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Morphology of testate amoeba *Difflugia australis* (Playfair, 1918) Gautier-Lièvre *et* Thomas, 1958 from a subtropical reservoir (southeast China)

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Abstract

Difflugia australis, first described by Playfair (1918), has a unique morphotype. However, in the absence of morphometric data, it has not yet been reliably classified within the largest testate amoeba genus *Difflugia*. In this study *D. australis* collected from a subtropical reservoir in southeast China was investigated by means of light and scanning electron microscopy. Basing on biometrical data, we provide an improved diagnosis of this little known species. *Difflugia australis* is different from other similar congeners (i.e., *D. bacillariarum* Perty, 1849 and *D. elegans* Penard, 1890) mainly by the combination of the following features: the shell is broadly ovate, with rounded dome and convex sides converging down to a very short distance from the aperture and diverging suddenly into a short rim (collar). It is usually more or less asymmetrical, with one side being more dilated than the other. The shell surface is slightly smooth, composed of flat siliceous plates of irregular shape and size, mixed with fine grains; microbial spores of comparable forms are spread on the shell surface; particles are often interspersed with a network of organic cement with unique mesh pattern; one (sometimes two) slanting spine-like posterior end of the shell is variable in form; collar is mainly formed by small plates of equal size. The dimensions of the shell are: total shell length 88–106 µm; shell width 53–88 µm; aperture diameter 19–28 µm; collar height 3–6 µm; spine length 3–23 µm. The size frequency distributions of both total shell length and shell width indicate that it is a size-monomorphic species with low variability.

Keywords: *Difflugia australis*; Arcellinida; testate amoebae; freshwater; Shidou Reservoir; China

Introduction

Reliable taxonomy is a necessary condition for studies of the diversity and ecology of any group of organisms (Todorov & Bankov 2019). However, inadequate taxonomy is one of the burning issues in the study of free-living protists (Mazei & Warren 2012, 2014, 2015; Kosakyan *et al*. 2016), requiring the revision of the classification and nomenclature of eukaryotic organisms (Adl *et al*. 2019; Lahr *et al*. 2019; Ruggiero *et al*. 2020). Testate amoebae are polyphyletic group of shelled amoeboid protists (Adl *et al*. 2019), which are routinely used as a model in evolutionary and ecological studies due to the unique single-cell microorganism with a rigid shell, and their ability to be fossilised, making them important in palaeoecology (Charman 2001; Patterson & Kumar 2002; Mitchell *et al*. 2008; Amesbury *et al*. 2016; Kosakyan *et al*. 2016; Swindles *et al*. 2020). However, the potential uses of testate amoebae in ecology and other related fields are still limited by existing uncertainties in the morphology based taxonomy of this group (Gomaa *et al*. 2015; Kosakyan *et al*. 2016).

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The existence of many inadequately defined species has been identified as one of the most important taxonomic problems or impediments of testate amoebae (Mazei & Warren 2012, 2014, 2015; Gomaa *et al*. 2015). Therefore, it is crucial to have detailed morphological description of as many species as possible (Mazei & Warren 2012; Kosakyan *et al*. 2016; Todorov & Bankov 2019). A focus of this study is on the most species-rich genus of the Arcellinida, namely *Difflugia* Leclerc, 1815, because many of the species descriptions are still insufficient, and consequently the identification to species level is extremely difficult (Mazei & Warren 2012, 2014, 2015).

Difflugia bacillariarum var. *australis* was described by Playfair (1918) from different freshwater and wetland habitats of Australia. Forty years later, Gautier-Lièvre & Thomas (1958) elevated this subspecies to species rank as *Difflugia australis* stating that it was clearly different from both *D. bacillariarum* and *D. elegans*. Chardez (1978) further showed high variability of *D. australis*, although he did not compare it directly with *D. bacillariarum* and *D. elegans*. Thus, based on the shell shapes and sizes of these three species, Mazei & Warren (2012) synonymised *D. australis* with *D. elegans*. Following a review of the available data, all previous findings were based on very limited number of specimens and the detailed description of this taxon is necessary to clarify its taxonomic position.

During the investigation of testate amoebae from organic sediment of Shidou Reservoir in subtropical China from 2016 to 2018, we found abundant population of *D. australis*. We carried out detailed light and scanning electron microscopy and morphometry examinations of the shell of this species. Our primary aims were: (1) to examine morphologically and biometrically *D. australis* in order to clarify its typical characteristics and (2) to compare it with *D. bacillariarum* and *D. elegans* in order to provide sufficient evidence to show that all three are separate species.

Materials and methods

Sample collection

The field sampling of *Difflugia australis* was performed in Shidou Reservoir (24°42′N, 118°00′E) located in Xiamen city, Fujian province, southeast China. The sampling station and general characteristics of Shidou Reservoir are shown in supplementary materials and methods (Supplementary Figs 1, 2, 3 and Supplementary Table 1, respectively) with details given in our previous studies (Ju *et al*. 2014; Yang *et al*. 2016, 2017; Nyirabuhoro *et al*. 2020). To collect samples, we employed sedimentation traps consisting of four cylindrical traps fixed to a single rope at a depth of about 1 m beneath the surface water level (Supplementary Fig. 2). The sediment traps were made from long acrylic pipes with an internal diameter of 8.4 cm and height of 50 cm. This type of trap was chosen on the basis of earlier *in situ* investigations of the process of sediment deposition (Szmytkiewicz & Zalewska 2014). All the sediment traps (four replicates) were retrieved every 29–66 days of exposure between January 2016 and December 2018. A total of twenty organic sediment samples with different volumes (Supplementary Fig. 2) from the epilimnion layer (water depth: 0–0.5 m) were used for *D. australis* analysis. In order to clean the material deposited in the sediment traps, the sediment (volume = 1 cm^3) was washed through a set of sieves with diameters of 300 and 25 µm following Ndayishimiye *et al*. (2019, 2020). All sediment samples were preserved at 4 ºC for further investigation (Mazei *et al*. 2015).

Microscope observations

Although living *D. australis* specimens may have been present at the time of sampling, the samples were not stained so the analysis of *D. australis* was performed on living plus dead specimens. Six morphological characteristics (total shell length, shell width, aperture diameter, collar height, body length and spine length) of 150 shells were analysed using a light microscope (SK240, Teron, Shenzhen, China) under 200–400X magnification (Fig. 1). The shell texture, organic cement pattern on the shell surface and chemical characterization of specimens were determined by scanning electron microscopy and an energy dispersive spectroscopy system. Scanning electron microscopy analysis used a focused beam of electrons to produce detailed, high magnification and high resolution images of a sample's surface topography, while energy dispersive spectroscopy was able to detect elements that possess the

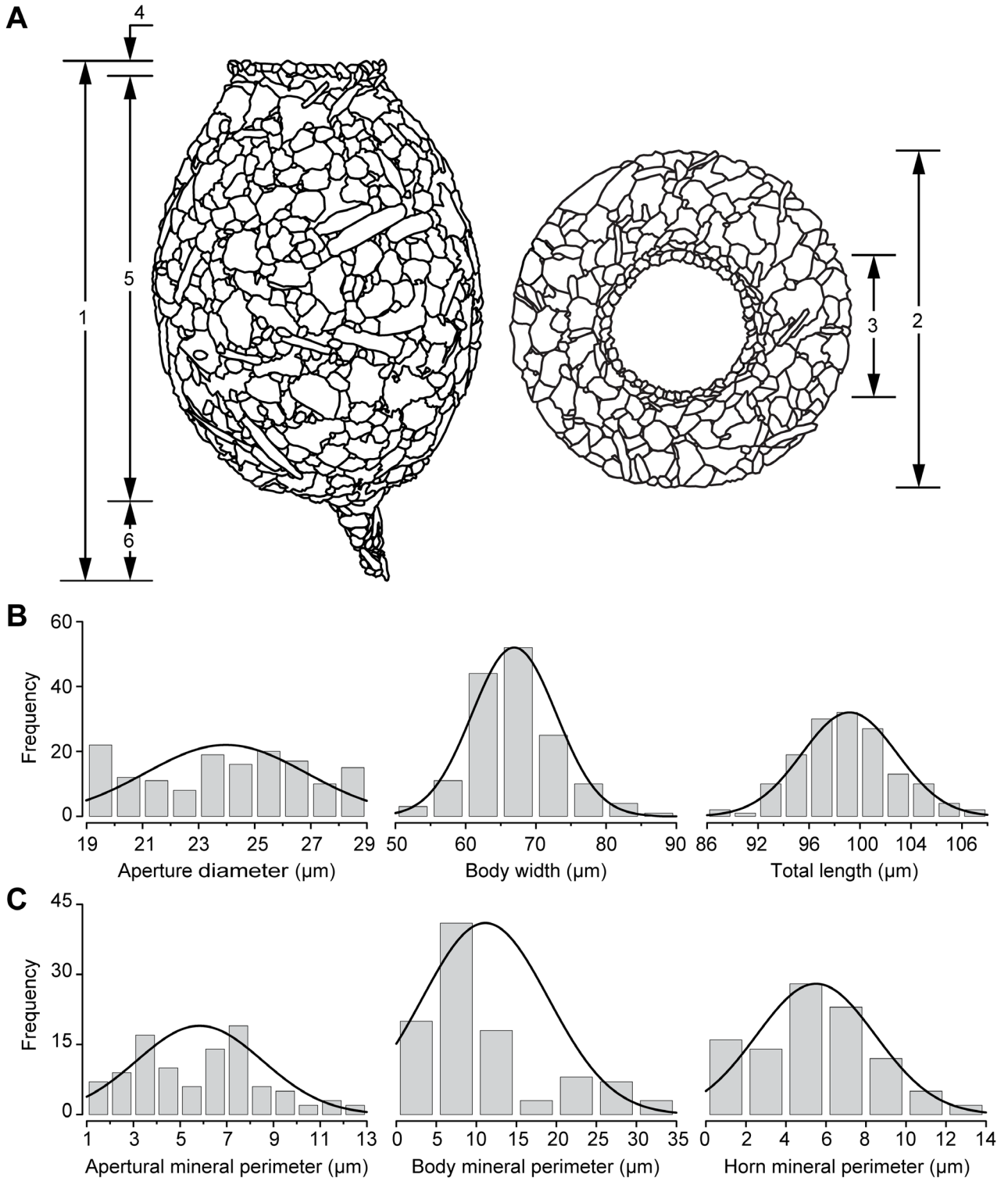

FIGURE 1. Morphometric characteristics of *Difflugia australis* from Shidou Reservoir, Xiamen, China. (**A**) Shell outline (lateral and apertural views) showing covering of irregularly shaped, flat particles and position of measured morphometric axes in this study. 1–total shell length, 2–shell width, 3–aperture diameter, 4–collar height, 5–body length, 6–spine length. (**B**) Frequency plots of shell dimensions. Histograms showing the size frequency of aperture diameter (left column), shell width (middle column) and total shell length (right column). (**C**) Frequency plots of mineral dimensions. Histograms showing the size frequency of apertural mineral perimeter (left column), body mineral perimeter (middle column) and spine mineral perimeter (right column).

atomic number of higher than boron and these elements can be detected at concentration of least 0.1%. We first isolated specimens (n = 9) by extracting them from the sediment in the cell culture dish using glass micropipette under a stereomicroscope (Model SMZ800N, Nikon, Tokyo, Japan). The individual shells were placed on double-sided adhesive tapes which were fixed on a standard aluminum stub, then air-dried and coated with the ultra-tiny layer of gold. The micrographs showing the morphology and structure of shell were captured using scanning electron microscope (Model S-4800, Hitachi, Tokyo, Japan). The line drawings of the shell outline (Fig. 1A) and dimensions of ellipsoidal particles (Supplementary Table 2) showing agglutinated grains at three main components of the shell (aperture, body and spine sections; n = 100 particles per section) were made from tracings from microscope projections using ImageJ 1.x (Schneider *et al*. 2012). The chemical composition of the shell (n = 9) at three main components of the shell surface (27 sections in total) was examined by scanning electron microscope equipped with an energy-dispersive X-ray spectrometer (Model S-4800, Hitachi, Tokyo, Japan). X-ray spectra were collected at 20 kv accelerating potential and calculated both semi-quantitatively (molecular weight percent) and in counts (live counting time of 100 seconds). Due to the similarity of results, only spectra for one specimen (aperture, body and spine sections) were presented.

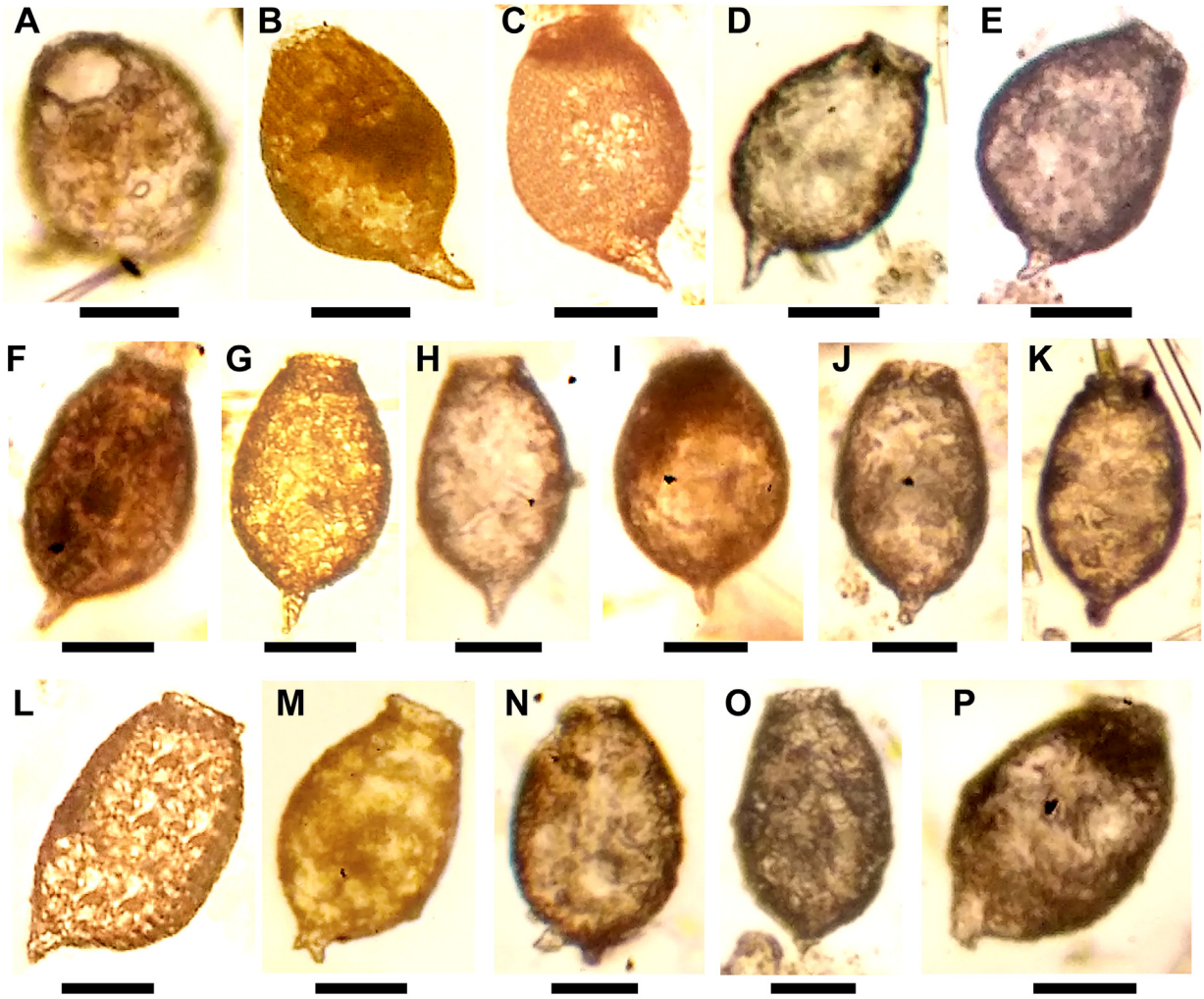

FIGURE 2. Light micrographs of *Difflugia australis*. (**A**) Apertural view. (**B–P**) Lateral views showing the shape of different specimens. Scale bars are 25 µm.

Statistical analysis

For each morphometric variable, we computed basic summary statistics: minimum, maximum, arithmetic mean, standard deviation, and coefficient of variation in % (CV). For some important morphometric variables and agglutinated grains, we also calculated the components of statistical distribution (its shape, center and spread). Statistical analyses were performed using the computer software PAST version 3.13 (Hammer *et al*. 2001).

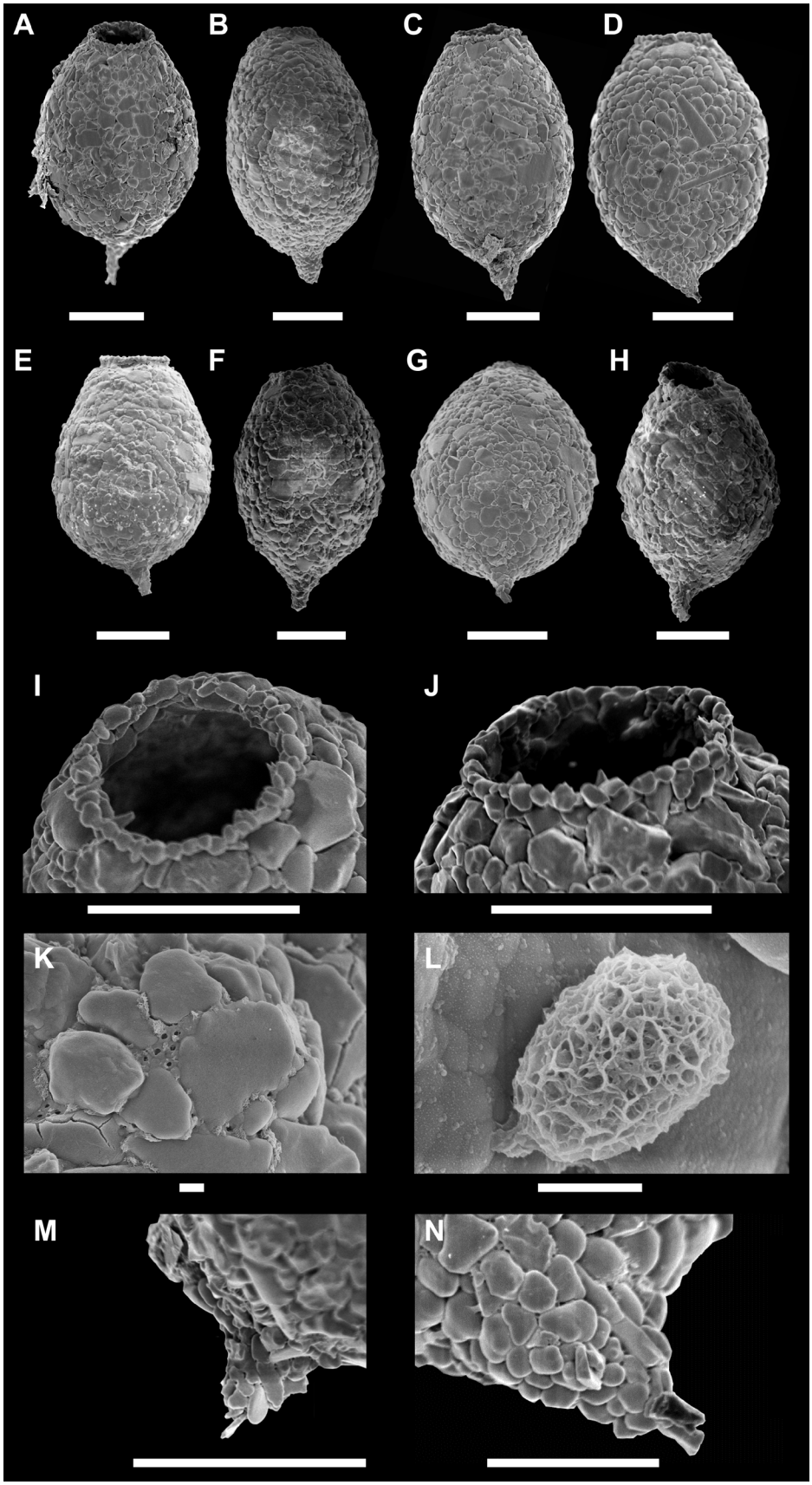

FIGURE 3. Scanning electron micrographs of *Difflugia australis*. (**A**, **B**, **I**, **J**) lateral-oblique view showing the aperture and collar. (**C**, **D**) lateral view showing the collar. (**E**–**H**) lateral view showing different shell shapes and the spine. (**K**) microstructure of organic cement. (**L**) agglutinated microbial spore. (**M**, **N**) details of the particles on the spine. Scale bars: A–J, 25 μm; K, 5 μm; L, 1 μm; M–N, 5 μm.

Results

Morphology of the shell

The shell of *D. australis* is light yellowish-brownish in colour, broadly ovate and dome-like with curved sides that converge down to a short-distance from the aperture, at that point they diverge abruptly forming a rim (collar). The shell is normally skewed, one side being more convex than the other. A slanting spine is clearly seen at the aboral end of the shell, and it is variable in form. The shell is rather smooth, made-up of flat siliceous plates of irregular shape and size, mixed with fine grains. The microbial spores are attached to the shell surface. Small areas of organic cement are clearly seen as a network with meshes and a broad connection membrane between meshes. The aperture is circular. The collar is composed of very small plates of equal size (Figs 2, 3).

Biometry

Table 1 shows the detailed morphometric characterization of *D. australis*. The dimensions of the shell are: total shell length 88–106 µm; shell width 53–88 µm; aperture diameter 19–28 µm; collar height 3–6 µm; body length 73–99 µm; spine length 3–23 µm. All measures are slightly variable (CV between 3.8 and 12.0%) with the exception of collar height (17.4%) and spine length (40.5%). Both total shell length and shell width are characterised by normal distribution (Fig. 1B), which shows that *D. australis* is a size-monomorphic species. The most stable morphometric character is the total shell length, having the lowest variability of any of the charteristics measured in this study.

Further, the morphometric dimensions in shell length and collar show significant correlation at $P < 0.05$ (Table 2), particularly total shell length is well correlated with body length ($r = 0.343$, $n = 150$) and spine length ($r = 0.354$, $n = 150$), while aperture diameter is positively correlated with collar height ($r = 0.167$, $n = 150$). However, the shell width does not show any significant correlation with other morphometric characters.

TABLE 1. Morphometric characteristics of *Difflugia australis* from Shidou Reservoir, Xiamen, China.

Feature	Min	Max	Mean	SD	CV (%)	N
Total shell length (1)	88	106	97	3.7	3.8	150
Shell width (2)	53	88	67	6.0	9.0	150
Aperture diameter (3)	19	28	24	2.9	12.0	150
Collar height (4)	3	6	3	0.6	17.4	150
Body length (5)	73	99	86	4.7	5.5	150
Spine length (6)	3	23	12	4.7	40.5	150

The numbers in parentheses correspond to the features of the shell outline in Fig. 1. The measurements in µm. Min–minimum; Max–maximum; SD–standard deviation; CV–coefficient of variation in percent; N–the total number of individuals examined.

TABLE 2. The relationship between morphometric characteristics of *Difflugia australis* from Shidou Reservoir, Xiamen, China.

	Total shell length	Shell width	Aperture diameter	Collar height	Body length	Spine length
Total shell length (1)	—					
Shell width (2)	0.038	—				
Aperture diameter (3)	0.158	0.056	—			
Collar height (4)	0.089	–0.027	0.167*	—		
Body length (5)	0.343**	0.095	0.143	0.087	—	
Spine length (6)	0.354**	–0.066	–0.057	–0.057	–0.719**	—

The numbers in parenthesis correspond to the features of the shell outline in Fig. 1. $n = 150$, $^{**}P<0.01$, $^{*}P<0.05$.

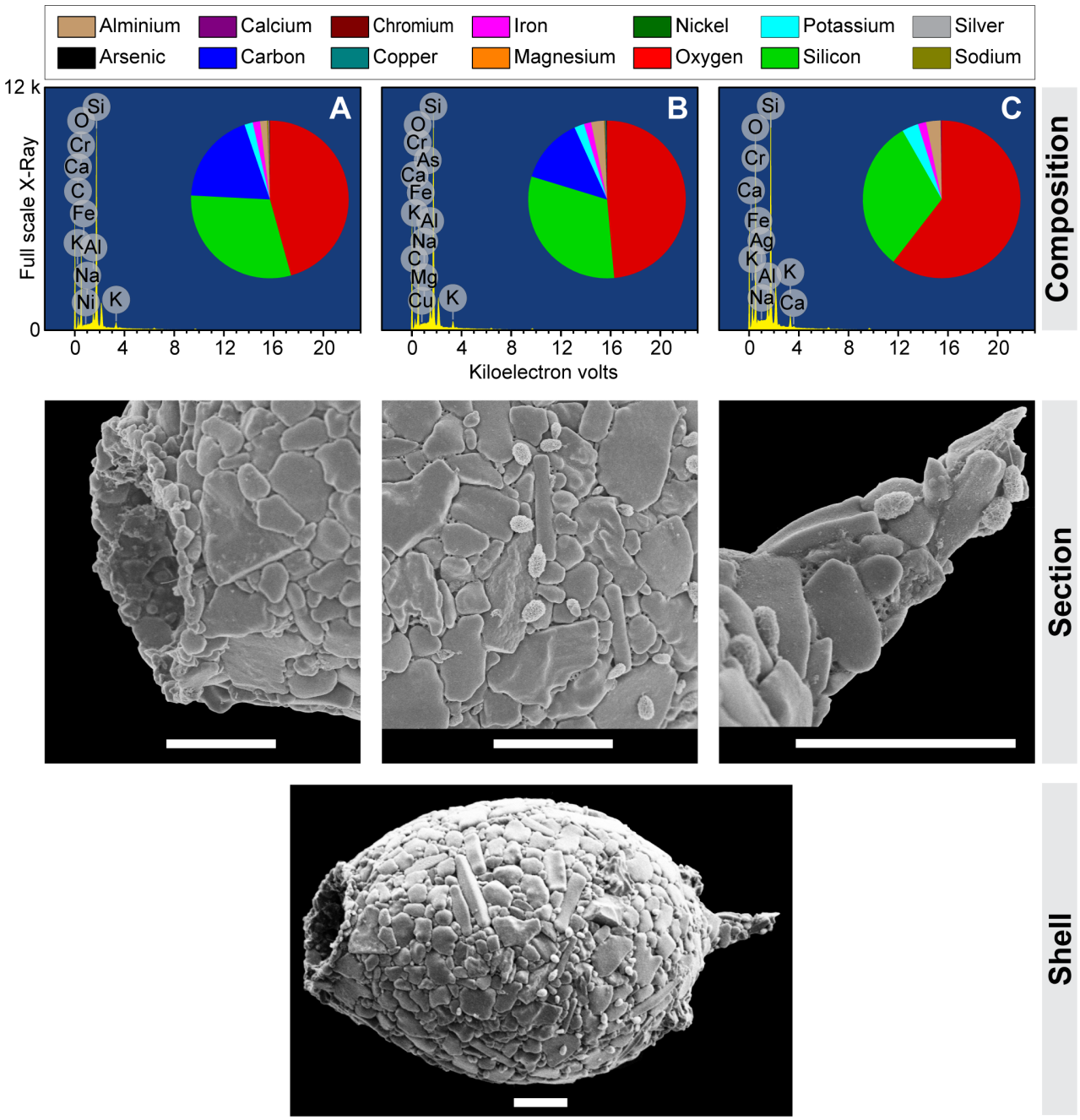

FIGURE 4. The shell elemental composition of *Difflugia australis* as measured in full scale X-Ray on aperture (**A**), body (**B**) and spine (**C**) sections. The scale bars are 10 µm.

Mineralogy of the agglutinated grains

Our energy dispersive spectroscopy was measured in percent elemental weight, and showed a similarity of spectra for selected specimens of *D. australis* ($n = 9$). The mineralogy of the agglutinated grains on aperture, body and spine sections of these specimens were different (Fig. 4). It seems that *D. australis* possesses an affinity for a mixture of silicates, oxides and carbonates (e.g., 51.57, 30.85 and 10.82%, respectively in Fig. 4) as the shell of many specimens was built of these materials. The grain shapes varied among the specimens and grain surface areas of the aperture section are usually small than that of the body and spine sections (Figs 3, 4). The grains ($n = 100$) fit ellipsoids (major x minor axes) of 2.1 x 1.3 µm at the aperture section, 4.1 x 2.3 µm at the body, and 2.0 x 1.2 µm at the spine section (Supplementary Table 2). Microbial spores (2.5–3.0 µm in length and 0.7–2.0 µm in width) are agglutinated

on the shell surface. Organic cement is a network with a mesh 0.14–0.32 μm in diameter and with a seemingly wide connection membrane between meshes (≥ half of mesh diameter based on few individuals) (Figs 3, 4).

TABLE 3. Comparisons of *Difflugia australis* with two similar species.

Character	*Difflugia australis*	*Difflugia bacillariarum*	*Difflugia elegans*
Total shell length	88–106	67–122	81–128
Shell width	53–88	40–59	54–95
Aperture diameter	19–28	22–31	22–41
Spine length	3–23	?	4.4–39.6
Shell shape	ovoid with rounded dome and convex sides	ovoid and circular in transverse section with slightly pronounced neck near the aperture	ovoid with slightly pronounced neck near the aperture
Shell color	light yellowish-brownish	light yellowish-brownish	yellowish-brownish
Aperture	circular, surrounded by short collar, made up of small siliceous plates	circular, masked by diatom shells	circular, masked by irregular particles and diatoms
Collar	present	absent	absent
Spine	aboral, tubular	?	aboral, tubular
Building materials	flattish siliceous plates	thin siliceous plates, diatom frustules	sand-grains, diatom frustules
Organic cement	network of small meshes	?	small network of meshes
Mesh diameter	0.14–0.32	?	0.30–0.45
Mesh wall thickness	≥ half of mesh diameter	?	0.15–0.28
SSU rRNA phylogeny	?	Species in a genus *Difflugia*	?
Sample location	China	Switzerland, UK	Bulgaria, UK
Reference	present study	Ogden & Hedley 1980; Gomaa *et al*. 2012	Mazei & Warren 2012; Todorov & Bankov 2019

All measurements are in μm.? Data or information is not available.

Discussion

Morphology of the shell and biometry

Difficulties are often discovered when trying to characterize specimens of testate amoebae belonging to the genus *Difflugia*, due to inadequate knowledge regarding the shape and structure of their shells (Yang *et al*. 2004, 2005a; Yang & Shen 2005). Playfair (1918), Gautier-Lièvre & Thomas (1958) and Chardez (1978) described *D. australis* as (1) broadly ovate and dome-like with convex sides that converge to within a small distance of the aperture, at which point they diverge sharply forming a rim collar, (2) built in flat siliceous plates of irregular shape and size, mixed with fine grains, (3) circular at the apertural zone, and (4) ended by a slanting spine. On the other hand, these illustrations lack satisfactory data for a statistical analysis and are insufficient to be employed in the robust separation of *D. australis* from other related taxa (Mazei & Warren 2012). Based on our observations of a large number of specimens (Fig. 1), the species can match well *D. australis* shown by Playfair (1918), Gauthier-Lièvre & Thomas (1958), Chardez (1978) and Meisterfeld & Mitchell (2008), and is similar to *D. bacillariarum* and *D. elegans* (Table 3). In fact, shell length, shell width, aperture diameter, collar height, body length and spine length are essential parameters of shell size in the classification of *Difflugia* species (Todorov & Bankov 2019). In this study, the size of collar height and spine length demonstrates a wider variability (CV between 17.4% and 40.5%), but body length and spine length strongly correlated ($r = –0.719$, $P < 0.05$, $n = 150$) possibly due to the fact that different populations of the same species differ in character of variability in both amplitude and correlativity of morphometric characteristics (Wanner 1999; Bobrov & Mazei 2004). This shows that morphology may provide numerous criteria for separation between *D. australis* and related species.

Minerals and organic cement of the shell

Testate amoebae are widely distributed and respond quickly to environmental changes (Yang *et al*. 2010; Ju *et al*. 2014; Ndayishimiye *et al*. 2019). Moreover, the specimens have the ability to select grains of different shapes and sizes to build their shells (Todorov & Bankov 2019). As a result, the nature of the agglutinated shells may rely on their sedimentological environment (Armynot du Châtelet *et al*. 2010, 2015). Investigation by scanning electron microscope armed with an energy dispersive spectroscopy has previously been shown to be successful for analyzing the grains of different specimens of the testate amoeba species (Patterson *et al*. 1996; Armynot du Châtelet *et al*. 2013, 2015; Fialkiewicz-Koziel *et al*. 2015), allowing one to examine in detail the composition and outlines of the shell surface, the nature of the organic cement, and anthropogenic particles that are built into the shell (Armynot du Châtelet *et al*. 2015; Fialkiewicz-Koziel *et al*. 2015). Likewise, our results show that *D. australis* is able to select grains of small size fraction of the sediment with a priority for low-density silicates as good as quartz (Fig. 4). In Fig. 3L and Fig. 4, organic grains that are agglutinated on *D. australis* shell seem to be microbial spores (about 2.5–3.0 µm in length and 0.7–2.0 µm in width) of similar forms. These microbial spores may confirm that *D. australis* agglutinates organic grains of particular shapes and sizes from its immediate environment (Armynot du Châtelet *et al*. 2013). This may suggest that the lack of diatom frustules or chrysomonad cysts and rough surface from the shell of *D. australis* (Figs 3, 4) may be an important feature that have not been seen in few specimens examined by Playfair (1918), Gauthier-Lièvre & Thomas (1958), Chardez (1978) and Meisterfeld & Mitchell (2008).

Comparison with similar species

The efficiency of morphometric characters for separating *Difflugia australis*, *D. bacillariarum* and *D. elegans* appears to be meaningful for taxonomic purposes, although *D. australis* was recently synonymized as *D. elegans* (Mazei & Warren 2012). Likewise, our results suggest that the shell length and shell width of *D. australis* is within the range of *D. bacillariarum* and *D. elegans* (Table 3). However, in comparison to both *D. bacillariarum* and *D. elegans*, *D. australis* differs considerably in several metric characteristics: First, *D. australis* has a collar, whereas both *D. bacillariarum* and *D. elegans* have short neck, and this might be main diagnostic feature for delimitation between *D. elegans* and *D. australis*. The apertural collar of *D. australis* is more close with *Netzelia* (Gomaa *et al*. 2017). In fact, *D. australis* having such collar unlike *D. bacillariarum* and *D. elegans* may suggest that *D. australis* is more closely related with *Netzelia* than with *Difflugia* (Figs 3, 4). Nevertheless, since we do not have enough data to discuss the possibility to transfer *D. australis* into *Netzelia*, a key priority for any future study is to investigate *D. australis* using phylogenetic analyses based on small subunit ribosomal ribonucleic acid (SSU rRNA) gene sequences and possibly revise the taxonomy of this species. Second, shell of *D. australis* is slightly smooth and consists mainly of small circular grains (flattish siliceous plates) embbeded in a thick layer of organic cement, while *D. elegans* normally has rough shell composed mainly of angular particles (sand-grains and diatom frustules) with small areas of organic cement regularly observe in shell matrix as a network. The details of this organic cement matrix might be another important diagnostic feature for delimitation among these three species as it has previously been suggested to be important in *Difflugia* (Ogden & Ellison 1988; Yang *et al*. 2005b; Mazei & Warren 2012; Todorov & Bankov 2019). In both *D. australis* and *D. elegans*, the organic cement matrices are clearly visible among particles. However, the dimensions shown by organic cement of *D. australis* is different from that of *D. elegans*. This organic cement is in the form of a network with small mesh diameter compared to that shown by *D. elegans*. Finally, our results show that the smallest shell of *D. bacillariarum* is shorter than both *D. australis* and *D. elegans*, pyriform, usually composed of thin siliceous plates overlaid by diatom frustules and unified by organic cement. In addition, the outline of the aperture of *D. bacillariarum* is different from these two species due to the fact that it is reliant on the arrangement and dimension of the diatoms which surrounds it (Ogden & Hedley 1980; Siemensma 2019).

The SSU rRNA gene sequence has been confirmed as valuable for high-level phylogeny of testate amoebae and *Difflugia* in particular (Nikolaev *et al*. 2005; Gomaa *et al.* 2012, 2015, 2017). However, we were unable to obtain molecular data since we investigated dead assemblages from sediment with age of 29–66 days. In fact, only a few molecular phylogenetic studies have been carried out on the testate amoebae to date (Nikolaev *et al*. 2005; Gomaa *et al.* 2012, 2015, 2017; Ruggiero *et al*. 2020), and phylogeny of these microorganisms is affected by typical under-

sampling artefacts, leading to a still mostly unresolved tree (Gomaa *et al*. 2012; Kosakyan *et al*. 2016). Published molecular data from *Difflugia* deposited in GenBank show clearly that *D. bacillariarum* is a member of genus *Difflugia* with distinct morphological characteristics (Gomaa *et al*. 2012). Unfortunately, the difficulty of obtaining living specimens of *D. australis* and *D. elegans* means that molecular data are still not available from these two species. Increasing sampling effort for living specimens is urgently required (Gomaa *et al*. 2012). Additionally, future molecular studies should focus on these two species because they may be found in abundance in inland waters and possibly revise the taxonomy of *D. australis* based on the SSU rRNA gene sequence.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at: https://doi.org/10.11646/zootaxa.4890.1.5

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