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Morphology, Biometry and Distribution of *Difflugia biwae* Kawamura, 1918 (Protozoa: Rhizopoda)

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Summary. The freshwater testate amoeba *Difflugia biwae* Kawamura, 1918, isolated from Mulan Lake, Hubei Province, China is investigated using light microscopy and scanning electron microscopy. The morphology, biometry and distribution of this little known species are supplied. After careful comparison with three other similar species, including *D. delicatula* Gauthier-Lièvre *et* Thomas, *D. elegans* Penard and *D. oblonga caudata* Štěpánek, we believe that the characteristics of smooth fusiform shell, conspicuous great collar flare (always larger than body-width) around the aperture, constriction behind the aperture and a somewhat curved aboral horn set *D. biwae* apart from all other *Difflugia* species. This species shows a great diversity in total length, collar height and aboral horn length which have high variability (CV between 11.76 and 24.52). However, body width, collar diameter, neck width, body length and aperture diameter are fairly constant with low variability (CV between 5.34 and 8.79) which shows a remarkable uniformity of *D. biwae*. Also, the size frequency distributions of both body width and body length yield bell-shaped (normally distributed) curves and indicate that *D. biwae* is a size-monomorphic species, characterized by a main-size class and a small size range. *D. biwae* is probably endemic to East Asia (China and Japan) because it has such a large size (165-306 µm) that it would have been easily found in Europe and North America, if it were there. Consequently, *D. biwae* must have a restricted geographical distribution, disproving the old hypothesis that microscopic organisms are cosmopolitan.

Key words: biometry, *Difflugia biwae*, distribution, morphology, Testacea.

INTRODUCTION

Difflugia biwae Kawamura, 1918 was first found in Lake Biwa, the largest freshwater lake in Japan (Kawamura 1918). Subsequently, "*D. biwae* is endemic to Lake Biwa, Japan" has been a fundamental paradigm for nearly a century (Toshihiko 1979, Nishino and Watanabe 2000, Tsugeki *et al.* 2003). However, *D. biwae* has not been seen since 1987 in the waterbodies of Lake Biwa (Ichise *et al.* 1996). More recently, Tsugeki *et al.* (2003) showed that the remains of *D. biwae* occurred abundantly before 1960, but decreased dramatically thereafter and were not found at all after 1980 in the sediments of Lake Biwa. Thus the fact that *D. biwae* seems to be extinct in Lake Biwa was affirmed in both the waterbodies and the sediments.

A very short description of *Difflugia biwae*, together with a simple line drawing, was originally supplied in Japanese by Kawamura (1918, 1927). Many years later, both the description and the line drawing of *D. biwae* have been presented in more than three monographs in

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Japanese (Abe *et al*. 1957, Toshihiko 1979, Okada *et al*. 1981). However, the later data gave us no more information than those reported by Kawamura in the original literature. Unfortunately, little emphasis has been given to detailed study of its morphometrical characterization in all previous investigations. Further, the comparisons of this species with similar species were never perfectly defined. Interestingly, we have observed abundant material of living specimens of *D. biwae* with a high population density in Mulan Lake, Hubei Province, China during our investigation on the testate amoebae of the Yangtse River Valley. The purposes of this work are (1) to characterize morphologically and biometrically *D. biwae* using abundant material, and (2) to discuss the geographical distribution of *D. biwae*.

MATERIALS AND METHODS

Difflugia biwae Kawamura was collected from Mulan Lake, Hubei Province, China on 24-25 July, 2003, and on 28-29 July, 2004. The information of the lake was given by Yang *et al.* (2004). Specimens for light microscopy (Zeiss Axioplan 2 imaging System, Germany) and scanning electron microscopy (X-650 HITACHI, Japan) observations were prepared following the procedure of Yang *et al.* (2004). To visualize the number and position of the nucleus, we incubated the samples with 1.0 µg/ml 4',6-diamidino-2-phenylindole hydrochloride (DAPI) in PBS for 10 min and viewed them under UV fluorescence.

Eight morphometric characters were portrayed in Figs 1 and 2. All measurements were made at middle magnification (320x) using an ocular micrometer. Statistical analysis was performed using the computer program STATISTICA, version 6.0.

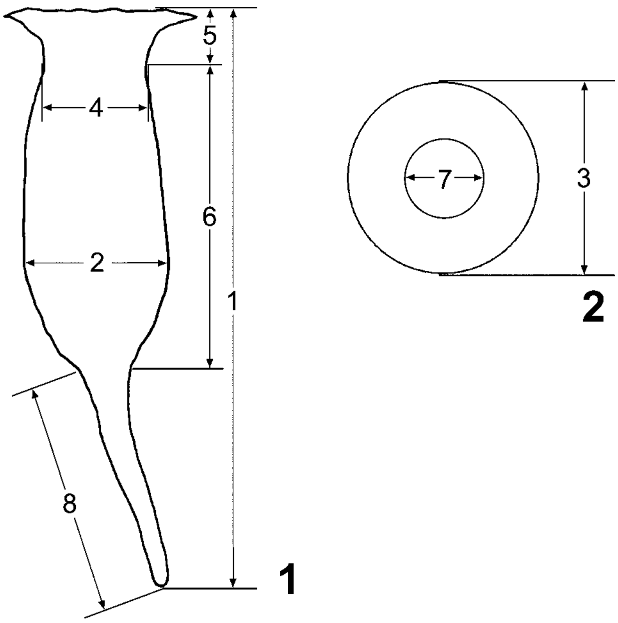
RESULTS AND DISCUSSION

Morphology

The shell is brown, fusiform, 2.1-3.7 collar diameters in body length (Figs 3-5, 15). Surrounding the circular oral aperture is a conspicuous low-funnel-shaped (95°-125°) collar with a ragged margin (Figs 3-6, 15); the body is circular in cross section, narrowest below the collar and gradually swelling to broadest in the position of the posterior 0.20-0.25 of the body length, then narrowing abruptly towards the aboral horn, its greatest diameter 0.60-0.98 collar diameter (Figs 3-5, 15); the aboral horn is stout, tubular and somewhat curved, variable in length, 0.16-0.50 of the total length, and gradually tapering to a blunt tip (Figs 3-5, 7, 8, 15). One to seven colourless long finger-like pseudopodia from the protoplasmic body may protrude through the aperture (Figs 8, 10). A gas vacuole was often observed in living specimens (Fig. 8). Such specimens can right themselves either by extrusion of the long pseudopodia attaching to the substrate (Fig. 10), or by a combination of the pseudopodia and gas vacuole formation. The cytoplasm commonly occupies the greater part of the shell cavity and usually attaches to the internal walls by one or more threads of ectoplasm (Fig. 11); sometimes the cytoplasm can extend into the aboral horn (Fig. 12). Reproduction was sometimes encountered, but the evidence (two shells joined at their oral apertures, Fig. 13) could not be used to determine if it was sexual or asexual because the high optical density of the shells obscured details of the nuclei in these specimens. Although the shell is only semitransparent or opaque, the fluorescent light micrograph stained with DAPI clearly shows that single spherical nucleus is generally located in the posterior of the protoplasmic body and a few tiny algae are sometimes attached to the surface of the shell (Fig. 14). The nucleus is ovular with many nucleoli and has a diameter range of 25-30 µm (Fig. 9).

Study under light microscopy reveals the shell wall is thin, uniform in thickness, composed of fine sand granules with flattish pieces of quartz and muddy particles to produce a smooth surface (Figs 3-5). It has been illustrated by scanning electron microscopy that shells are covered with variously shaped particles, apparently of exogenous origin as determined from the irregularity of the shapes of included particles (Figs 16-18). However, the details of the surface of the shell are visibly different along the anterior body, the posterior body and the aboral horn. The reason is because the particles constituting the anterior portion of the shell covering are small to medium, angular and polymorphic to give a smooth appearance (Fig. 16); the posterior region is composed of small angular particles and medium flattish pieces of quartz to give a smoother appearance (Fig. 17); the aboral horn is made of small flattish pieces of quartz to give the smoothest appearance (Fig. 18). It appears that *Difflugia biwae* is able to select and arrange the building material according to size and shape to construct a species-specific shell. This is in good agreement with that reported by Meisterfeld (2000) in the genus *Difflugia*. No cement structures are recognizable in the scanning electron microscope.

In this study, we found that the typical shape of the shell of *D. biwae* in lateral view is fusiform in outline with a straight neck, a conspicuous great collar flare and a long aboral horn (Fig. 3). Furthermore, the morphological characters of the typical specimens from Mulan



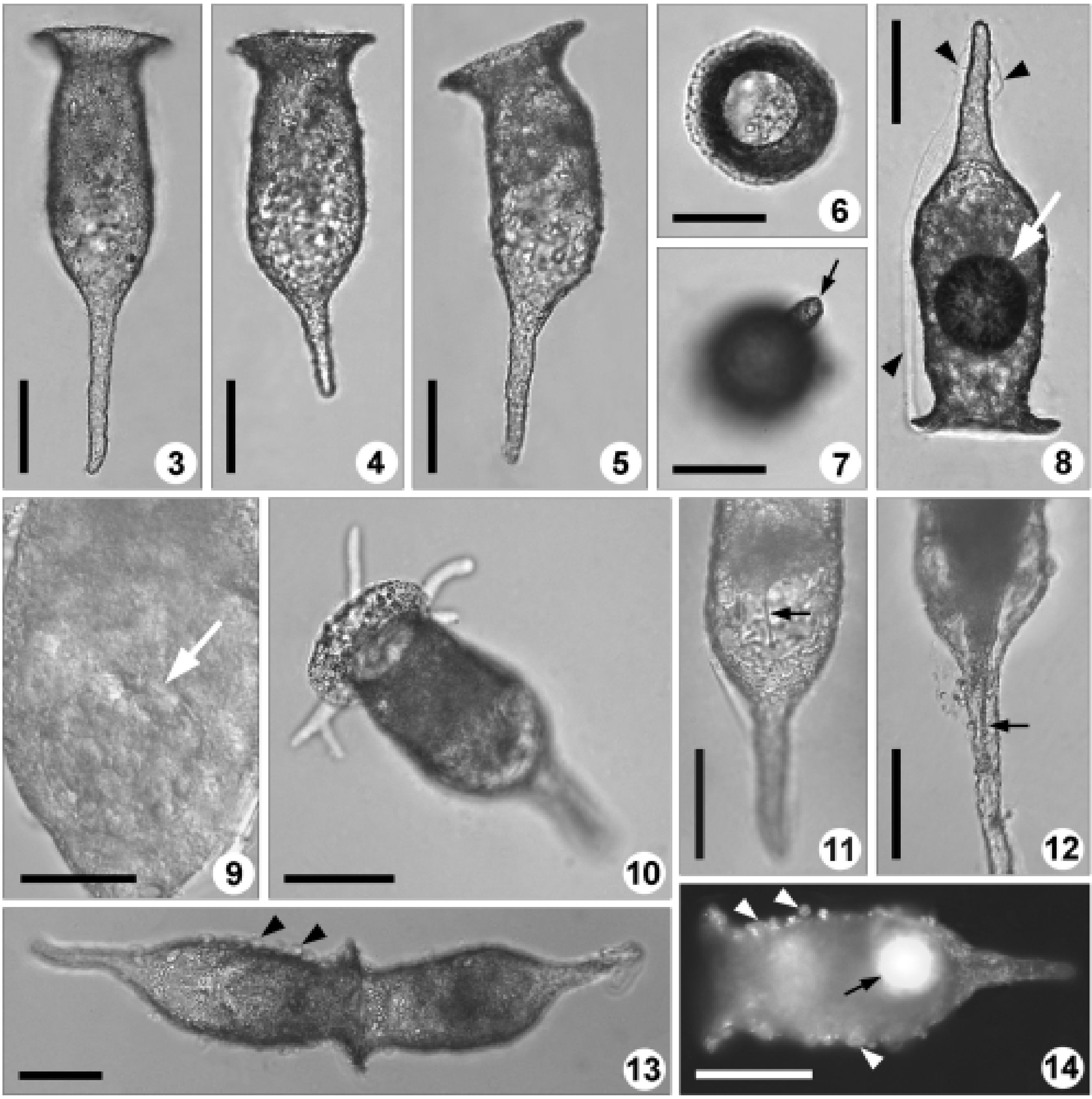
Figs 1, 2. Shell outline and position of measured axes used in this study. 1 - total length; 2 - body width; 3 - collar diameter; 4 - neck width; 5 - collar height; 6 - body length; 7 - aperture diameter; 8 - aboral horn length.

Lake agree well with those reported from Lake Biwa (Kawamura 1918, 1927). However, none of the previous descriptions touched on the atypical shapes of the shell of *D. biwae* (Kawamura 1918, 1927; Abe *et al.* 1957; Toshihiko 1979; Okada *et al.* 1981). Our examination suggests that among the atypical shapes found are those with a short aboral horn (Fig. 4) and those with an oblique neck (Fig. 5). Nevertheless, the population from Mulan Lake bears a strong resemblance to the original in terms of the following characteristics: its fusiform smooth shell furnished with a conspicuous great collar flare (always larger than body-width) around the aperture and a usually curved aboral horn, its long thin pseudopodia, its single spherical nucleus located in the posterior position and its lacustrine habitat (Kawamura 1918, 1927). Unfortunately, Kawamura (1918, 1927) was not very precise in the original illustration of the pseudopodia of living specimens in *D. biwae*, and because of this its typical locomotive form on the substrate was overlooked. The resting form of the specimens as shown in Fig. 8 is similar to that already illustrated by Kawamura (1918, 1927). One or two pseudopodia always extend from the aperture to the back of the shell. In fully extended condition, the pseudopodia may exceed the point of the aboral horn along the shell (Fig. 8). During locomotion two to seven long, sometimes branched, finger-like pseudopodia are seen to be projected out from the aperture (Fig. 10).

Biometry

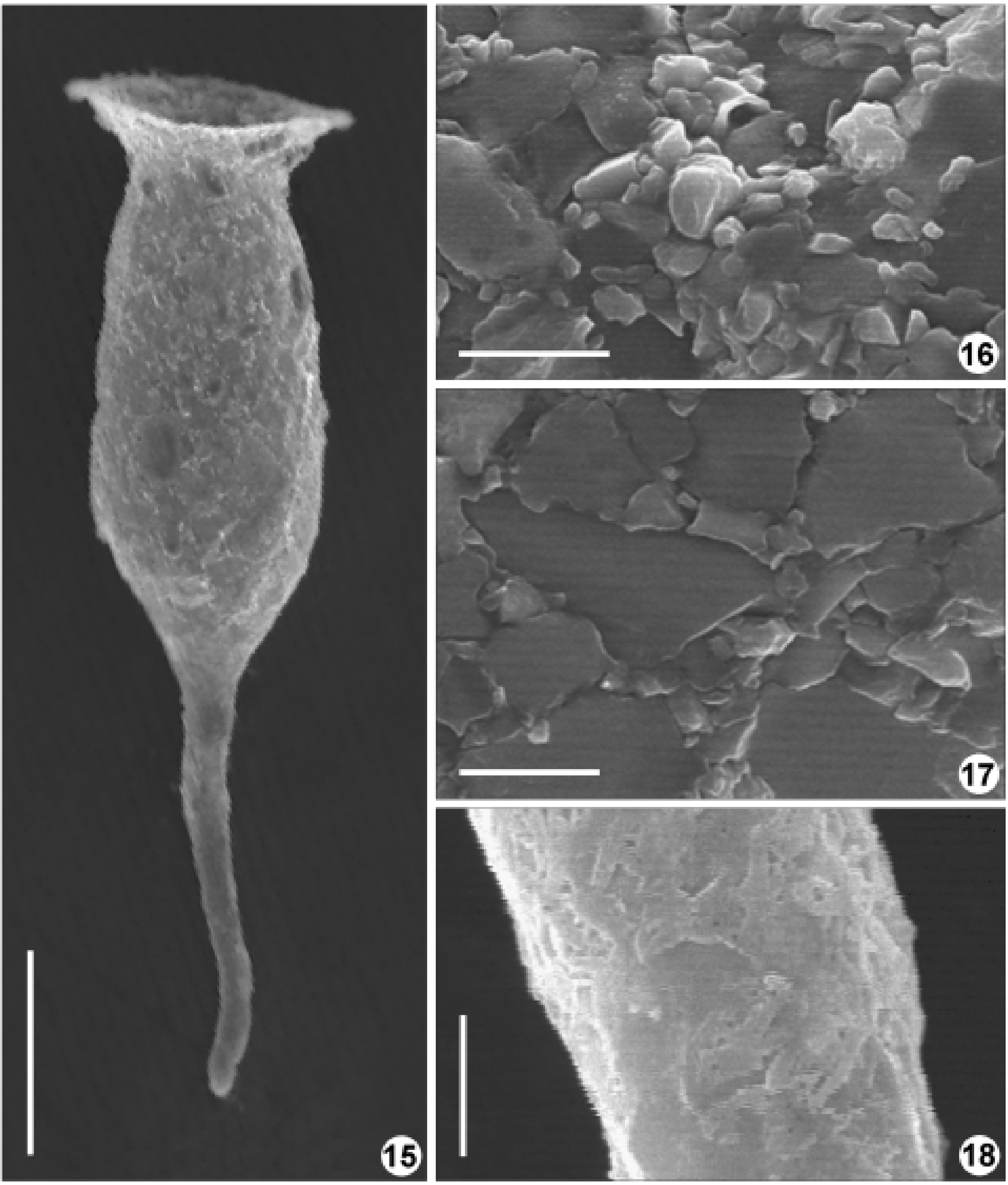
Table 1 shows the detailed morphometric characterization of *Difflugia biwae* according to our studies. The species shows a great diversity in total length, collar height and aboral horn length which have high variability (CV between 11.76 and 24.52). However, body width, collar diameter, neck width, body length, and aperture diameter are fairly constant and have low variability (CV between 5.34 and 8.79) (Table 1). The coefficients of variation of body width and body length are the lowest, and show a remarkable uniformity of *D. biwae* in body size. Also, the size frequency distributions yield bell-shaped (normally distributed) curves and indicate that *D. biwae* is a size-monomorphic species, characterized by a main-size class and a small size range (Figs 19, 20). Table 2 illustrates that more than half (57%) of all the relationships between morphometric characteristics in *D. biwae* are positively correlated at $p < 0.05$, especially TL is strongly positively correlated with HL at $p < 0.001$ ($r = 0.9383$; $n = 100$), while NW is highly positively correlated with AD at $p < 0.001$ ($r = 0.9432$; $n = 100$).

Biometric analysis is the most important part of phenotypic analysis of testate amoebae, as organisms with few morphological characters (Bobrov and Mazei 2004). However, we learned that the size of *D. biwae* is 300-400 µm in total length (including aboral horn) from previous studies (Kawamura 1918, 1927; Abe *et al.* 1957; Okada *et al.* 1981). Thus, total length in the population from Lake Biwa is greater than that in the population from Mulan Lake (300-400 µm *vs.* 165-306 µm). This discrepancy may mainly be due to differences in the variation of the investigated specimens. In the current study, 100 specimens including one kind of typical shape of shell of *D. biwae* and two kinds of atypical shapes (specimens with a short aboral horn and those with an oblique neck) were randomly selected for the morphometric investigation, whereas only a few specimens with typical shape of shell were probably involved in Kawamura's study (Kawamura 1918, 1927). Furthermore, the variation of the aboral horn length was not mentioned at all in any previous studies (Kawamura 1918, 1927; Abe *et al.* 1957; Toshihiko 1979; Okada *et al.* 1981). However, our results clearly showed that the aboral horn length (HL) has the highest value of the coefficient of variation (24.52%), and it is strongly



Figs 3-14. LM photographs of *Difflugia biwae*. **3-5** - lateral views of three different specimens with a long aboral horn (3), a short aboral horn (4) and an oblique neck (5); **6** - apertural view showing the circular aperture is surrounded by a conspicuous great collar flare; **7** - bottom view showing the aboral horn (arrow); **8** - lateral view showing the pseudopodia (arrowheads) and the gas-vacuole (arrow); **9** - view showing the ovular nucleus (arrow); **10** - lateral-oblique view showing the pseudopodia; **11** - living specimen showing the cytoplasm attached to the internal walls of the shell (arrow); **12** - living specimen showing the cytoplasm extended into the aboral horn; **13** - view showing reproduction (shells joined at their oral apertures) and some algae attached to the surface of the shell (arrowheads); **14** - lateral view showing the single nucleus (arrow) and the algae (arrowheads) (Fluorescence microscopy with DAPI stain). Scale bars 20 µm (9); 50 µm (3-8, 10-14).

positive with total length (TL) (Tables 1, 2). Accordingly, the total length of *D. biwae* is largely dominated by the length of the aboral horn. The intrinsic reason for the variation of the aboral horn length in *D. biwae* is still not well understood. One possibility is that it was due to the direct and indirect effect of the local environmental



Figs 15-18. SEM photographs of *Difflugia biwae*. **15** - lateral view; **16** - detail of the surface of the anterior portion of the shell covering, note the close interlocking of small to medium angular and polymorphic particles to give a smooth surface; **17** - detail of the surface of the posterior portion of the shell covering, note the close interlocking of small angular particles and medium flattish pieces of quartz to give the smoother surface; **18** - detail of the surface of the aboral horn, note the close interlocking of small flattish quartz to give the smoothest surface. Scale bars 5 µm (16-18); 50 µm (15).

Table 1. Morphometric characteristics of *Difflugia biwae* from Mulan Lake, China.

Characters[1]	x̄	M	SD	SE	CV	Min	Max	n
Total length (1)	227.6	227	26.76	2.68	11.76	165	306	100
Body width (2)	60.5	60	3.23	0.32	5.34	53	69	100
Collar diameter (3)	78.2	78	6.76	0.68	8.64	63	101	100
Neck width (4)	45.1	45	3.04	0.30	6.74	37	56	100
Collar height (5)	22.9	23	5.08	0.51	22.18	11	36	100
Body length (6)	121.3	122	7.90	0.79	6.51	100	142	100
Aperture diameter (7)	35.4	35	3.11	0.31	8.79	27	45	100
Aboral horn length (8)	85.0	83	20.84	2.08	24.52	29	153	100

[1]Numbers 1-8 in parenthesis designate features as shown in Figs 1 and 2. Data based on random selection of specimens. Measurements in µm. CV - coefficient of variation in %, M - median, Max - maximum, Min - minimum, n - number of individuals investigated, SD - standard deviation, SE - standard error of the mean, x̄ - arithmetic mean.

Table 2. Correlation coefficients between morphometric characteristics in *Difflugia biwae* from Mulan Lake, China. TL - total length; BW - body width; CD - collar diameter; NW - neck width; CH - collar height; BL - body length; AD - aperture diameter; HL - aboral horn length (see Figs 1 and 2).

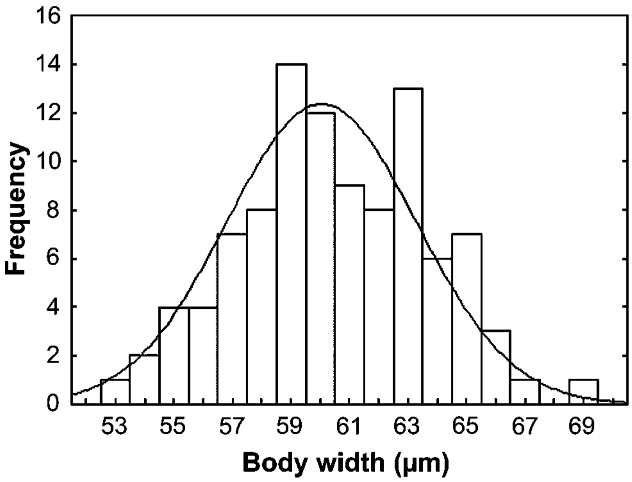
Characters	TL (1)	BW (2)	CD (3)	NW (4)	CH (5)	BL (6)	AD (7)	HL (8)
TL (1)	——							
BW (2)	0.1935	——						
CD (3)	0.4607***	0.2618**	——					
NW (4)	0.1326	0.2763**	0.2700**	——				
CH (5)	0.3824***	0.1659	0.5128***	0.0487	——			
BL (6)	0.5994***	0.1155	0.1907	0.2278*	-0.0205	——		
AD (7)	0.1264	0.2665**	0.3222**	0.9432***	0.1356	0.2109*	——	
HL (8)	0.9383***	0.1604	0.3712***	0.0917	0.2266*	0.4052***	0.0594	——

Significant relationship *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

factors (e.g. water chemistry and trophic status) of the habitat where the population was formed. Another possibility is that the aboral horn length likely represents a normal variation in growth form of this species because all other morphometric characteristics of the shell, with the exception of collar height and total length, and the basic shape of the shell are consistent with the existence of just a single phenotype.

Comparison with similar species

In terms of the shell shape and the possession of an aboral horn, *Difflugia biwae* resembles *D. delicatula* Gauthier-Lièvre *et* Thomas, 1958 (Gauthier-Lièvre and Thomas 1958), *D. elegans* Penard, 1890 (Ogden 1979) and *D. oblonga caudata* Štěpánek, 1952 (Štěpánek 1952). However, *D. biwae* differs from *D. elegans* and *D. oblonga caudata* in having smooth appearance of the shell (*vs.* rough in *D. elegans* and *D. oblonga caudata*) and in having a longer aboral horn (more than 25% of the body length *vs.* less than 20% in *D. elegans* and *D. oblonga caudata*) (Table 3). Although the shell shape and structure of *D. biwae* are most similar to those of *D. delicatula* in having a smooth and fusiform shell with a long aboral horn, it can be clearly distinguished by having a greater total length in excess of 165 µm (*vs.* 75-100 µm in *D. delicatula*) and by having the wide flat collar which is always larger than its body-width in diameter (*vs.* always less than body-width in diameter in *D. delicatula*) (Table 3). So *D. biwae* is considered to be a distinct species in having smooth fusiform shell with a wide flat collar, constricted neck, and long aboral horn.



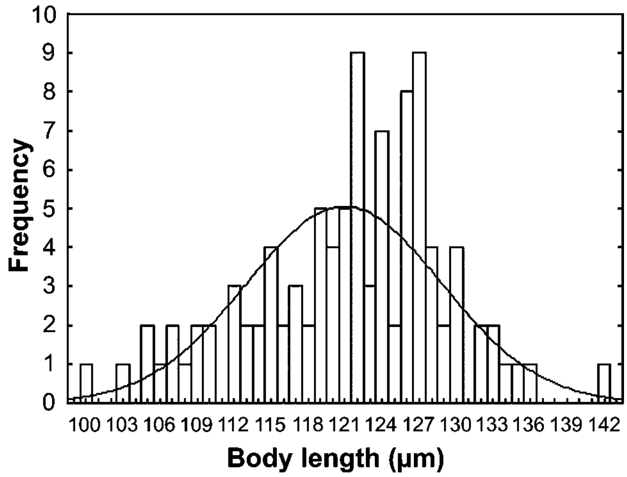


Fig. 19. Histogram showing the size frequency of body width (character 2 as shown in Fig. 1) in *Difflugia biwae*.
Fig. 20. Histogram showing the size frequency of body length (character 6 as shown in Fig. 1) in *Difflugia biwae*.

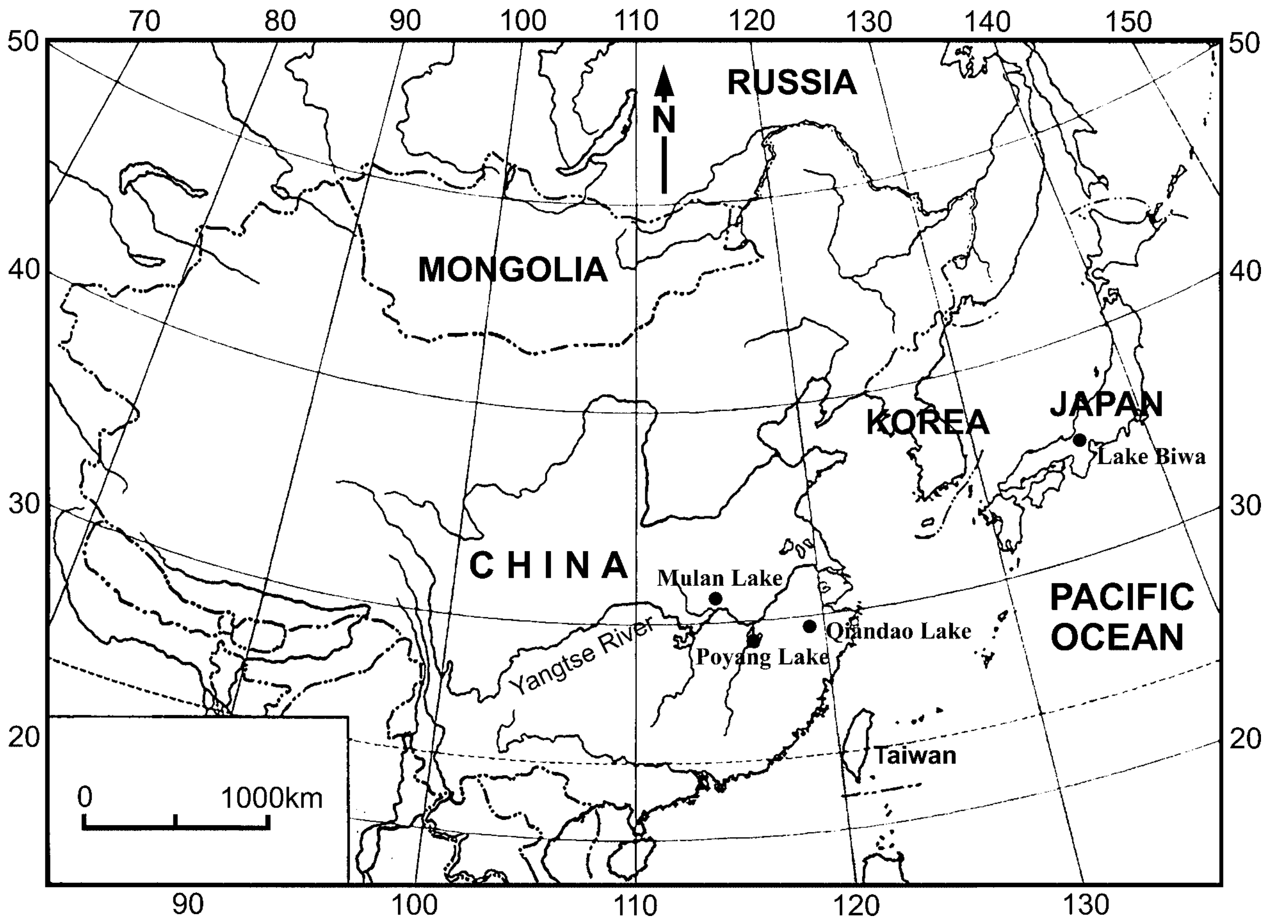


Fig. 21. Distribution of *Difflugia biwae* indicated by filled circles in the East Asia.

Table 3. Comparison of *Difflugia biwae* with three closely-related species. All measurements in µm. (? - data not available)

Characters	*Difflugia biwae*	*D. delicatula*	*D. elegans*	*D. oblonga caudata*
Total length	165-306	75-100	113-158	287
Body width	53-69	28-35	69-99	105
Collar diameter	63-101	?	?	90
Neck width	37-56	?	?	56
Collar height	11-36	8-9	?	?
Body length	100-142	?	?	?
Aperture diameter	27-45	15	35-55	70
Aboral horn length	29-153	20-22	?	42
Shell outline	smooth; elongate fusiform with a conspicuous collar flare and a long aboral horn	smooth; elongate fusiform with a small collar flare and a long aboral horn	rough; elongate oviform with a small collar flare and a short aboral horn	rough; elongate oviform with a conspicuous collar flare and a long aboral horn
Shell composition	small to medium polymorphic particles and flattish quartz	small to medium polymorphic particles and flattish quartz	small to large pieces of angular quartz and often diatoms	medium sherds and sand grains
Collar diameter > Body width	yes	no	no	no
Neck	long and constricted	short and constricted	short and constricted	long and constricted
Sample location	China	Africa	England	Slovakia
Data resource	present study	Gauthier-Lièvre and Thomas 1958	Ogden 1979	Štěpánek 1952

Distribution

Many testate amoebae species have cosmopolitan distribution, at least at the morphological level, but others have restricted distribution (Foissner 1987, 1999; Beyens and Meisterfeld 2001). At the beginning of the twentieth century, the aquatic testacean species *Difflugia biwae* Kawamura was first found in Lake Biwa, Southern Honshu, Japan (Kawamura 1918, Tsugeki *et al.* 2003), but it was not found everywhere other than in three Chinese lakes: Qiandao Lake, Zhejiang Province (Li and Yu 2001), Poyang Lake, Jiangxi Province (Wang *et al.* 2003) and Mulan Lake, Hubei Province (present work) up to now (Fig. 21). Furthermore, it is so large (165-306 µm) that it would have been easily found in Europe and North America, if it were there. Distribution of the *D. biwae* is limited, as far as it is known at present, to only the deep lakes in East Asia (Japan and China). Consequently, *D. biwae* must have a restricted geographical distribution, disproving the old hypothesis that microscopic organisms are cosmopolitan (Finlay 2002). According to Finlay (2002), the abundance of individuals in microbial species is so great that dispersal is rarely restricted by geographical barriers, but geographical barriers to dispersal apply increasingly to organisms above 1-10 mm in size, thus excluding nearly all protists. However, Wilkinson (2001) argued that the ubiquity-biogeography transition gradient starts at 100-150 µm, rather than 1 mm, thus including some larger testate amoebae. It is safe to affirm that our results agree with the opinion of Wilkinson because *D. biwae* always exceeds 150 µm in size.

Regarding its typical habitat, *D. biwae* is considered to be among the typical inhabitants of the deep lake (Kawamura 1927). Recently, Tsugeki *et al.* (2003) showed that *D. biwae* has been extinct in Lake Biwa due to eutrophication caused by nutrient input. So far, only China has the living organism of this special and interesting species.

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