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Evidence for two different morphotypes of *Difflugia tuberspinifera* from China

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Abstract

Difflugia is a morphologically diverse genus of testate amoebae, which are common components of freshwater ecosystems. We observed a new morphotype similar to *Difflugia tuberspinifera* but without spine in four Xiamen reservoirs, Fujian Province, southeast China. We investigated its morphology and biometry using light and scanning electron microscopy. The linear discriminant analysis and principal component analysis of biometric characters revealed that the spiny and spineless forms of *D. tuberspinifera* differed only in the presence or absence of spine. Shell height, shell diameter, aperture diameter and collar height did not differ significantly between the two morphotypes. The number of conical spines varies from 0 to 8. However, the distribution of spine numbers showed two main modes at 0 (spineless form 45.1% of individuals) and 4–6 (38.9%), suggesting the possible existence of two genetically distinct lineages. Spines may have ecological and evolutionary significance. Our results suggested that the spiny and spineless morphotypes of *D. tuberspinifera* represent either a single variable taxon with different ecotypes or sibling species. Further morphological studies on clonal variations and molecular approaches are needed to clarify if the spineless morph represents an independent species or not.

Keywords: Biometry; New morphotype; Taxonomy; Testate amoebae; Xiamen reservoirs

Introduction

Testate amoebae are a diverse polyphyletic group of shelled amoeboid protozoa, which are important components of aquatic ecosystems including lakes, rivers, reservoirs, and wetlands (Beyens and Meisterfeld 2001; Chardez 1960; Mitchell et al. 2008). Research on testate amoebae during the past three decades has resulted in great progress on realizing the potential of this group as a sensitive indicator of local hydrology and surface moisture conditions (Booth 2001; Charman 2001; Elliott et al. 2012). Although the higher level classification of protist have been updated based on ultrastructural and molecular phylogenetic studies, the phylogenetic picture at lower taxonomic levels are only slowly starting to become clearer (Adl et al. 2012; Gomaa et al. 2012).

Reliable taxonomy is essential for studies of the biodiversity, ecology and evolution of testate amoebae. The genus

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Difflugia Leclerc, 1815 is the earliest described taxon and the most diverse group of testate amoebae. More than 300 species and subspecies in *Difflugia* have been described based on variations in the morphology, composition and size of the shell although these microorganisms have few morphological characters (Meisterfeld 2002). A hundred year ago, variations of both apertural teeth and spines in *Difflugia corona* were reported by Jennings (1916) under cultural conditions. Extreme morphological variations in field populations and laboratory cultures have been reported for several *Difflugia* species (Lahr and Lopes 2006; Medioli et al. 1987; Štěpánek 1952; Todorov et al. 2009). However, the accepted degree of intraspecific morphological variation appears to be variable among authorities because the intraspecific variability of shell morphology in most species are complicated and poorly known (Mazei and Warren 2012; Medioli et al. 1987; Meisterfeld 2002; Mitchell et al. 2008). For both taxonomic and ecological reasons, it is important and challenging, to fully understand the range of variability within a given taxon because morphological or genetic variability is often related to environmental conditions (Bobrov et al. 1999; Heger et al. 2013; Wanner 1999). In fact, some morphologically similar taxa are genetically distinct, and therefore that there is evidence for both phenotypic plasticity and pseudo-cryptic species in testate amoebae (Heger et al. 2013; Medioli et al. 1987).

Difflugia tuberspinifera is an endemic species of East Asia and is common in freshwater lakes and reservoirs in China (Han et al. 2011; Liu et al. 2010; Yang et al. 2004). This species was first observed and described by Hu et al. (1997) based on a few empty shells from the Wujiang River, China. Subsequently, the living specimens were redescribed to modern standards and compared with six other similar species in relation to its morphology, biometry and ecology (Yang et al. 2004). The main characters which allow this species to be easily recognized from other species are the sub-spherical to spherical shell which has a mulberry-shaped appearance, tooth-like structures on the inside of the circular aperture, and conspicuous conical spines at the upper equatorial region of the shell. Recently, Liu et al. (2010) investigated the morphometric variability of six natural populations based on 374 individuals and showed this species to be size-monomorphic with normal distribution of shell height, shell diameter and aperture diameter. Although the shell height and shell diameter were relatively constant and had low variability, all shells had conical spines numbering between 3 and 8 in previous studies (Hu et al. 1997; Liu et al. 2010; Yang et al. 2004). During our investigations of the protist diversity in Xiamen reservoirs, we found abundant material of living specimens of *D. tuberspinifera* in 2010. More interestingly, a new morphotype without spine was first identified in four reservoirs of Xiamen. We performed detailed morphometric investigation and statistical analysis of the shell. The aim of the study is to test, by morphometric analyses, whether the spiny type and spineless type are of different species or different morphotypes of a single species.

Material and Methods

Study site and sample collection

The *Difflugia* samples were collected from four different reservoirs of Xiamen, Fujian Province, China in 2010, including Shidou Reservoir (118°00′ E, 24°42′ N), Bantou Reservoir (118°01′ E, 24°40′ N), Tingxi Reservoir (118°08′ E, 24°48′ N) and Hubian Reservoir (118°10′ E, 24°30′ N). The environmental characteristics and locations of these reservoirs have been described by Yang et al. (2012). The samples were taken from the surface water by horizontal hauls with a plankton net (64 μm mesh size). After observation of the living specimens, they were fixed with Bouin's fluids until further investigation.

Microscopy and measurement

Light microscopy (LM) and scanning electron microscopy (SEM) observations were conducted following the procedure of Yang et al. (2004). Hundreds of individuals, representing a wide range of different conical spine numbers, were randomly sampled for examination and measurement. Following Yang et al. (2004), six morphometric characters were measured in present study, namely shell height, shell diameter, aperture diameter, collar height, number of aperture tooth-like structures, and number of conical spines. In total 791 specimens (525 spineless and 266 spiny) were examined and measured morphometrically. All measurements were made at 400× magnification using an ocular micrometer.

Statistical analyses

The statistical analyses were performed on 1165 individuals including 374 previously studied specimens (Liu et al. 2010). In order to quantitatively assess the degree of separation between the two morphotypes, both linear discriminant analysis (LDA) and principal component analysis (PCA) of the morphological variables measured on all specimens (1165 individuals) were conducted. All measurements were log-transformed to improve normality and homoscedastcity prior to the PCA based on covariance matrix. All statistical analyses were performed using the software STATISTICA 6.0.

Results

Morphology

The shape of shell of *D. tuberspinifera* from four different reservoirs was similar except for the conical spines (Fig. 1). All specimens had a sub-spherical or spherical shell in outline with mulberry-shaped wall structure (or regular blunt protuberances). In lateral view, the aperture was terminal and surrounded by a distinct short collar, and the shell

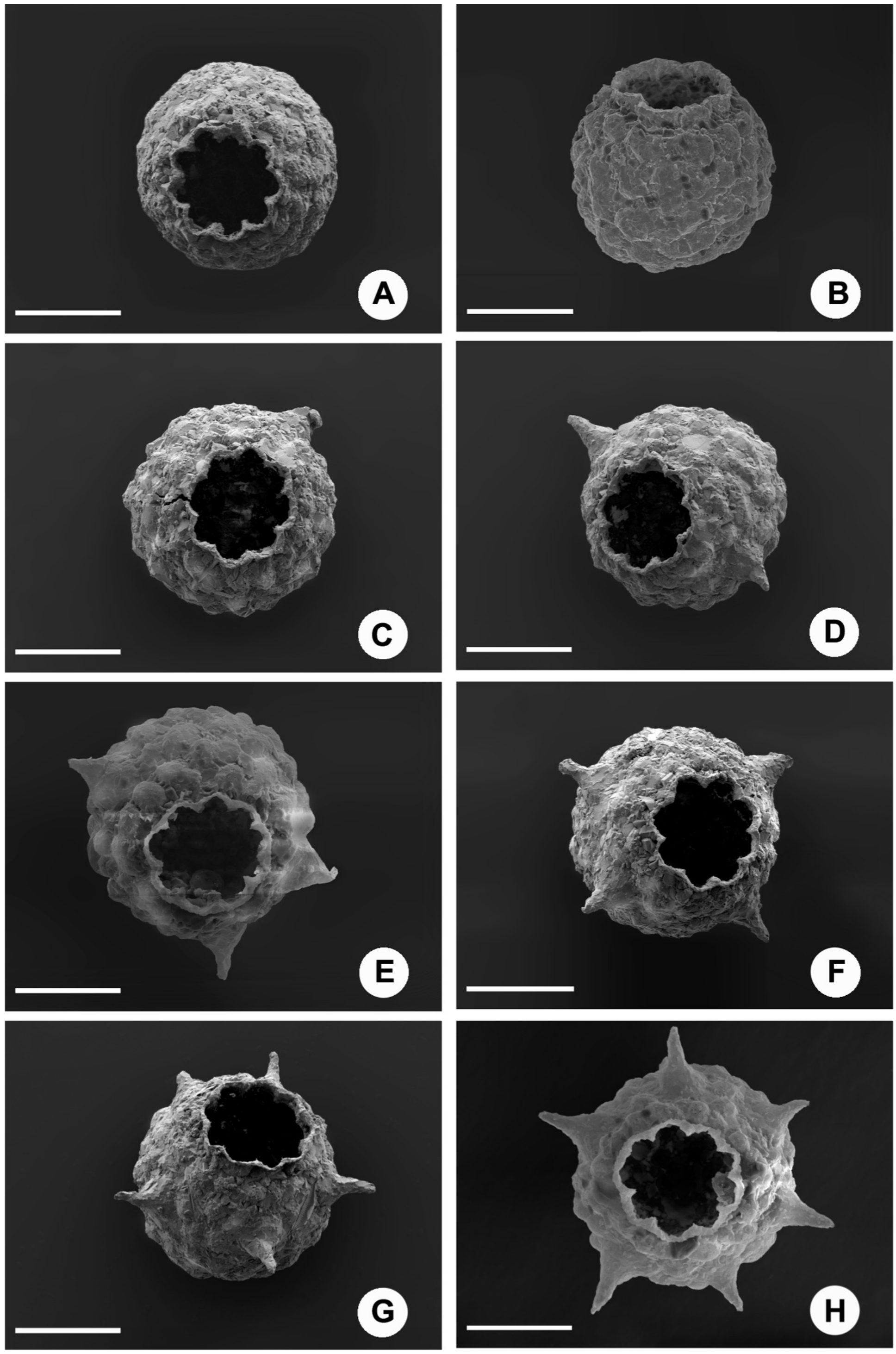

Fig. 1. SEM photographs of the different morphotypes of *Difflugia tuberspinifera*. (A) and (B) Spineless type; (C)–(H) spiny type showing the variation in number and length of the spines. Scale bars 50 μm.

was furnished with conical spines at the upper equatorial region for spiny type. In apertural view, the shell was circular and had a variable number (0–8) of conical spines. The aperture was circular and bordered by an organic collar, the inner margin of which was denticulated, forming tooth-like structures. The shell was brown and composed of fine sand granules, flattish pieces of quartz and muddy particles (Fig. 1). Figure 2 shows the relative frequencies of *D. tuberspinifera* with different numbers of conical spines based on 791 individuals from four Xiamen reservoirs and 374 individuals from the Yangtze River and Pearl River valleys. About 45.1% (525) of the *D. tuberspinifera* individuals examined in this study had no spines. Individuals with 4, 5 and 6 spines accounted for 10.3%, 17.3% and 11.3% of the total number

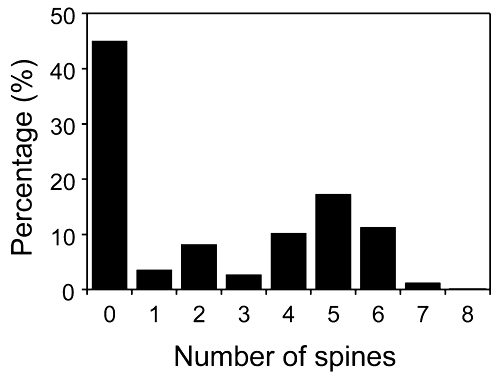

Fig. 2. Relative frequencies of *Difflugia tuberspinifera* with different numbers of spines from China ($n = 1165$).

of *D. tuberspinifera*, respectively (Fig. 2). While these estimates are not directly comparable in absolute values, they indicate the existence of two distinct morphotypes, namely spiny and spineless types, within *D. tuberspinifera*.

Biometry

Table 1 shows the detailed morphometric characteristic of two morphotypes of *D. tuberspinifera* from four reservoirs of Xiamen. The values of shell height, shell diameter and aperture diameter were similar for the two morphotypes. Both linear discriminant analysis (LDA) and principal component analysis (PCA) yielded similar results, because both analyses based on six metric variables clearly divided the 1165 specimens into two distinct groups, spineless and spiny morphotypes (Fig. 3). More importantly, the first two axes of PCA explained 96.9% of the total variability and effectively captured the main patterns of variation in the original variables. However, the PCA on all measured variables but excluding spine number did not allow differentiation of spineless type from spiny type, indicating that other morphometric characters were undistinguishable between the two morphotypes. Further, shell height, shell diameter and aperture diameter had highly positive load leveling. Different populations had their own main size range. The size of shell height, shell diameter and aperture diameter in *D. tuberspinifera* (spiny type) from Yangtze River and Pearl River valleys was somewhat larger than that of Xiamen populations (Fig. 4). Teeth number in the spineless type was slighter lower than in the spiny type. However, both spiny and spineless specimens from Xiamen reservoirs had similar range in shell height, shell diameter and aperture diameter (Fig. 4).

Discussion

This study provides the basis for further investigation into ecomorphological variation in the Asian endemic *D. tuberspinifera*. As most early taxonomic studies of *Difflugia* lack type material and involve small sample sizes, the identities of most species is not clear (Lahr and Lopes 2006). The morphology of *Difflugia* displays high diversity and great variation, including the presence or absence of spines, the shape and composition of tests and morphological features associated with the aperture, such as collars, lobes, and teeth (Meisterfeld 2002; Todorov and Golemansky 2007). Species within the group are normally distinguished by their size, morphology and the composition (and ultrastructure) of the shell. *D. tuberspinifera* is a size-monomorphic species with a small size range (Liu et al. 2010). A very important characteristic distinguishing *D. tuberspinifera* from all other species of mulberry-shaped *Difflugia* is the size of the shell (Yang et al. 2004). Shell height, shell diameter, aperture diameter and collar height are important parameters of shell size in the classification of *Difflugia*. In this study, the shell dimensions of all morphotypes were relatively constant and the shell architecture in all these specimens ($n = 791$) was nearly identical (Table 1). The character of variability in amplitude and correlativity differs not only among different species, but also among populations of the same species (Bobrov and Mazei 2004). Our morphometric results indicated that on all metric variables but excluding spine number did not allow differentiation of spineless type from spiny type. In the populations of Yangtze River valley, Yang et al. (2004) reported that *D. tuberspinifera* had a number of spines varying from 4 to 8 at the upper equatorial region. Our current study found a wider range of variation (from 0 to 6). It appears that the only character distinguishing the two morphotypes of *D. tuberspinifera* is the number of spines. These two morphotypes of *D. tuberspinifera* either could be considered as two different morphotypes of the same species or two closely related species. Morphological data on clonal variations and molecular data would be useful to test these two hypotheses. In support of the distinct species hypothesis, numerous examples now exist of cryptic species or pseudo-cryptic species among various taxonomic groups of protists including testate amoebae (Heger et al. 2013). In this study, the distribution of spine numbers showed two major modes: one with zero spine (about 45.1% of the recorded individuals) and a second, relatively large one (38.9%) with 4–6 spines, and a third, smaller one (about 8.2%) with two spines. This distribution pattern suggests the existence of two distinct taxa and a small proportion of intermediate forms, possibly hybrids between the two closely-related taxa. At the beginning of the twentieth century, Penard (1902) observed a similar pattern of morphological variation in *Difflugia varians* in number of spines (zero, two, three or four) and their position on the shell. Moreover, high morphological variability of a very widespread species (*Difflugia urceolata*) was also reported by Todorov and Golemansky (2007). *D. corona tuberculata* is perhaps the most similar organism to *D. tuberspinifera*, because its spines also have variable number (Lahr and Lopes 2006; Yang et al. 2004). However, the spines in *D. corona tuberculata* are located in the aboral region and they are short in length, while in *D. tuberspinifera* the spines are conical and located at the upper equatorial region (Yang et al. 2004).

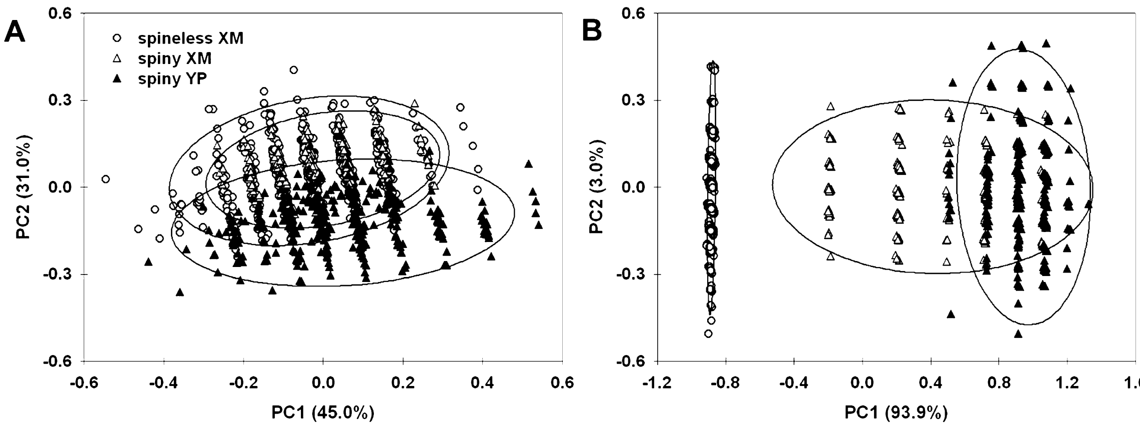

Fig. 3. Plots of principal component 1 (PC1) versus principal component 2 (PC2) for *Difflugia tuberspinifera* with 95% confidence ellipses. (A) Principal component analysis (PCA) based on five morphological traits (shell height, shell diameter, aperture diameter, collar height, and number of aperture tooth-like structures) of three *Difflugia tuberspinifera* populations, showing no significant separation between different types. (B) Principal component analysis (PCA) based on six morphological traits (shell height, shell diameter, aperture diameter collar height, number of aperture tooth-like structures and number of conical spines), showing a sharp distinction between spineless and spiny types. Spineless XM, *D. tuberspinifera* without spine from Xiamen reservoirs; spiny XM, *D. tuberspinifera* with spines from Xiamen reservoirs; spiny YP, *D. tuberspinifera* with spines from Yangtze River and Pearl River valleys.

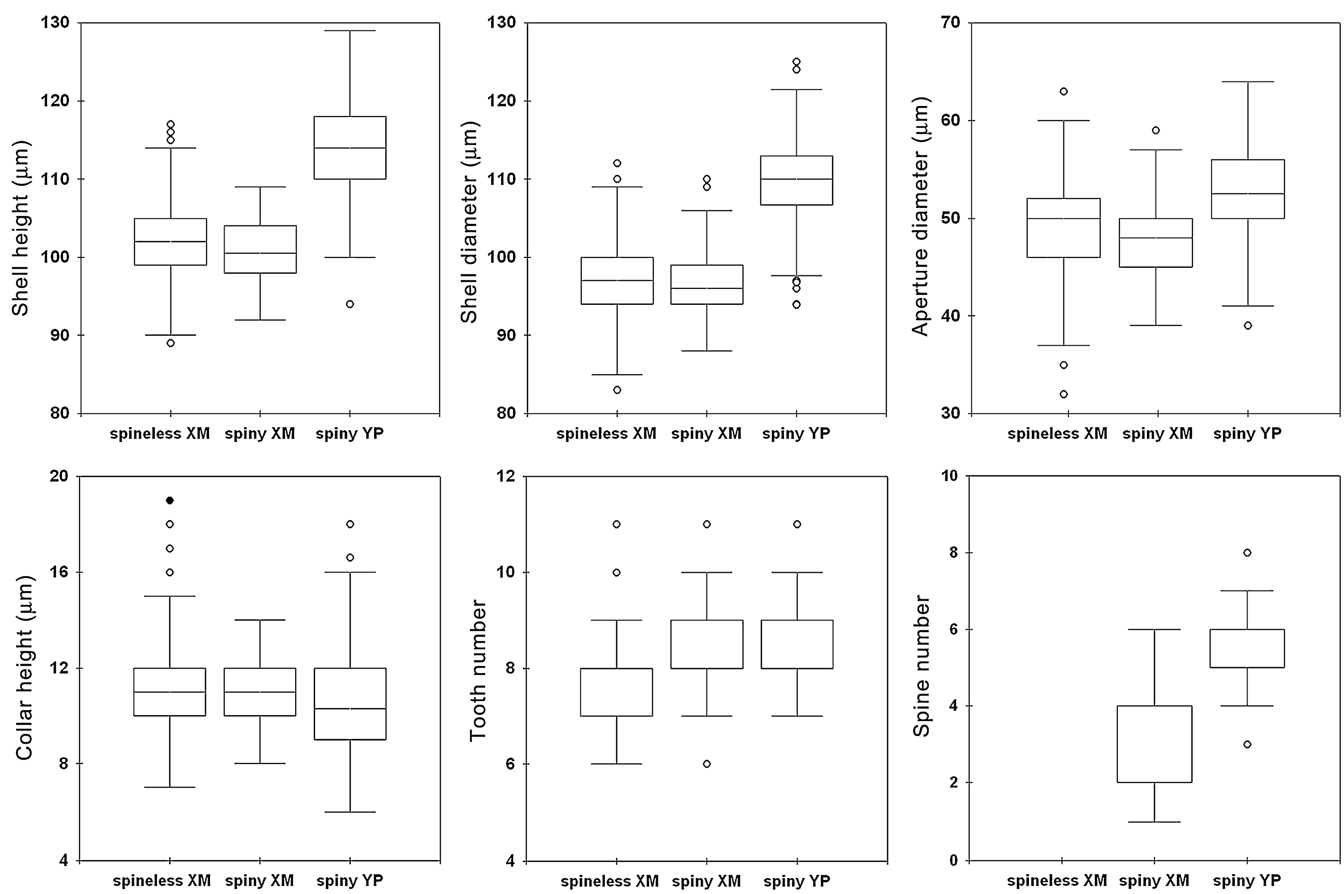

Fig. 4. Box plots for all measured characters in two morphotypes of *Difflugia tuberspinifera*. (Spineless XM, *D. tuberspinifera* without spine from Xiamen reservoirs; spiny XM, *D. tuberspinifera* with spines from Xiamen reservoirs; spiny YP, *D. tuberspinifera* with spines from Yangtze River and Pearl River valleys). Boxes contain the center 50% of values, box edges are at the first and third quartiles. Whiskers contain values within 1.5 midranges. Empty circles represent outside values and full circles represent extreme outliers.

Table 1. Morphometric characteristics of two morphotypes of *Difflugia tuberspinifera* from Xiamen reservoirs, China.

Characters	*X*	*M*	SD	SE	CV	Min	Max	*n*
All populations								
Shell height	101.8	102.0	4.55	0.16	20.66	89.0	117.0	791
Shell diameter	96.7	97.0	4.63	0.16	21.45	79.0	112.0	791
Aperture diameter	48.8	49.0	4.30	0.15	18.52	32.0	63.0	791
Collar height	11.1	11.0	1.69	0.06	2.86	7.0	19.0	791
Tooth number	7.9	8.0	0.92	0.03	0.85	6.0	11.0	791
Spine number	1.0	0.0	1.63	0.06	2.65	0.0	6.0	791
Spineless type								
Shell height	102.4	102.0	4.78	0.21	22.83	89.0	117.0	525
Shell diameter	96.9	97.0	5.00	0.22	24.97	79.0	112.0	525
Aperture diameter	49.4	50.0	4.63	0.20	21.43	32.0	63.0	525
Collar height	11.2	11.0	1.80	0.08	3.25	7.0	19.0	525
Tooth number	7.8	8.0	0.93	0.04	0.87	6.0	11.0	525
Spine number	0.0	0.0	0.00	0.00	0.00	0.0	0.0	525
Spiny type								
Shell height	100.7	100.5	3.83	0.23	14.64	92.0	109.0	266
Shell diameter	96.4	96.0	3.79	0.23	14.38	88.0	110.0	266
Aperture diameter	47.8	48.0	3.36	0.21	11.28	39.0	59.0	266
Collar height	10.8	11.0	1.41	0.09	1.99	8.0	14.0	266
Tooth number	8.2	8.0	0.84	0.05	0.71	6.0	11.0	266
Spine number	2.9	2.0	1.47	0.09	2.16	1.0	6.0	266

Measurements in μm. *X*, arithmetic mean; *M*, median; SD, standard deviation; SE, standard error of the mean; CV, coefficient of variation in %; Min, minimum; Max, maximum; *n*, number of investigated specimens.

The function of spines is unclear in the planktonic *D. tuberspinifera*. Their length and number increases the surface area and may potentially increase buoyancy of planktonic species. Moreover, spines can provide protection from predation (Lampert and Sommer 1997). The morphology of testate amoebae often shows changes in response to variation in environmental conditions (Bobrov and Mazei 2004). Bobrov et al. (1999) pointed out that spiny forms within the genera *Euglypha* and *Placocista* consistently occurred in wetter habitats whereas spineless forms, or those with short spines, were more prevalent in drier habitats. Interestingly, the four reservoirs were located in one city in this study, but the spiny forms of *D. tuberspinifera* were only found in Hubian Reservoir where NH_4-N concentrations were highest during the sampling period (0.975 ± 0.535 mg/L vs. 0.123 ± 0.077 mg/L in the other three reservoirs). This pattern is intriguing enough to deserve further study. Although individual species have characteristic size ranges, there are often considerable variations within species, which may be attributed to genetic or environmental influences (Medioli and Scott 1988). Several traits of testate amoebae, such as small size, high sensitivity to environmental conditions, make them increasingly used as biomarkers for environmental biomonitoring (Booth and Meyers 2010; Nguyen et al. 2004). Therefore, if we can understand the reasons for the presence or absence of spines in *D. tuberspinifera*, and their number, this may have useful ecological and evolutionary significance.

Both geographic factors and local environmental variables play important role in shaping population structure and in determining population distribution of testate amoebae (Lahr and Lopes 2006; Yang et al. 2006). Globally, many testate amoebae species have a cosmopolitan distribution at the morphological species level (Yang et al. 2010), but *D. tuberspinifera* has a restricted distribution in Eastern Asia. At a national scale, the normal spiny type of *D. tuberspinifera* was the dominant taxon and its density was usually higher than the other testate amoebae especially in the Yangtze River and Pearl River valleys (Liu et al. 2010; Yang et al. 2004). At a regional scale, however, the spineless type of *D. tuberspinifera* was first reported from Xiamen reservoirs and the transitions between two typical morphotypes were found in Hubian Reservoir. Further research for these coexisting forms is required for details on the biogeographic patterns and processes based on their genetic sequences and ecological data.

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