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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