Species Diversity **26**: 171–186
Published online 6 August 2021
DOI: 10.12782/specdiv.26.171

Neotypification of *Difflugia biwae* (Amoebozoa: Tubulinea: Arcellinida) from the Lake Biwa, Japan

Satoshi Ichise[1], Yositaka Sakamaki[2], and Satoshi D. Shimano[3,4]

[1] *Shiga Prefectural Institute of Public Health and Environmental Science, 13-45 Gotenhama, Otsu, Shiga 520-0834, Japan*
[2] *Entomological Laboratory, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan*
[3] *Science Research Center, Hosei University, 2-17-1 Fujimi, Chiyoda, Tokyo 102-8160, Japan*
E-mail: sim@hosei.ac.jp
[4] *Corresponding author*

(Received 6 November 2020; Accepted 22 April 2021)

http://zoobank.org/E60E69CD-3523-4542-AFD4-2FD88DC90DE4

Difflugia biwae Kawamura, 1918 has been redescribed. Here, the neotype of *D. biwae*, which was newly collected from its type locality, Lake Biwa, is designated to the species to clarify its taxonomic status. Morphometric characterization of *D. biwae* was performed. A statistically significant correlation between body length and shell collar diameter was identified in the population of the Lake Biwa, but this correlation was not significant in the population from the Mulan Lake. The PCA scatterplot indicates that the Mulan Lake population could be a separate taxon identified by shorter protuberance length and body length.

Key Words: Arcellinida, Difflugiidae, morphology, testate amoeba, Tubulinea.

Introduction

Difflugia biwae Kawamura, 1918 is a large and morphologically peculiar testate amoeba. This aquatic amoeba species was first isolated and described from Lake Biwa, the largest and oldest lake in Japan, by Kawamura in 1918 (Kawamura 1918). Lake Biwa originated about 4 million years ago and gradually moved northwest to its present location about 400 thousand years ago (Takaya 1963; Satoguchi 2012). Over 1000 species, including 57 endemic species (50 species, five subspecies, two varieties) of organisms have been recorded from Lake Biwa (Nishino and Watanabe 2000). Around half of these species were recorded to be small invertebrates. Since 1990, 11 species have been newly described (Nishino and Watanabe 2000; Ichise et al. 2004). These new species were identified as protists (eukaryotic unicellular organisms), as some endemic species of microalgae (Mori and Miura 1980) and the ciliate *Levicoleps biwae* Foissner, Kusuoka, and Shimano, 2008 (Foissner et al. 2008), and were described as new genera from Lake Biwa.

The genus *Difflugia* Leclerc, 1815 includes the greatest number of species in the order Arcellinida, with around 300 nominal species and varieties (Ogden 1983; Meisterfeld 2002; Meisterfeld and Mitchell 2008). The morphology (Mazei and Warren 2012, 2014, 2015) and molecular phylogeny (Gomaa et al. 2012) of this genus treat it as a polyphyletic taxon.

Regarding the taxonomy of *D. biwae,* Kawamura (1918, 1927) provided only a very short description in Japanese along with a simple line drawing. The type material of *D. biwae* investigated in Kawamura (1918) was not deposited anywhere (Kenichiro Negoro, personal communication). Many years later, both the description and the line drawing of *D. biwae* were presented at least in three Japanese monographs (Kawamura 1952; Kawamura and Hada 1965; Mizuno 1977). Unfortunately, the later data gave us no more information than that reported by Kawamura in the original literature (Kawamura 1918, 1927).

Difflugia biwae has been considered to be endemic to Lake Biwa for nearly a century (Mizuno 1977; Nishino and Watanabe 2000; Tsugeki et al. 2003). As evidence for the endemism of *D. biwae*, Tanaka (1992, 2002, 2004) investigated and made detailed lists of freshwater plankton from major lakes and marshes in all areas of Japan; however, Tanaka did not find *D. biwae* himself—a finding reported in his literatures that was reflected in other faunistic reports from other parts of Japan [also see Japanese protist literature list, including distribution in Shimano and Miyoshi (2008)].

Regular interval investigations and samples of freshwater plankton have still been performed in Lake Biwa by the Shiga Prefectural Institute of Public Health and Environmental Science from 1952 (e.g., Ichise et al. 1996, 2004). According to Kawamura (1927), *D. biwae* was found in the whole area of Lake Biwa, in all water layers from mid-September to the end of November. However, Ichise et al. (1996) reported that the species was the sub-dominant plankton species in August each year until the 1960s. When this species was abundant in the 1960s, it was detected in whole water column, from the surface to the bottom of the lake (Ichise et al. 1996). Subsequently, the population of *D. biwae* decreased rapidly and disappeared from Lake Biwa in the 1970s and no living individuals has been found since April 1994 (Ichise et al. 2004). It seems that this species would be extinct in Lake Biwa.

Difflugia biwae was also recorded from three Chinese lakes: Qiandao Lake, Zhejiang Province (Li and Yu 2001), Poyang Lake, Jiangxi Province (Wang et al. 2003), and Mulan Lake, Hubei Province (Yang and Shen 2005). Yang and Shen (2005) described the morphological details of *D. biwae* along with biometrical data based on living amoebae from Mulan Lake, where it was found in high abundance. Fortunately, one of the authors (Jun Yang, personal communication) provided raw measurement data for *D. biwae*.

The aims of the present paper are (1) to redescribe *D. biwae* from preserved specimens collected from Lake Biwa and to designate the neotype of this species, and (2) to morphometrically compare the population of *D. biwae* from Mulan Lake (China) with the population from Lake Biwa (Japan).

Materials and Methods

Sample collection. *Collection of regular interval investigations of plankton*. The Shiga Prefectural Institute of Fisheries Experimental Station and the Shiga Prefectural Institute of Public Health and Environmental Science carried out regular interval investigations (once in the middle of each month) of plankton from 1952 to 2004, for 57 years in five sites (St. I–V; Fig. 1) in the Northern Basin (Ichise et al. 2004; regular interval investigations have still been performed). Sampling sites were designed as the five dividing points on a line of about 15 km from point St. I (entrance of Hikone port: 35°17′06″N, 136°14′39″E) to point St. V (Funakizaki small peninsula, Adogawa-cho: 35°19′12″N, 136°04′48″E) to three points St. II (35°17′35″N, 136°12′08″E), St. III (35°18′04″N, 136°09′29″E), and St. IV (35°18′36″N, 136°07′09″E), respectively (Ichise et al. 2004). Samples were taken using a plankton net with a NXX14 Muller gauze (sieve size 95 μm: net diameter 25 cm: Rigo Co. Ltd.) in four layers (0–10 m, 10–20 m, 20–40 m, and 40–75 m depth) from the investigation boat. All plankton samples were fixed by 5% formalin solution and stored in the Shiga Prefectural Institute of Fisheries Experimental Station. In the present study, the specimens were mainly obtained from this collection.

Specimens from the collection for this study. The neotype and "voucher specimen series I (VSS-I) and II (VSS-II) (non-type specimens)" were taken from a plankton sample fixed by in 5% formalin solution from a 0–10 m depth at St. III (GPS data mentioned above: Fig. 1) on 15 August 1961 by Mr. Kan-ichi Mita and Mr. Kenji Naka (Ichise et al. 2004).

The sampling data of specimens for morphological studies (non-type specimens) were as follows, code Morph#1: the middle of Seta River (34°59′13″N, 135°54′43″E), southernmost area of Lake Biwa, on unknown day in October 1977 by Satoshi Ichise (SI) (Fig. 1: St. 6 in Ichise et al. 2004; see also Ichise et al. 1998); St. III, 0–10 m depth on 15 August 1961 (Ichise et al. 2004); code Morph#2: same data as the neotype and VSS-I and II; code Measurement#1: 10–20 m depth at St. III (Fig. 1) on 15 August 1961, by Mr. Kan-ichi Mita and Mr. Kenji Naka; code Measurement#2: 0–10 m depth at St. IV (Fig. 1) on 18 September 1963, by Mr. Kenji Naka, (Ichise et al. 2004).

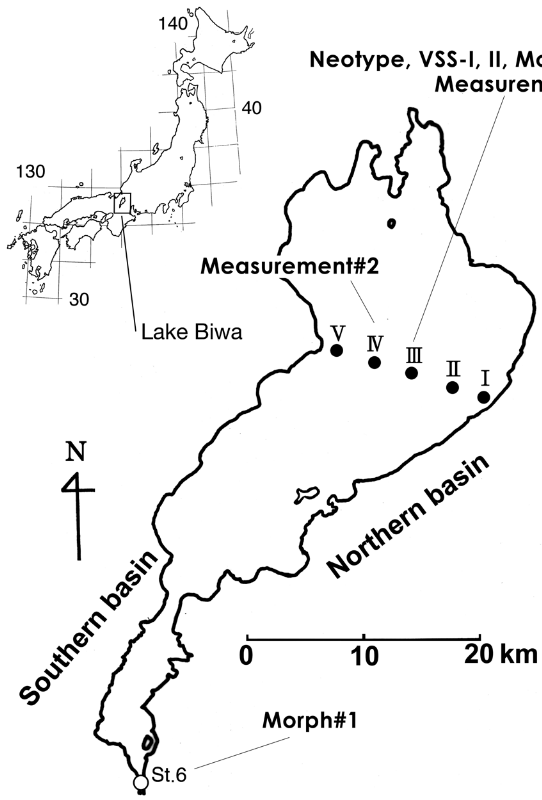

Fig. 1. Maps illustrating the longtime sampling in the Lake Biwa. Left upper map provides an overview of the area with frames indicating the position of the map of Lake Biwa. Codes refer to sample sites given in the Materials and Methods section; filled symbols refer to St. I–V; an open symbol refers to St. 6.

The raw data measurements of 100 shells of *D. biwae* in Mulan Lake, China [the same raw data of Yang and Shen (2005)] were provided courtesy of Dr. Yang, based on the species collected from Mulan Lake, Hubei Province, China on 24–25 July 2003 and on 28–29 July 2004 (Yang and Shen 2005). The information regarding the lake is given in Yang et al. (2004).

Neotype and voucher specimen series. The neotype and specimens of the series VSS-I were mounted with Euparal on mounted slides under stereoscopic microscope (SMZ745, Nikon). Specimens of the series VSS-II were sorted by hand under the stereoscopic microscope and stored in vials/jars filled with 5% formalin solution (see Appendix 2).

Light micrographs and scanning electron micrographs. Light micrographs of specimens (Morph#1) were taken in 5% formalin solution with an Optiphot I microscope (Nikon) in the Shiga Prefectural Institute of Public Health and Environmental Science, by SI. Scanning electron micrographs of specimens (Morph#2) were taken with a JSM-6380LV SE microscope (JEOL) in the Kyoto Municipal Waterworks Bureau by SI. Before the scanning electron microscopic observation, shells of *D. biwae* fixed by 5% formalin solution, which were washed by distilled water sev-

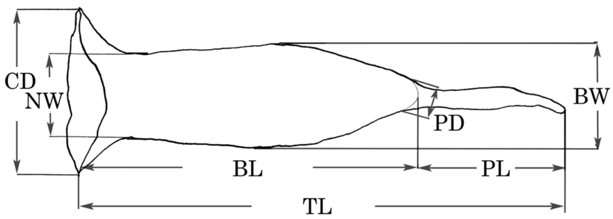

Fig. 2. Shell outline and positions of measured axes used in present study: TL, total length; BW, body width; CD, collar diameter; NW, neck width; BL, body length; PD, protuberance diameter; PL, protuberance length.

eral times, sputtered with gold, and placed on a sample stage with carbon double-sided tape.

Measurement of specimens. Measurements of *D. biwae* were performed on the following specimens according to axes shown in Fig. 2 under a light microscope by SI: the neotype, 18 shells of VSS I (details in Appendix 1), 161 shells of Measurement#1, and 70 shells of Measurement#2 (Ichise et al. 2004).

The raw measurement data of Chinese population of *D. biwae* [the same raw data used by Yang and Shen (2005)] was provided courtesy of Dr. Yang, based on 100 shells of specimens collected from Mulan Lake, Hubei Province, China on 24–25 July 2003, and on 28–29 July 2004 (Yang and Shen 2005).

Morphometric analyses. Basic statistical analysis and calculation of correlation coefficients were performed using the BellCurve software for Excel (Social Survey Research Information Co., Ltd., Japan). A principal component analysis (PCA) using a variance-covariance matrix with a 95% confidential ellipse was conducted to investigate the differences among the datasets (the neotype with VSS I, Measurement#1, and Measurement#2). The analyses were performed on size-corrected data, which was divided by the geometric mean of the respective specimens. No rotation was applied to the multivariate data.

For the investigation of divergence among the different datasets, Tukey–Kramer's multiple comparisons were conducted to clarify if single variables differed significantly between the populations or datasets. Additionally, a linear discriminant analysis (LDA) was performed on the size-corrected data to reveal size and shape differences between the sample sets. All analyses were performed with JMP version 8.0 (SAS).

Results

Taxonomy

Order **Arcellinida** Kent, 1880
Suborder **Arcellina** Haeckel, 1894
Family **Difflugiidae** Wallich, 1864
Genus ***Difflugia*** Leclerc, 1815
Difflugia biwae Kawamura, 1918
[Japanese standard name: Biwako-tsubokamuri]
(Figs 3–5)

Difflugia biwae Kawamura, 1918: 114–115, fig. 174; Kawamura 1927: 2054, fig. 3886; Kawamura 1952: 1735, fig. 4855; Kawamura and Hada 1965: 33, fig. 98-1; Mizuno 1977: 26, fig. 7; Li and Yu 2001: 117, table 1; Wang et al. 2003: 347, table 1; Yang and Shen 2005: 103–107, figs 3–18.

Material examined. Neotype: TNS-AL–63110 (VSS-I-10 in Appendix 1) in TNS (Department of Botany, National Museum of Nature and Science), on mounted slide with Euparal, 0–10 m in depth at St. III (35°18′04″N, 136°09′29″E) on 15 August 1961 by Mr. Kan-ichi Mita and Mr. Kenji Naka (Ichise et al. 2004), deposited at Department of Botany, National Museum of Nature and Science, previously known as the National Science Museum.

Voucher specimen series, VSS I: The 19 specimens were individually mounted on slides. Fifteen specimens were deposited at TNS under the catalogue numbers TNS-AL-63101 to 63116 (VSS-I-1 to 16), and the remaining four were deposited at the Lake Biwa Museum in Shiga Prefecture, Japan, under the catalogue numbers LBM 2040000001–2040000004 (VSS-I-17 to 20). The collection data for all the specimens is same as the neotype.

VSS II: TNS-AL-63117 to 63140 in TNS (see Appendix 2 with vial data), a total of 51 specimens kept in 5% formalin solution in vials (see Appendix 2). The collection data is same as the neotype.

The specimens of Morph#1, Morph#2, Measurement#1 and Measurement#2 were lost from a laboratory collection in Shiga Prefectural Institute of Public Health and Environmental Science. Sampling data were shown in Materials and methods part.

Designation of neotype and voucher specimens. The authors could not confirm the existence of the type series (holotype and paratypes) of *D. biwae*. The type series or any observed specimens are believed to have been lost in the past because no specimens were deposited by Kawamura (1918), which was confirmed by interviews of several of his colleagues (e.g., Kenichiro Negoro, personal communication); this was also the case for his simultaneous studies.

The original very short description of this species is incomplete, and thus its taxonomic status must be clarified. Furthermore, because the population of the type locality is considered to be extinct, a name-bearing type for *D. biwae* is deemed to be needed to verify their conservation status in the long term. Thus, the specimen (TNS-AL–63110), which was collected from Lake Biwa as the type locality assigned by Kawamura (1918) (Fig. 3A), is herein designated as the neotype for *D. biwae*. The species was abundant with morphological variation in Lake Biwa population as the subdominant plankton species in August each year in that collection period in 1961. Accordingly, the present neotypication meets the conditions of Article 75 of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature 1999).

Diagnosis. Shell fusiform, transparent, and brown in fixed specimens, composed of mineral particles or diatom frustules as xenosomes, from their environment. The circu-

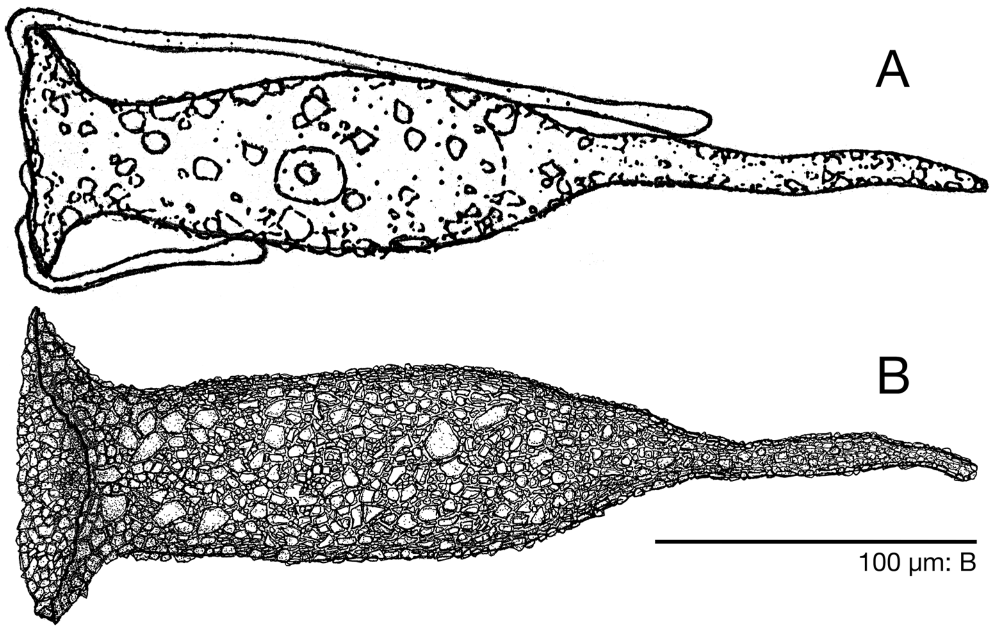

Fig. 3. Line drawings of *Difflugia biwae*. A, Lateral view with cytoplasm (original line drawing, originally no scale: after Kawamura 1918); B, lateral view of shell (present study, scale 100 μm for B).

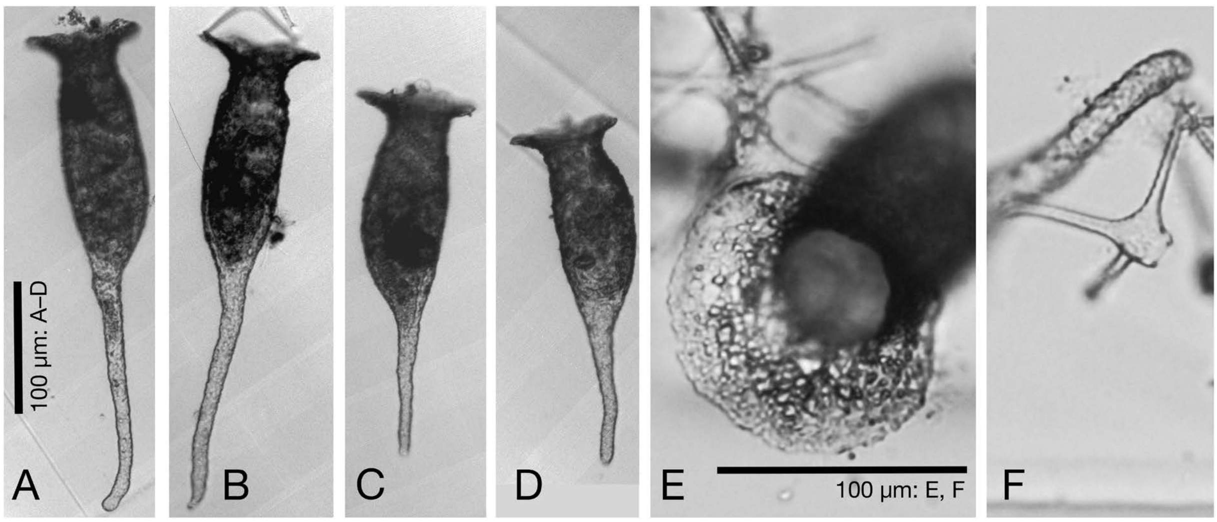

Fig. 4. Light micrographs of the shell of *Difflugia biwae*. A–D, Lateral views of whole shells of four different specimens with variations (A after "Photo. 1" in Ichise et al. 2004); E, apertural view showing the circular aperture is surrounded by a conspicuous great collar flare; F, apertural view showing the protuberance. Sampling data, Morph#1 in the Materials and Methods section. Scale 100 μm in A for A–D; scale 100 μm in E for E and F.

lar oral aperture surrounded by a conspicuous low funnel-shaped collar with a ragged margin. Shell circular in cross-section, narrowest at the base of the collar and gradually swelling to broadest at the posterior, then narrowing gently toward the aboral protuberance. The protuberance gently sinuous and pointed at the tip. One or two pseudopodia extended from the aperture to the back of the shell.

Redescription. Shell fusiform, transparent, and brown in fixed specimens, composed of mineral particles (Figs 3B, 4E, 5A–G) or diatom frustules [typically *Praestephanos suzukii* (A. Tuji and Kociolek, 2000), same one species as previous "*Stephanodiscus suzukii*" and "*S. pseudosuzukii*" in Ichise et al. (2004)] as xenosomes, captured from their environment (Fig. 5D).

Body length [(TL) in Fig. 2, all measurement in VSS-I, maximum–minimum, followed by the measurement of the neotype in parenthesis, the same hereinafter] 359–230 (312) μm, broadest body width (BW) 79–54 (59) μm, narrowest body width (NW) 52–40 (42) μm, broadest diameter of collar (CD) 97–78 (83) μm, broadest diameter of aboral protuberance (PD) 26–19 (24) μm, length of protuberance (PL) 157–84 (155) μm (Figs 4A–D, 5A). Shell circular in cross-section (Fig. 5B), narrowest below the collar and gradually swelling to broadest 1.98–1.29 (1.40), (BW/NW in Fig. 2, the same hereinafter) in the position of the posterior 0.19–0.27 (0.19), (BW/TL) of the body length, then narrowing

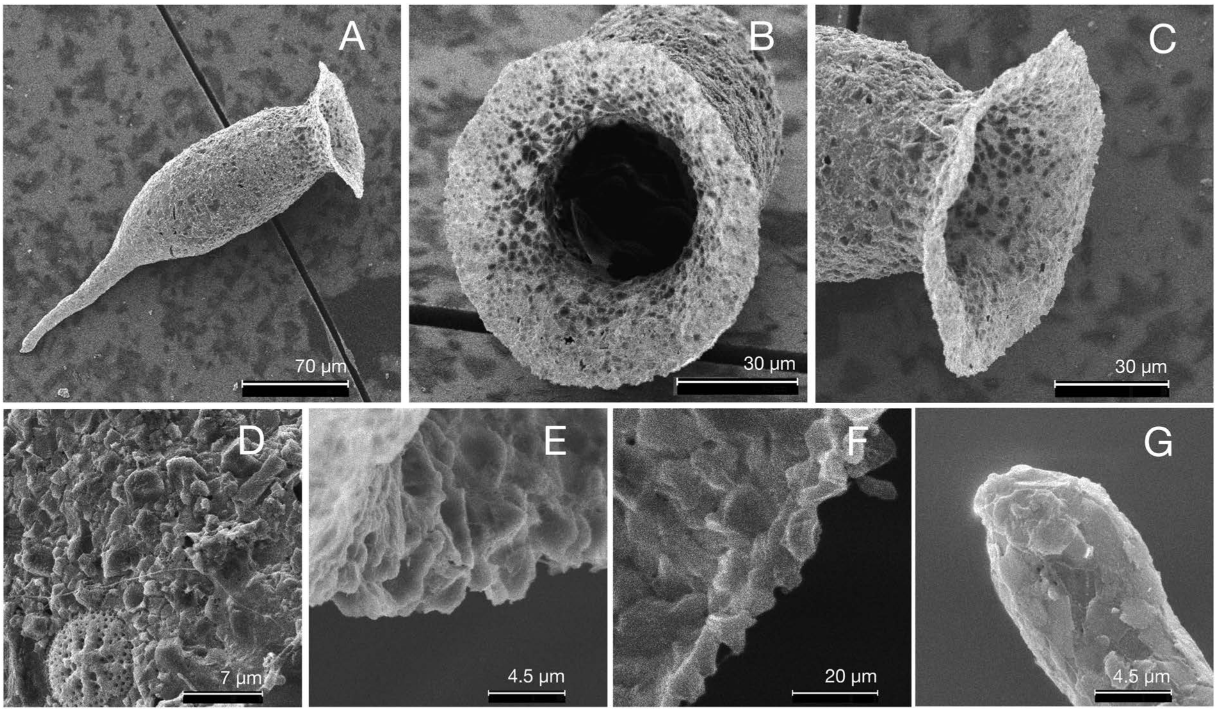

Fig. 5. Scanning electron micrographs of the shell of *Difflugia biwae*. A, Lateral view of whole shell; B, apertural view showing the circular aperture is surrounded by a conspicuous great collar flare; C, lateral-apertural view of collar flare; D, composed of mineral particles or diatom frustules (typically *Praestephanos suzukii*, endemic diatom of Lake Biwa) as xenosomes, captured from their environment; E, detail of surface of collar flare rim (inside) with small angular particles and flattish pieces of quartz; F, detail of surface of collar flare rim (outside); G, tip of the protuberance with flattish pieces of quartz. Sampling data of these specimens was given as Morph#1 in the Materials and Methods section. Scales are given in each photo.

abruptly toward the aboral protuberance (Figs 4A–D, 5A). A conspicuous low-funnel-shaped collar surrounds the circular oral aperture (Figs 4A–E, 5A–C). Collar margin thin and slightly wavy in the outer periphery (Figs 4E, 5A–C, E, F). Broadest diameter of body 0.90–1.59 (1.41), collar diameter (CD/BW). The aboral protuberance, gently sinuous and pointed in the tip (Figs 4A–D, F, 5A, G). Broadest diameter of the protuberance is in the position of the posterior attachment part of body, at 0.07–0.05 (0.08) of the body lengths (PD/TL) (Figs 4A–D, 5A). All raw measurement data shown in Appendix 1 [one obviously irregular shell (VSS-I-19) excluded from all size discussion and morphometric analyses]. One or two pseudopodia extend from the aperture to the back of the shell (Fig. 3A; Kawamura 1918). Food predominantly diatoms such as *P. suzukii* (Fig. 5D; *P. suzukii* in shell of this species, as previously in cytoplasm).

Distribution. Lake Biwa, Shiga Prefecture, Japan (e.g., Kawamura 1918) and three Chinese lakes: Qiandao Lake, Zhejiang Province (Li and Yu 2001); Poyang Lake, Jiangxi Province (Wang et al. 2003); Mulan Lake, Hubei Province, China. However, Tsugeki et al. (2003) and Ichise et al. (2004) reported that *D. biwae* is extinct in Lake Biwa due to eutrophication caused by nutrient input from its surrounding river systems.

Remarks. *Difflugia biwae* resembles *D. delicatula* Gauthier-Lièvre and Thomas, 1958, *D. elegans* Penard, 1890 and *D. oblonga caudata* Štěpánek, 1952 in its shell shape and aboral protuberance. However, *D. biwae* can be clearly distinguished from these species by the feature that its collar diameter is longer than its body width. Additionally, *D. biwae* differs from these species in that it has a long neck (vs. short neck in *D. delicatula* and *D. elegans*) and the smooth appearance of its elongated fusiform shell (vs. rough appearance of elongated oviform shells in *D. elegans* and *D. o. caudata*). Yang and Shen (2005) have discussed the comparisons of these conjugates.

Yang and Shen (2005) referred to "its typical locomotive form on the substrate." However, the authors did not observe pseudopodia of *D. biwae* in the present study. The description of pseudopodia in the redescription section is based on the original line drawings (Fig. 3A; Kawamura 1918) and the observations of Yang and Shen (2005).

Ichise et al. (2004) reported that when the body of *D. biwae* was crushed, shells of Centrales diatom, mainly *P. suzukii*, exuded from the cells, suggesting that this species mainly preyed predominantly on diatoms, such as *P. suzukii* (Fig. 5D).

In the present paper, we restore the Japanese standard name as "Biwako-tsubokamuri" written in the original description (Kawamura 1918). Although Mizuno (1977) used "Biwa-tsubokamuri" as the Japanese name for the species, it was a miss-spelling at first. "Biwako" and "ko" represent "Lake Biwa" and "Lake" respectively in Japanese.

Biometry

Divergence and correlation of each character. Table 1 shows the detailed morphometric characterization of *Difflu-*

Table 1. Morphometric characteristics of *Difflugia biwae* from Lake Biwa (taken in 1961: neotype and VSS I, n=19: left, 1961: Measurement#1, n=161: middle left, 1963: Measurement#2, n=70: middle right), and from Mulan Lake (n=100: right)[1]. Measurements in μm. Minimum (Min), maximum (Max), arithmetic mean (X), standard deviation (SD), and coefficient of variation in % (CV).

Characteristics[2]	X[5]				M				SD				SE			
Total length (TL)	317.1 *a*	293.2 *b*	311.2 *a*	227.5 *c*	315	290	310	227	24.43	24.81	34.22	26.76	5.76	1.96	4.12	2.68
Body width (BW)	63.2 *a*	60.8 *b*	59.9 *b*	60.5 *b*	62	40	60	60	5.57	2.52	2.77	3.23	1.31	0.20	0.33	0.32
Collar diameter (CD)	87.8 *a*	81.2 *b*	82.3 *b*	78.2 *c*	89	80	83	78	6.07	6.00	4.74	6.76	1.43	0.47	0.57	0.68
Neck width (NW)	43.2 *b*	40.8 *c*	42.7 *b*	45.1 *a*	42	40	42	45	3.17	2.52	2.63	3.04	0.75	0.20	0.32	0.30
Body length (BL)[3]	183.7 *a*	184.8 *a*	176.1 *b*	144.1 *c*	184	190	180	144	14.80	15.73	15.97	9.83	3.49	1.24	1.92	0.99
Protuberance diameter (PD)[4]	45.3	17.5	18.3	—	42	20	18	—	9.43	3.61	1.88	—	2.22	0.29	0.23	—
Protuberance length (PL)	133.3 *a*	108.4 *b*	135.2 *a*	84.1 *c*	135	110	130	83	20.27	18.54	24.94	20.84	4.78	1.47	3.00	2.08

Characteristics[2]	CV				Min				Max			
Total length (TL)	7.71	8.47	10.99	11.76	269	240	250	165	359	350	380	306
Body width (BW)	8.81	6.16	4.62	5.34	54	35	52	53	79	50	68	69
Collar diameter (CD)	6.91	7.40	5.76	8.64	71	70	70	63	97	92	92	101
Neck width (NW)	7.34	6.16	6.15	6.74	40	35	40	37	52	50	52	56
Body length (BL)[3]	8.05	8.52	9.07	6.82	156	120	130	120	206	230	200	171
Protuberance diameter (PD)[4]	20.80	20.61	10.26	—	40	10	15	—	83	30	22	—
Protuberance length (PL)	15.21	17.12	18.45	24.52	84	70	100	29	160	170	190	153

[1] Data after Yang and Shen (2005); [2] Alphabetical letters in Fig. 2; [3] Calculated raw dataset provided from Dr. Yang; [4] Data not measured in Yang and Shen (2005); [5] Different italic alphabets after arithmetic means (X) indicate significant differences among means in each row by Tukey–Kramer multiple comparison (α=0.05).

Table 2. Correlation coefficients between morphometric characteristics in *Difflugia biwae* from Lake Biwa (1961: neotype and VSS I, n=19). Abbreviations (the same hereinafter in Tables 3–5), TL, total length; BW, body width; CD, collar diameter; NW, neck width; BL, body length; PD, protuberance diameter; PL, protuberance length. Significant relationship: *, p<0.05; **, p<0.001 (the same hereinafter in Tables 3, 4).

Characteristics[1]	TL	BW	CD	NW	BL	PD
TL	—					
BW	0.0042	—				
CD	0.3857	−0.4987*	—			
NW	−0.2369	−0.0019	0.0889	—		
BL	0.5599*	0.1233	0.4347	0.1974	—	
PD	−0.2581	0.2785	−0.1150	0.2522	0.1477	—
PL	0.7963**	−0.0850	0.1475	−0.4296	−0.0553	−0.4189

Table 3. Correlation coefficients between morphometric characteristics in *Difflugia biwae* from Lake Biwa (1961: Measurement#1, n=160).

Characteristics[1]	TL	BW	CD	NW	BL	PD
TL	—					
BW	0.1792*	—				
CD	0.2029**	0.1893*	—			
NW	0.1027	0.2999**	0.3028**	—		
BL	0.6598**	0.1466	0.2264*	0.1433	—	
PD	0.3104**	0.1555*	0.1630*	0.1347	0.1058	—
PL	0.7728**	0.1146	0.0786	0.0155	0.0330	0.3236**

Table 4. Correlation coefficients between morphometric characteristics in *Difflugia biwae* from Lake Biwa (1963: Measurement#2, n=70).

Characteristics[1]	TL	BW	CD	NW	BL	PD
TL	—					
BW	0.0191	—				
CD	0.2802*	0.2309	—			
NW	0.0814	0.1273	0.4711**	—		
BL	0.7356**	−0.1229	0.2649*	−0.0016	—	
PD	0.1174	−0.0708	0.0365	−0.0588	0.1823	—
PL	0.9011**	0.1049	0.2148	0.1127	0.3690**	0.0444

gia biwae from four sample series: Lake Biwa in 1961 ("the neotype and VSS I" and "Measurement#1"), 1963 ("Measurement#2") and from Mulan Lake. The species shows great diversity in protuberance length (PL), which has high variability (coefficient of variation in %, CV between 15.21 and 24.52). While body width (BW), collar diameter (CD), neck width (NW), and body length (BL) are fairly constant and have low variability (CV between 4.62 and 8.81, between 5.76 and 8.64, between 6.15 and 7.34, and between 6.82 and 9.07, respectively).

In three sample series from Lake Biwa (Tables 2–4), positive correlations ($p<0.05$) were found among the measurement characteristics of *D. biwae* only in two relationships: total length (TL) and body length (BL), and TL and protuberance length (PL). Neck width (NW) was highly positively correlated with CD at $p<0.01$, while TL and BL were positively correlated with CD (at least $p<0.05$) (Tables 3, 4).

Comparison among sample series. Tukey–Kramer multiple comparison tests indicated significant differences among each arithmetic mean (X), but did not show a clear trend in each of the sample series (Table 1).

Multiple comparison revealed that all variables of the Mulan Lake sample series were significantly different from the three series of the Lake Biwa population. The PCA scatterplot (Fig. 6) indicated that the Mulan Lake and Lake Biwa populations tended to differ, with some overlaps. Eigen vectors of PC1 and PC2 axes indicated that they are mainly contributed by PL and BL (Table 5).

Overlap rate was evaluated by LDA (Table 6). Three sample series from Lake Biwa ("the neotype and VSS 1", "1961: Measurement#1" and "1963: Measurement#2") included many misclassifications. Some samples from Lake Biwa (1961: Measurement#1 and 1963: Measurement#2) were misclassified as samples which belonged to the Mulan Lake population. Conversely, 5% of samples from Mulan Lake were misclassified as samples from Lake Biwa.

Discussion

The correlation between BL and CD was statistically significant in the Lake Biwa sample series, but was not significant in the sample series from Mulan Lake (Table 2; Yang and Shen 2005). Slight differences in the values of correlation coefficient were found between Lake Biwa and Mulan Lake individuals.

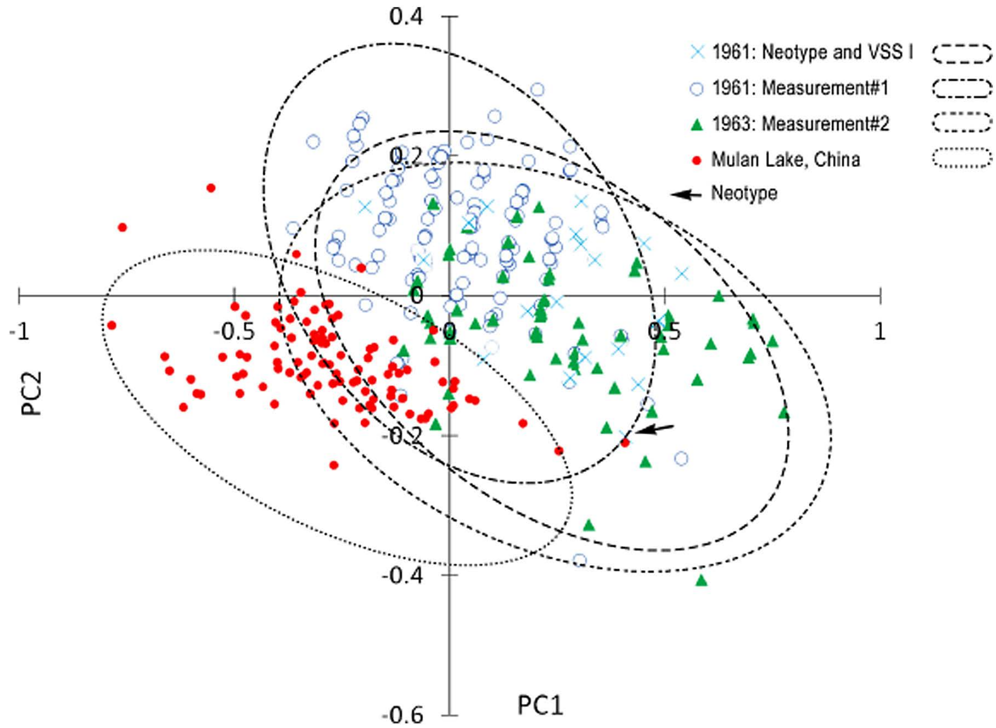

Fig. 6. Scatter plot of principal component scores (PC1 and PC2) performed with size-corrected data of four different sample series from Lake Biwa (1961: neotype and VSS I, 1961: Mesurement#1 and 1963: Mesurement#2) and Mulan Lake. Crosses, neotype and VSS 1; open circles, Measurement#1; filled triangles, Measurement#2; filled circles, Mulan Lake, China; arrow, neotype. Each broken line circle indicates 95% confidence limit. Table 1. Morphometric characteristics of *Difflugia biwae* from Lake Biwa (taken in 1961: neotype and VSS I, n=19: left, 1961: Measurement#1, n=161: middle left, 1963: Measurement#2, n=70: middle right), and from Mulan Lake (n=100: right)[1]. Measurements in μm. Minimum (Min), maximum (Max), arithmetic mean (X), standard deviation (SD), and coefficient of variation in % (CV).

Table 5. Eigen vectors of principal component analysis from characteristics of *Difflugia biwae* from Lake Biwa and from Mulan Lake.

	PC1	PC2	PC3	PC4	PC5
BW	0.0191	0.02312	0.27602	0.48468	0.82946
CD	0.11336	0.13665	0.68089	−0.68987	0.17012
NW	−0.05639	−0.2593	0.6725	0.48076	−0.49619
BL	0.27204	0.91021	0.06174	0.23868	−0.19164
PL	0.95373	−0.29166	−0.06431	0.03264	−0.0115
Contribution (%)	73.5	13.6	6.9	3.3	2.7

Table 6. Numbers of sample predicted by linear discriminant analysis from characteristics of *Difflugia biwae* from Lake Biwa and from Mulan Lake.

Sample series	n	Sample series predicted*			
		1961: neotype and VSS I	1961: Measurement#1	1963: Measurement#2	Mulan Lake
1961: neotype and VSS I	19	11 (58)	1 (5)	7 (37)	0
1961: Measurement#1	160	21 (13)	11 (71)	22 (14)	3 (2)
1963: Measurement#2	70	11 (16)	11 (16)	42 (60)	6 (8)
Mulan Lake	100	1 (1)	1 (1)	3 (3)	95 (95)

* numbers between parentheses are percentage.

The scatter plot based on the PC1 and PC2 in the PCA with five traits excluding TL and Protuberance diameter (PD) shows that the Mulan Lake sample is out of variation among three sample series ("the neotype and VSS 1", "1961: Measurement#1" and "1963: Measurement#2") from Lake Biwa, although there is a slight overlap between them. The PC1 and PC2 have the most positive contributions to PL and BL, respectively, indicating that the Mulan Lake population tends to have shorter PL and BL than the Lake Biwa population. Indeed, as shown in Table 1, the arithmetic means for PL and BL were significantly smaller in the Mulan Lake sample. This PCA indicates that the Mulan Lake population could be a separate taxon identified by shorter PL and BL.

Based on the present measurements (Lake Biwa) and the provided measurement data (Mulan Lake), the multiple comparison tests revealed that all variables of the population from the Mulan Lake were significantly different from the three series of the Lake Biwa population. Morphometric analysis showed that the two populations were differentiated at the subspecies level, with some overlaps.

However, because we could not obtain a living individual of this species in Lake Biwa, we would like to suspend this decision. If living individuals are obtained from Lake Biwa in the future, we could make a taxonomic decision by determining its genetic distance using DNA analysis between the Lake Biwa and Mulan Lake populations.

We have not seen *D. biwae* from the other two lake populations, Qiandao Lake (Li and Yu 2001) and Poyang Lake (Wang et al. 2003) from China, but we hope that further study on all populations may make the species of *D. biwae* more clear. However, these three lakes in China are also threatened by pollution (Dr. Jun Yang, personal communication), meaning that the species is at risk of extinction.

Acknowledgements

We thank to Professor Dr. Jun Yang, Aquatic EcoHealth Group, Institute of Urban Environment, Chinese Academy of Sciences, China, for providing raw data of measurement of *D. biwae* from Mulan Lake. We also thank to Drs Taisuke Ohtsuka, Minako Ashiya, and Yasushi Kusuoka, curators, the Lake Biwa Museum, Japan, for their valuable comments on the manuscript and Dr. Tobias Pfingstl, Department for Biodiversity and Evolution, Institute of Biology, University of Graz, Austria for helping us with the statistical analysis. We would also like to express our sincere gratitude to Mr. Takashi Morita of the Shiga Prefectural Fisheries Experimental Station for providing us with fixed samples of *D. biwae*, Mr. Takeshi Negoro of the Kyoto Municipal Waterworks Bureau for allowing us to use the scanning electron microscope for *D. biwae*, and all the researchers of Lake Biwa Environmental Research Institute, Shiga Prefecture, which was newly established in 2005.

We would like to express our appreciation to Dr. Yuri Mazei, Department of Hydrobiology, Faculty of Biology, Lomonosov Moscow State University, Moscow, Russia for his critical reading of the manuscript in the early stages of manuscript preparation. And we would like to be grateful to two anonymous reviewers and associate editor Dr. Hiroshi Yamasaki for their constructive comments and invaluable suggestions on the manuscript.

This research was partly supported by the JSPS KAKENHI (grant numbers 15H02858) to SS.

References

Foissner, W., Kusuoka, Y., and Shimano, S. 2008. Morphology and gene sequence of *Levicoleps biwae* n. gen., n. sp. (Ciliophora, Prostomatida), a proposed endemic from the ancient Lake Biwa, Japan. Journal of Eukaryotic Microbiology 55: 185–200.

Gomaa, F., Todorov, M., Heger, T. J., Mitchell, E. A. D., and Lara, E. 2012. SSU rRNA phylogeny of Arcellinida (Amoebozoa) reveals that the largest Arcellinida genus, *Difflugia* Leclerc 1815, is not monophyletic. Protist 163: 389–399.

Haeckel, E. 1894. *Systematische Phylogenie der Protisten und Pflanzen. Erster Theil des Entwurfs einer systematischen Stammesgeschichte*. Verlag von Georg Reimer, Berlin, xvi+400 pp.

Ichise, S., Wakabayashi, T., Matsuoka, Y., Yamanaka, S., Fujiwara, N., and Nomura, K. 1996. Succession of phytoplankton in northern basin of Lake Biwa, 1978–1995. Report of the Shiga Prefectural Institute of Public Health and Environmental Science 31: 84–100. [In Japanese]

Ichise, S., Wakabayashi, T., Matsuoka, Y., Yamanaka, S., Fujiwara, N., and Nomura, K. 1998. Succession of zooplankton in the southern basin of Lake Biwa, 1982–1997. Report of the Shiga Prefectural Institute of Public Health and Environmental Science 33: 144–160. [In Japanese]

Ichise, S., Wakabayashi, T., Morita, T., Kusuoka, Y., and Nishino, M. 2004. Disappearance and its past distribution of the endemic protista *Difflugia biwae* Kawamura, 1918 in Lake Biwa, central Japan. Report of the Shiga Prefectural Institute of Public Health and Environmental Science 39: 57–63. [In Japanese]

International Commission on Zoological Nomenclature 1999. *International Code of Zoological Nomenclature, Fourth edition*, International Trust for Zoological Nomenclature, London, xxix+306 pp.

Kawamura, T. 1918. Amoebida. Pp. 112–116. *In*: Kawamura, T. (Ed.) *Nihon Tansui Seibutsugaku Jyoukann [Japanese Freshwater Biology First Volume]*. Syoukabou, Tokyo. [In Japanese]

Kawamura, T. 1927. *Difflugia biwae* Kawamura. P. 2054. *In*: Hirase, S., Hozawa, S., Izuka, A., Kawamura, T., Kishida, K., Komai, T., Kuroda, N., Marukawa, H., Marumo, S., Nakazawa, K., Oka, A., Okada, Y., Ohshima, H., Sakaki, M., Shinohara, T., Tanaka, S., Uchida, K., Uchida, S., Uchida, T., Yokoyama, K., and Yoshida, S. (Eds) *Figuraro de Japanaj Bestoj* (*An Illustrated Encyclopedia of the Fauna of Japan*). Hokuryukan, Tokyo. [In Japanese]

Kawamura, T. 1952. *Difflugia biwae* Kawamura. P. 1735. *In*: Abe, T., Aoyagi, H., Asanuma, S., Ii, N., Iwasa, M., Iizuka, K., Ueno, M., Uchida, K., Uchida, S., Uchida, T., Ezaki, T., Oshima, H., Obuchi, S., Oka, A., Okada, Y., Okukawa, K., Okuda, S., Kato, K., Kawamura, T., Kaburaki, T., Kishida, K., Kumano, M., Kubo, I., Kuroda, N., Kuroda, T., Koga, T., Komai, T., Sasaki, N., Sato, H., Saito, S., Sakai, T., Shiino, S., Shinohara, Y., Sugiyama, T., Tanaka, S., Takakuwa, Y., Takasu, N., Takasu, N., Taki, Y., Tokioka, T., Toriumi, A., Nakazawa, T., Nozawa, K., Hada, Y., Baba, K., Hatai, K., Hirase, S., Hozawa, S., Matsubara, K., Maruyama, H., Miyake, S., Mori, T., Yamaguchi, E., Yamaguti, S., and Yoshida, S. (Eds) *Illustrated Encyclopedia of the Fauna of Japan, Revised Edition.* Hokuryukan, Tokyo. [In Japanese]

Kawamura, T. and Hada, Y. 1965. *Difflugia biwae* Kawamura. P. 33. *In*: Okada K., Uchida S., and Uchida T. (Eds) *New Illustrated Encyclopedia of the Fauna of Japan I.* Hokuryukan, Tokyo. [In Japanese] [as the first edition cited as Okada et al. (1981) in Yang and Shen (2005)]

Kent, W. S. 1880. *A Manual of the Infusoria: Including a Description of All Known Flagellate, Ciliate, and Tentaculiferous Protozoa, British and Foreign, and an Account of the Organization and the Affinities of the Sponges. Vol. 1.* David Bogue, London, 472 pp.

Leclerc, M. 1815. Note sur la *Difflugie*, nouveau genre de Polype amorph. Mémoires du Muséum d'Histoire Naturelle 2: 474–478.

Li, G. G. and Yu, Z. M. 2001. A study on the diversity of a zooplankton community in Lake Qiandaohu, Zhejiang. Biodiversity Science 9: 115–121. [In Chinese with English abstract]

Mazei, Yu. and Warren, A. 2012. A survey of the testate amoeba genus *Difflugia* Leclerc, 1815 based on specimens in the E. Penard and C.G. Ogden collections of the Natural History Museum, London. Part 1: Species with shells that are pointed aborally and/or have aboral protuberances. Protistology 7: 121–171.

Mazei, Yu. and Warren, A. 2014. A survey of the testate amoeba genus *Difflugia* Leclerc, 1815 based on specimens in the E. Penard and C.G. Ogden collections of the Natural History Museum, London. Part 2: Species with shells that are pyriform or elongate. Protistology 8: 133–171.

Mazei, Yu. and Warren, A. 2015. A survey of the testate amoeba genus *Difflugia* Leclerc, 1815 based on specimens in the E. Penard and C.G. Ogden collections of the Natural History Museum, London. Part 3: Species with shells that are spherical or ovoid. Protistology 9: 3–49.

Meisterfeld, R. 2002. Order Arcellinida Kent, 1880. Pp. 827–860. *In*: Lee, J. J., Leedale, G. F., and Bradbury, P. (Eds) *The Illustrated Guide to the Protozoa. Vol. 2, Second edition*. Society of Protozoologists, Kansas.

Meisterfeld, R. and Mitchell, E. 2008. *Difflugia* Leclerc 1815. Version 02 September 2008 (under construction). http://tolweb.org/Difflugia/124487/2008.09.02 in The Tree of Life Web Project, http://tolweb.org/ (22 October 2020).

Mizuno, T. 1977. *Illustrations of the Freshwater Plankton of Japan (Revised Edition)*. Hoikusha, Osaka, viii+353 pp., 32 pls. [In Japanese] [cited as "Toshihiko (1979)" in Yang and Shen (2005)]

Mori, S. and Miura, T. 1980. List of plant and animal species living in Lake Biwa. Memoirs of the Faculty of Science. Kyoto University 8: 1–3.

Nishino, M. and Watanabe, N. C. 2000. Evolution and endemism in Lake Biwa, with special reference to its gastropod mollusk fauna. Pp. 151–180. *In*: Rossiter, A. and Kawanabe, H. (Eds) *Ancient Lakes*: *Biodiversity, Ecology and Evolution*. Academic Press, San Diego and Tokyo.

Ogden, C. G. 1983. Observations on the systematics of the genus *Difflugia* in Britain (Rhizopoda, Protozoa). Bulletin of the British Museum (Natural History). Zoology 44: 1–73.

Satoguchi, Y. 2012. Geological history of Lake Biwa. Pp. 9–16. *In*: Kawanabe, H., Nishino, M., and Maehata, M. (Eds) *Lake Biwa: Interactions between Nature and People*. Springer, Berlin Heidelberg.

Shimano, S. and Miyoshi, N. 2008. A bibliography of publications on free-living protists (ciliates and testate amoebae) recorded from Japan. —preliminary version, April 2008. Japanese Journal of Protozoology 41: 133–152. (updated version 1.5, 9 May 2010) https://sites.google.com/site/bibliographyjapan/ (22 October 2020).

Takaya, Y. 1963. Stratigraphy of the Paleo-Biwa group and the paleogeography of Lake Biwa with special reference to the origin of the endemic species in Lake Biwa. Thesis of Kyoto University, Kyoto.

Tanaka, M. 1992. *The Lakes in Japan*. The University of Nagoya Press, Nagoya, v+530 pp., 4 pls. [In Japanese]

Tanaka, M. 2002. *An Illustrated Guide of Animal and Plant Planktons in Japan*. The University of Nagoya Press, Nagoya, viii+584 pp., 4 pls. [In Japanese]

Tanaka, M. 2004. *The Lakes in Japan II*. The University of Nagoya Press, Nagoya, vii+396 pp., 4 pls. [In Japanese]

Tsugeki, N., Oda, H., and Urabe, J. 2003. Fluctuation of the zooplankton community in Lake Biwa during the 20th century: a paleolimnological analysis. Limnology 4: 101–107.

Yang, J. and Shen, Y. 2005. Morphology, biometry and distribution of *Difflugia biwae* Kawamura, 1918 (Protozoa: Rhizopoda). Acta Protozoologica 44: 103–111.

Yang, J., Beyens, L., Shen, Y. F., and Feng, W. S. 2004. Redescription of *Difflugia tuberspinifera* Hu, Shen, Gu et Gong, 1997 (Protozoa: Rhizopoda: Arcellinida: Difflugiidae) from China. Acta Protozoologica 43: 281–289.

Wallich, G. C. 1864. On the extent, and some of the principal causes, of structural variation among the Difflugian Rhizopods. The Annals and Magazine of Natural History Third Series 13: 215–245.

Wang, J. Q., Wu, J. P., Yu, Y. B., Wang, T. Y., and Cheng, G. 2003. The specific list, quantitative distribution and change of zooplankton in the season of spring and autumn in Poyang Lake. Journal of Lake Sciences 15: 345–352. [In Chinese with English abstract]

Appendix 1. Measurements, raw data, of neotype and voucher specimen series I (VSS-I, 1961, n=20) on slide glass. Abbreviations, TL, total length; BW, body width; CD, collar diameter; NW, neck width; BL, body length; PD, protuberance diameter; PL, protuberance length.

Specimen number	Measured axes in Fig. 2										
	TL	BW	CD	NW	BL	PD	PL	BW/NW	BW/TL	CD/BW	PD/TL
VSS-I-1	322	58	92	41	167	19	155	1.41	0.18	1.59	0.06
VSS-I-2	341	61	91	40	184	22	157	1.53	0.18	1.49	0.06
VSS-I-3	330	69	96	42	191	19	139	1.64	0.21	1.39	0.06
VSS-I-4	328	63	78	41	178	22	150	1.54	0.19	1.24	0.07
VSS-I-5	269	62	85	46	185	20	84	1.35	0.23	1.37	0.07
VSS-I-6	308	65	86	49	182	19	126	1.33	0.21	1.32	0.06
VSS-I-7	341	69	91	52	206	21	135	1.33	0.20	1.32	0.06
VSS-I-8	338	68	89	40	204	20	134	1.70	0.20	1.31	0.06
VSS-I-9	351	63	94	41	201	26	150	1.54	0.18	1.49	0.07
VSS-I-10 [neotype]	312	59	83	42	157	24	155	1.40	0.19	1.41	0.08
VSS-I-11	315	60	85	43	182	21	133	1.40	0.19	1.42	0.07
VSS-I-12	305	61	88	45	166	19	139	1.36	0.20	1.44	0.06
VSS-I-13	310	60	91	46	198	20	112	1.30	0.19	1.52	0.06
VSS-I-14	274	62	90	42	156	25	118	1.48	0.23	1.45	0.09
VSS-I-15	330	60	92	41	196	21	134	1.46	0.18	1.53	0.06
VSS-I-16	315	79	71	40	172	19	143	1.98	0.25	0.90	0.06
VSS-I-17	359	54	97	42	199	19	160	1.29	0.15	1.80	0.05
VSS-I-18	278	58	86	45	178	20	100	1.29	0.21	1.48	0.07
VSS-I-19*	230	61	79	43	175	25	55	1.42	0.27	1.30	0.11
VSS-I-20	298	70	84	42	189	19	109	1.67	0.23	1.20	0.06

* One obviously irregular shell (VSS-I-19*) removed from Redescription part, all size discussion and morphometric analyses.

Appendix 2. Preservation detail of voucher specimen series II (VSS-II, 1961, n=51) stored in vials/jars filled with 5% formalin solution, added the catalogue numbers in TNS (Department of Botany, National Museum of Nature and Science).

Catalogue number in TNS	Jar number	Vial number	Number of amoeba shells
TNS-AL-63117	J-1	VSS-II-1	1
TNS-AL-63118	J-1	VSS-II-2	1
TNS-AL-63119	J-1	VSS-II-3	1
TNS-AL-63120	J-1	VSS-II-4	1
TNS-AL-63121	J-1	VSS-II-5	1
TNS-AL-63122	J-1	VSS-II-6	1
TNS-AL-63123	J-1	VSS-II-7	1
TNS-AL-63124	J-1	VSS-II-8	1
TNS-AL-63125	J-2	VSS-II-9	1
TNS-AL-63126	J-2	VSS-II-10	1
TNS-AL-63127	J-2	VSS-II-11	1
TNS-AL-63128	J-2	VSS-II-12	1
TNS-AL-63129	J-2	VSS-II-13	1
TNS-AL-63130	J-2	VSS-II-14	1
TNS-AL-63131	J-2	VSS-II-15	1
TNS-AL-63132	J-3	VSS-II-16	1
TNS-AL-63133	J-3	VSS-II-17	1
TNS-AL-63134	J-3	VSS-II-18	1
TNS-AL-63135	J-3	VSS-II-19	1
TNS-AL-63136	J-3	VSS-II-20	1
TNS-AL-63137	J-3	VSS-II-21	1
TNS-AL-63138	J-3	VSS-II-22	10
TNS-AL-63139	J-3	VSS-II-23	10
TNS-AL-63140	J-3	VSS-II-24	10
Total			51

Appendix 3. Measurements, raw data, of Measurement#1 (1961, n=161) and Measurement#2 (1963, n=70). Abbreviations, TL, total length; BW, body width; CD, collar diameter; NW, neck width; BL, body length; PD, protuberance diameter; PL, protuberance length.

Specimen number	Shell number	Measured axes in Fig. 2						
		TL	BW	CD	NW	BL	PD	PL
Measurement#1	1	320	60	90	40	200	20	120
	2	300	60	80	40	170	15	130
	3	320	62	80	45	190	18	130
	4	300	62	90	40	180	20	120
	5	290	60	85	50	200	20	90
	6	320	60	90	40	190	20	130
	7	275	55	80	40	175	10	100
	8	285	60	80	40	185	20	100
	9	295	60	90	40	185	20	110
	10	310	60	90	40	205	13	105
	11	310	60	80	40	170	15	140
	12	290	60	90	42	180	15	110
	13	260	60	80	38	180	15	80
	14	320	60	80	40	200	18	120
	15	320	80	80	40	210	20	110
	16	320	70	80	45	200	20	120
	17	280	60	80	40	180	10	100
	18	270	60	80	40	180	15	90
	19	300	60	90	40	190	15	110
	20	260	60	80	40	160	15	100
	21	260	60	80	40	170	15	90
	22	295	62	90	40	190	18	105
	23	300	60	80	40	180	20	120
	24	320	62	80	40	200	20	120
	25	300	65	80	40	190	20	110
	26	300	60	80	40	190	18	110
	27	300	60	70	35	200	15	100
	28	290	60	80	40	190	18	100
	29	300	60	80	40	190	20	110
	30	330	62	70	40	170	20	160
	31	320	60	90	40	190	20	130
	32	260	60	80	40	170	15	90
	33	280	60	80	40	190	15	90
	34	300	70	90	50	190	20	110
	35	240	65	90	40	160	15	80
	36	280	70	90	50	180	20	100
	37	290	60	80	40	170	15	120
	38	290	65	90	45	190	20	100
	39	280	60	80	40	170	20	110
	40	340	60	80	50	190	20	150
	41	310	60	80	45	200	20	110
	42	300	60	80	40	190	20	110
	43	280	60	90	40	170	20	110
	44	280	63	80	45	190	15	90
	45	240	65	90	45	140	20	100
	46	260	60	80	40	190	15	70
	47	280	60	85	40	200	18	80
	48	330	62	90	45	220	20	110
	49	280	60	80	45	200	15	80
	50	280	60	80	40	170	20	110
	51	350	70	80	45	210	20	140
	52	340	68	90	40	200	20	140
	53	300	65	85	40	190	15	110
	54	320	60	80	40	190	20	130
	55	320	60	90	42	200	15	120

Appendix 3. Continued.

Specimen number	Shell number	Measured axes in Fig. 2						
		TL	BW	CD	NW	BL	PD	PL
Measurement#1	56	280	60	70	40	190	15	90
	57	350	60	80	42	210	20	140
	58	330	70	70	40	160	20	170
	59	340	70	80	45	200	20	140
	60	340	65	90	40	210	20	130
	61	320	65	85	45	200	20	120
	62	300	60	90	40	200	20	100
	63	310	60	80	42	180	15	130
	64	340	70	90	40	210	20	130
	65	280	60	85	40	200	15	80
	66	300	60	70	40	185	20	115
	67	300	62	80	38	160	20	140
	68	290	60	92	48	200	20	90
	69	300	60	80	40	180	25	120
	70	290	60	80	40	180	20	110
	71	290	62	90	45	200	20	90
	72	270	60	80	40	180	15	90
	73	330	60	90	42	210	15	120
	74	310	60	80	40	200	20	110
	75	320	60	80	40	190	30	130
	76	280	60	80	40	180	20	100
	77	270	60	80	40	180	20	90
	78	280	60	90	40	190	18	90
	79	250	60	80	42	150	15	100
	80	260	60	80	40	170	20	90
	81	300	60	80	40	190	20	110
	82	310	70	90	42	200	20	110
	83	240	60	70	40	170	20	70
	84	270	60	80	40	180	20	90
	85	280	60	80	45	180	20	100
	86	310	58	80	40	170	20	140
	87	270	60	80	40	120	15	150
	88	300	60	80	42	190	20	110
	89	250	60	80	40	170	15	80
	90	270	60	80	40	170	15	100
	91	310	60	90	40	200	15	110
	92	290	60	80	40	190	20	100
	93	250	60	80	40	165	13	85
	94	290	60	80	40	190	20	100
	95	250	70	70	40	170	15	80
	96	280	60	80	40	190	15	90
	97	270	60	80	40	170	20	100
	98	280	58	70	40	180	15	100
	99	280	68	80	40	180	15	100
	100	295	60	80	40	195	15	100
	101	320	60	70	40	200	15	120
	102	310	60	80	45	190	20	120
	103	300	60	80	45	180	20	120
	104	310	60	80	45	190	20	120
	105	310	58	80	42	190	20	120
	106	290	58	70	38	170	20	120
	107	260	60	80	40	160	20	100
	108	270	60	90	43	180	15	90
	109	280	60	90	45	170	20	110
	110	280	60	90	45	170	20	110
	111	270	60	80	40	180	20	90

Appendix 3. Continued.

Specimen number	Shell number	Measured axes in Fig. 2						
		TL	BW	CD	NW	BL	PD	PL
Measurement#1	112	270	60	80	40	170	20	100
	113	270	60	80	40	170	20	100
	114	280	60	80	40	180	10	100
	115	300	60	80	40	170	20	130
	116	310	60	90	45	190	10	120
	117	290	60	70	40	170	10	120
	118	270	65	92	40	170	10	100
	119	310	60	80	40	200	10	110
	120	270	60	80	40	190	10	80
	121	330	55	80	40	200	20	130
	122	330	60	80	40	180	20	150
	123	320	60	80	40	190	20	130
	124	280	60	70	40	200	10	80
	125	250	60	70	40	160	10	90
	126	290	60	80	40	190	10	100
	127	280	60	80	40	200	10	80
	128	280	60	80	40	180	15	100
	129	310	50	80	40	190	10	120
	130	310	60	90	35	180	20	130
	131	280	60	80	40	170	10	110
	132	300	60	80	40	200	15	100
	133	270	60	80	40	190	18	80
	134	320	55	90	40	200	20	120
	135	280	60	70	40	200	20	80
	136	320	60	80	40	200	20	120
	137	260	60	70	40	170	10	90
	138	280	60	80	40	180	20	100
	139	280	60	80	40	200	20	80
	140	270	55	70	38	170	15	100
	141	270	60	80	40	160	20	110
	142	320	60	80	40	190	20	130
	143	270	60	70	40	180	20	90
	144	250	60	70	40	170	10	80
	145	300	60	80	40	200	10	100
	146	320	55	80	35	190	20	130
	147	270	60	85	42	170	20	100
	148	300	60	90	40	200	20	100
	149	270	60	80	40	180	20	90
	150	320	60	80	35	190	20	130
	151	300	60	80	35	180	20	120
	152	350	60	80	40	220	10	130
	153	280	55	85	40	160	20	120
	154	290	60	80	40	180	15	110
	155	280	60	70	40	170	20	110
	156	300	55	70	40	200	20	100
	157	300	60	80	40	170	20	130
	158	250	55	70	40	150	15	100
	159	350	60	80	40	230	22	120
	160	300	60	85	40	200	20	100
	161	250	60	70	40	250	15	—
Measurement#2	1	380	58	85	42	200	20	180
	2	370	60	90	42	190	20	180
	3	370	60	92	43	180	18	190
	4	320	58	75	40	180	15	140
	5	350	61	85	42	200	15	150
	6	330	60	82	45	170	18	160

Appendix 3. Continued.

Specimen number	Shell number	Measured axes in Fig. 2						
		TL	BW	CD	NW	BL	PD	PL
Measurement#2	7	290	60	75	40	170	15	120
	8	320	65	85	49	160	16	160
	9	310	60	80	43	180	20	130
	10	330	59	80	40	180	18	150
	11	310	57	84	45	180	18	130
	12	320	58	86	47	175	18	145
	13	320	60	89	48	180	16	140
	14	310	54	85	45	180	16	130
	15	320	58	85	46	190	18	130
	16	310	58	78	40	190	20	120
	17	290	62	91	48	185	20	105
	18	290	62	84	42	190	20	100
	19	380	62	80	40	200	18	180
	20	270	59	82	42	160	16	110
	21	300	60	80	40	170	15	130
	22	270	60	80	40	160	15	110
	23	320	63	85	45	170	15	150
	24	350	60	71	42	190	20	160
	25	320	52	70	45	140	18	180
	26	270	60	80	40	160	20	110
	27	300	60	80	40	180	18	120
	28	290	61	84	42	160	15	130
	29	270	62	82	42	160	16	110
	30	310	60	80	40	170	18	140
	31	320	62	85	45	170	20	150
	32	270	60	70	40	165	20	105
	33	310	60	80	42	170	19	140
	34	320	62	85	45	200	18	120
	35	315	60	81	42	190	20	125
	36	350	58	85	43	190	20	160
	37	305	59	86	45	170	19	135
	38	350	57	83	43	200	20	150
	39	350	60	76	40	180	20	170
	40	350	58	83	43	200	18	150
	41	370	62	86	52	200	20	170
	42	310	68	82	45	160	18	150
	43	310	65	80	45	180	16	130
	44	270	55	70	40	165	18	105
	45	300	55	75	40	190	19	110
	46	280	68	84	42	130	18	150
	47	300	66	80	40	170	20	130
	48	250	60	84	45	140	18	110
	49	270	60	75	42	170	15	100
	50	262	56	82	45	150	20	112
	51	370	62	86	42	190	20	180
	52	300	58	85	42	170	16	130
	53	285	62	82	45	170	15	115
	54	280	60	85	45	170	20	110
	55	300	60	80	40	180	22	120
	56	310	55	85	42	180	18	130
	57	285	60	92	45	180	18	105
	58	310	60	85	42	180	15	130
	59	310	60	85	45	170	20	140
	60	370	62	90	42	200	20	170
	61	350	60	82	40	190	20	160
	62	325	55	85	40	200	20	125

Appendix 3. Continued.

Specimen number	Shell number	Measured axes in Fig. 2						
		TL	BW	CD	NW	BL	PD	PL
Measurement#2	63	270	60	82	42	170	20	100
	64	265	60	80	40	160	20	105
	65	250	60	80	40	150	20	100
	66	265	60	85	45	160	20	105
	67	340	60	85	40	180	18	160
	68	380	62	85	40	195	18	185
	69	320	60	85	45	190	20	130
	70	250	60	80	40	150	20	100