*, note column openings (arrow) on eight successive columnar units by tailed arrow, ELI-AJH 8-2-1 AE09. **F-J**, *Eohadrotreta zhenbaensis*. **F**, relatively higher columns (ca. 20 µm), ELI-AJH 8-2-1 acro16. **G**, Apatite spherules of granule aggregations in columnar layer, note column openings (arrows) on both stratiform lamella surface, ELI-AJH S05 E18. **H**, cross section show column openings on 4 successive units by tailed arrow, ELI-WJP 7 AB98. **I**, poorly phosphatised columns (arrows), note openings of organic canals on stratiform lamella by tailed arrows, ELI-AJH S05 I76. **J**, stacked columnar layers in imbricated pattern, ELI-WJP 6 R47. Scale bars: **A**, **E**, **I**, 10 µm; **B**, 100 µm; **C**, **F**, 20 µm; **G**, 2 µm; **H**, 5 µm; **J**, 50 µm.

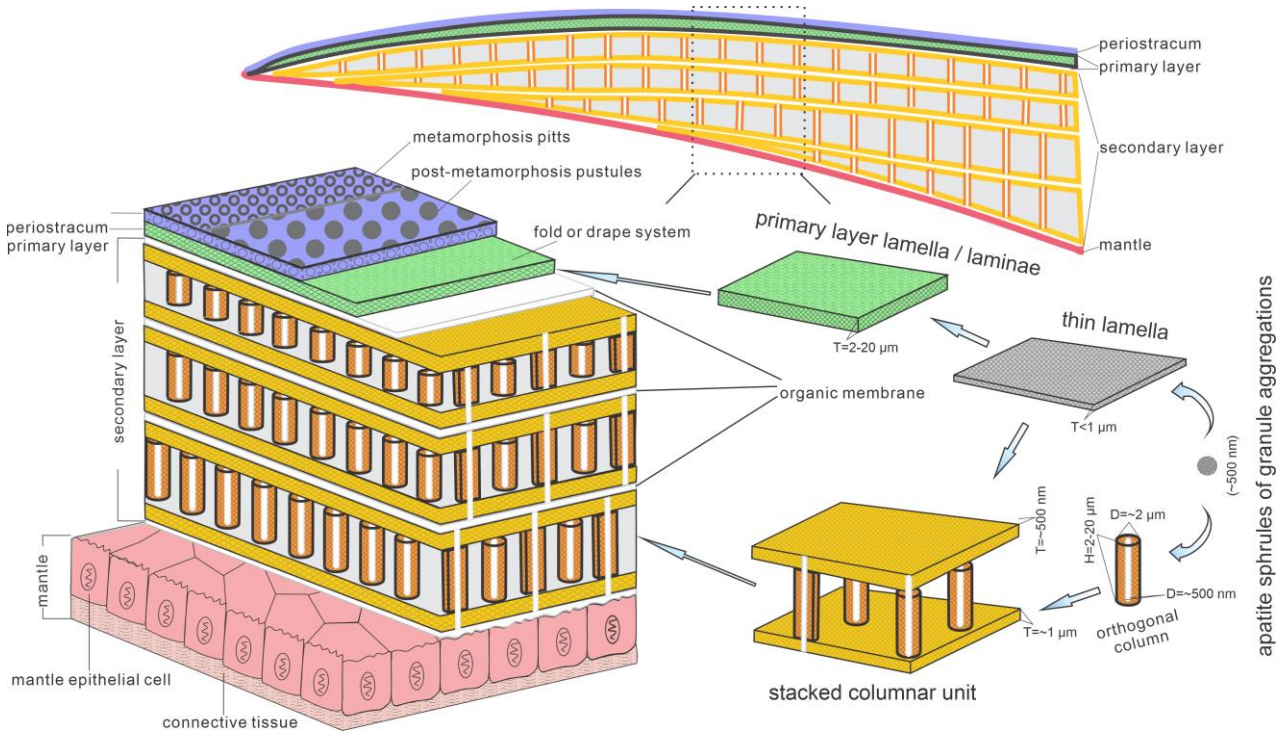

Figure 5. Biomineralization process of typical columnar layer by the conveyor-belt system of phosphatic-shelled brachiopods. Abbreviation: D=Diameter; H=height; T=Thickness. (modified from (Williams and Holmer, 1992; Z. L. Zhang et al., 2016))

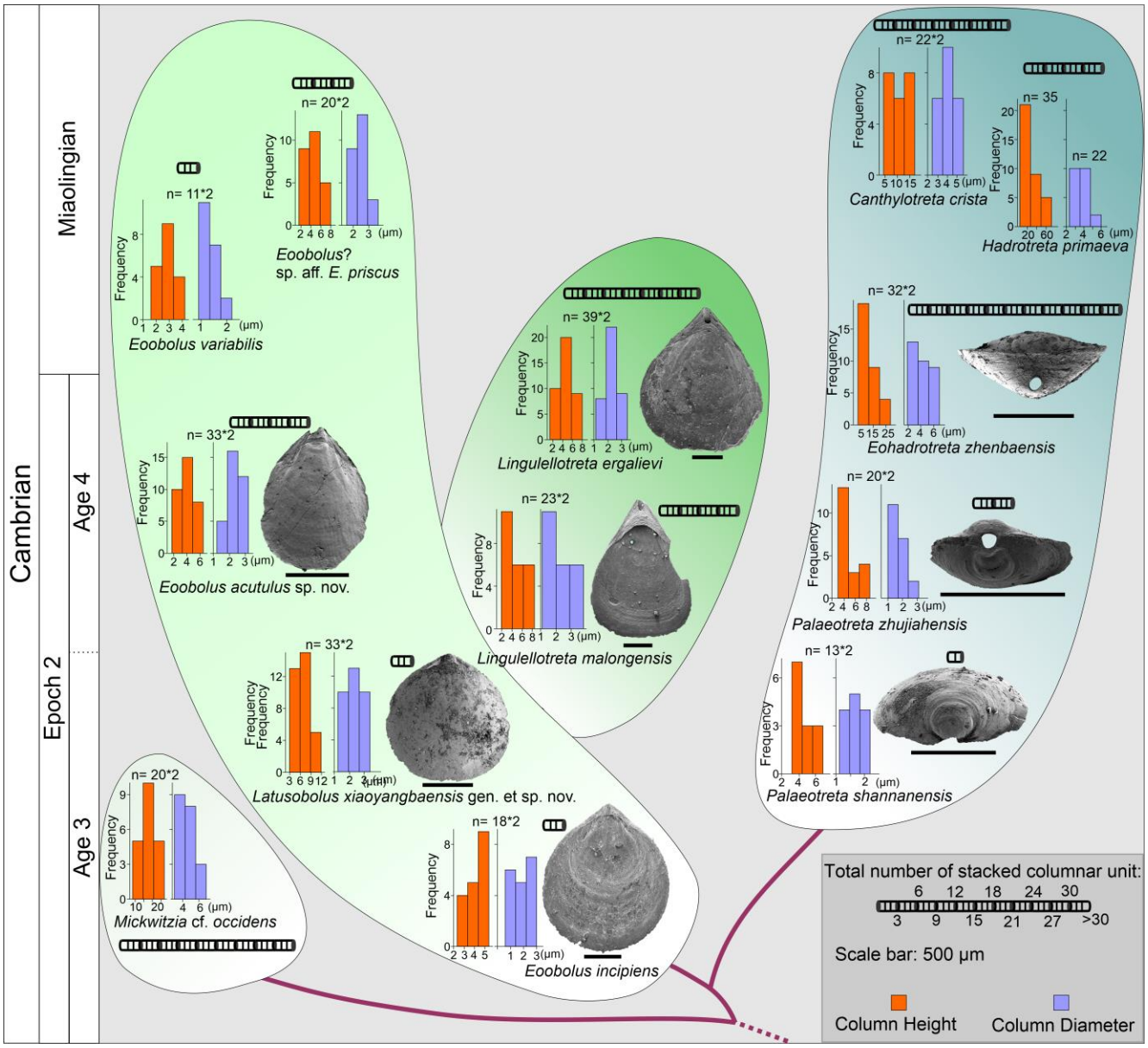

Figure 6. The evolutionary growth of stacked columnar shells in early Eoobolidae *Eoobolus incipiens*, *Latusobolus xiaoyangbaensis* gen. et sp. nov., *Eoobolus acutulus* sp. nov., *Eoobolus variabilis*, *Eoobolus*? aff. *priscus*, Lingulellotretidae *Lingulellotreta malongensis*, *L. ergelievi*, Acrotretida *Palaeotreta shannanensis*, *Palaeotreta zhujiahensis*, *Eoohadrotreta zhenbaensis*, *Canthylotreta crista* and stem group *Mickwitzia* cf. *occidens*. The height and diameter of column is from the literature (Skovsted and Holmer, 2003; Streng et al., 2007; Streng and Holmer, 2006; Ushatinskaya and Korovnikov, 2014; Z. L. Zhang et al., 2020a, 2020b, 2016).