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Title:

Phylogenetic divergence within the Arcellinida (Amoebozoa) is congruent with test size and metabolism type

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Abstract

Arcellinida (lobose testate amoebae) are abundant and diverse in many ecosystems, especially in moist to aquatic environments. Molecular phylogeny has shown that overall test morphology (e.g., spherical or elongate) is generally conserved in Arcellinida lineages, but the taxonomic value of other traits (e.g., size, ornamentation, mixotrophy/heterotrophy metabolism type) has not been systematically evaluated. Morphological and physiological traits that correspond to genetic differences likely represent adaptive traits of ecological significance. We combined high-resolution phylogenetics (NAD9-NAD7 genes) and advanced morphometrics to assess the phylogenetic signal of morphological traits of a group of elongate *Diffflugia* species (Arcellinida). The phylogenetic analyses revealed two clades which could be reliably separated by test size and the presence/absence of mixotrophy. Differences in test size may reflect trophic level, with smaller organisms occupying lower trophic levels. In addition to having larger tests, elongate mixotrophic *Diffflugia* are characterised by wide, flat bases and an inflation of the lower two thirds of their test. These morphological traits may provide additional volume for endosymbionts and/or increased surface area to aid light transmission. Our results showcase greater diversity within the elongate *Diffflugia* and highlight morphological traits of ecological and evolutionary significance.

Keywords

Diffflugia; Centroid size; Geometric morphometrics; Molecular barcoding; Morphometry; NADH gene complex

Introduction

Arcellinida Kent, 1880 are an order of testate (shelled) Amoebozoa, characterized by broad and thick (lobose) pseudopods used for feeding and locomotion. Like other testate amoebae they enclose their cell body within a test, the morphology and composition of which can be used to identify species (Meisterfeld, 2002). Several features make Arcellinida valuable ecological indicators: (1) they occur in great abundances in moist habitats, from forest soils to peatlands to lakes and even brackish waters (Mitchell et al., 2008; Ogden and Hedley, 1980); (2) a relatively fast reproduction rate and a large scope of morphological plasticity within species could result in relatively rapid evolutionary change (Charman, 2001); (3) the tolerance of some taxa to harsh environmental conditions (Pérez-Juárez et al., 2017); (4) the presence of a simple test facilitates identification and has resulted in several key taxonomic publications over two centuries of study (Leclerc, 1815; Mitchell et al., 2008); and (5) they occupy several trophic levels according to their feeding behaviour (i.e., bacterivory, detritivory, predatory and mixotrophy; Jassey et al. 2013). In addition, the excellent preservation potential of their test (Lahr et al., 2019; Mitchell et al., 2008; Swindles and Roe, 2007), especially in Holocene and acidic sediments, means they are an excellent proxy for paleoenvironmental reconstructions.

Poor taxonomy remains a major obstacle for the systematic use of Arcellinida as bioindicators for present and past environmental conditions (Kosakyan et al., 2016). Uncertainty as to the range of phenotypic plasticity – i.e., the natural variation of test morphologies not mirrored by variation in the genomes (Mulot et al., 2017) – lowers taxonomic precision in the Arcellinida. Mulot et al. (2017) demonstrated that test shape and pore numbers varied with environmental conditions in wild and cultured populations of *Hyalosphenia papilio* Leidy, 1874. Jennings (1916), in an early study of a clonal culture of *Diffugia corona* (now *Netzelia corona* (Wallich, 1864) Gomaa et al., 2017), observed a variation in both number and placement of spines that was independent of environmental conditions. Porfírio-Sousa et al. (2017) observed very little variability in the test or aperture diameter in clonal cultures of *Arcella intermedia*, but found that wild populations occupied a distinct morphometric space compared to clonal populations, corroborating previous studies (Wanner, 1999). Tremendous phenotypic plasticity present in wild Arcellinida populations was illustrated by Bobrov and Mazei (2004). For each species, results of test length and breadth measurements formed diffuse clouds of transitional morphotypes plotting around weak central peaks rather than dense distributions. Thus, differences in test Arcellinida morphology may not be the result of genomic differences, but merely reflect a species' range of phenotypic variability. Notwithstanding this, Jennings (1916) showed that specific traits (e.g., number of spines and aperture crenulations) could be artificially selected across successive generations, possibly highlighting a pathway for speciation (West-Eberhard, 1989).

Speciation events could result from specific morphotypes that have greater success at exploiting a novel niche (parapatric speciation) (Weisse, 2008). One such pathway could be the development of mixotrophy, whereby testate amoeba host symbiotic green algae (Treboxyiophyceae) in their cytoplasmic vacuoles (Gomaa et al., 2014; Lara and Gomaa, 2017). Mixotrophic taxa represent valuable ecological indicators as they signal nutrient poor conditions, especially in peatlands (Jassey et al., 2013). Jassey et al. (2013) demonstrated that species with large body sizes occupied higher trophic levels and consumed many food sources as compared to smaller species that shared low trophic

levels with more specific food strategies. Thus, morphological changes that correspond to genetic differences could be of ecological relevance. Greater taxonomic precision would lead to more robust ecological inferences.

Over a decade of molecular research (Nikolaev et al., 2005) has demonstrated several morphological features to be reliable predictors of genetic relatedness among the Arcellinida: (1) aperture outline (e.g., circular vs. slit); (2) presence of a neck; (3) shell outline (in both apertural and lateral view); and (4) shell composition (Gomaa et al., 2012; Kosakyan et al., 2016; Lahr et al., 2019). Lahr et al. (2019) presented a phylogenomic reconstruction based on 250 genes, obtained using single-cell transcriptomic techniques, which divided Arcellinida into five infraorders: (1) Sphaerothecina Kosakyan et al., 2016; Longithecina Lahr et al., 2019; Excentrostoma Lahr et al., 2019; Hyalospheniformes Lahr et al., 2019; and Vonustoma Lahr et al., 2019. For the Hyalospheniformes, ongoing molecular research has demonstrated that other test morphological features (e.g., compression, size, girth) can be used to reliably distinguish taxa at the genus and species level and how these features might be related to an organism's autecology. This remains to be done for the other infraorders of Arcellinida, such as Longithecina, containing the family Diffugiidae Wallich, 1864, one of the most diverse and abundant in wild assemblages.

To better define the boundary between baseline phenotypic plasticity and evolutionarily significant morphological differences, we require faster evolving genetic markers that can distinguish at the genus or species level and advanced morphometrics to characterise all features of the Arcellinida test. Blandenier et al. (2017) developed the application of the mitochondrial nicotinamide adenine dinucleotide dehydrogenase gene marker (NADH) to construct Arcellinida molecular phylogenies that could both characterise deep phylogenetic relationships as well as finer (i.e., genus and species level) relationships. They were able to replicate the topology of the small subunit ribosomal gene (18S rRNA) trees but could also discriminate between two isolates of a single species (i.e., *Diffugia nodosa* (Leidy, 1879) and *Diffugia pyriformis* Perty, 1849). Previous morphometric analyses used the average measurements ($n = 20-30$) of several Arcellinida test features (e.g., length, breadth, width) to construct "ideal-individuals" (e.g., Wanner, 1999). This approach does not separate size variation from shape variation and these measurements are often correlated since they overlap in what they are measuring (Zelditch et al., 2012). Geometric morphometric analysis provides a detailed summary of morphological variability across the entire specimen with the added benefit of separating size as an independent variable (Adams and Otárola-Castillo, 2013). Shapes are defined by the geometric configuration of landmarks (fixed anatomically definable locations found on all specimens) and by boundary curves drawn between the landmarks. Geometric morphometrics has been applied to paleontological studies (Adams and Otárola-Castillo, 2013), including microfossils (e.g. diatoms; Beszteri et al. 2005) but has yet to be applied to Arcellinida.

By combining high-resolution molecular markers with advanced morphometrics analysis and applying them to Arcellinida with similar test morphologies we aim to further explore the boundary between phenotypic variability and evolutionarily significant morphological differences. We focus on the elongate type diffugiids as they are abundant in lacustrine habitats, and, like most diffugiids, are poorly characterised by current phylogenetic reconstructions. The results will improve ecological inferences based on morphotype assemblages, as a clearer distinction can be made on which morphological features are

evolutionarily significant and likely represent an important ecological function. This can further be used to better understand the functional ecological role of Arcellinida and give a better estimate of the true diversity of this group.

Material and Methods

Sample collection and documentation

Diffflugia specimens were isolated from *Sphagnum* mosses on Pirin Mountain (*Diffflugia acuminata* Ehrenberg, 1830) and lacustrine submerged vegetation from Sofia Southern Park Artificial Lake (*Diffflugia lanceolata* Penard, 1890 and *Diffflugia oblonga* Ehrenberg, 1838), both in Bulgaria (Table 1). Cells were identified based on several reference articles (e.g., Chardez 1967; Ogden 1979, 1980, 1983; see Appendix A Table A.1) with an Axio Imager M2-Carl Zeiss compound microscope with a digital camera (ProgRes C7) and specialised software (CapturePro Software 2.8). Tests were documented using scanning electron microscopy as described previously (Todorov et al., 2010). We analyzed a single cell extraction for both *D. lanceolata* and *D. oblonga*, and a sample consisting of five morphologically identical cells from the same site for *D. acuminata* (Table 1).

DNA extraction, PCR amplification and sequencing

DNA extraction was performed with a thiocyanate guanidinium solution following the protocol of Duckert et al. (2018) adapted from Chomczynski and Sacchi (1987). New sequences of NADH dehydrogenases were obtained with a novel nested PCR protocol using forward and reverse primers developed by Blandenier et al. (2017) (Table 2). The *D. acuminata* sequence was obtained using the nested PCR product, whereas we cloned the nested PCR product of *D. lanceolata* and *D. oblonga* using a PCR2.1 Topo TA cloning vector and transformed into *Escherichia coli* TOP10' One Shots cells (Invitrogen kit) following the manufacturer's instructions. For all three taxa, the final PCR product was purified using Millipore kit and sent for sequencing with an ABI3730XL DNA sequencer (Applied Biosystems) at Macrogen, Amsterdam, The Netherlands. Sequences were deposited in GenBank with the following accession numbers: MN524965-MN524967.

Alignment and phylogenetic analyses

Using BioEdit (Hall, 1999), we aligned the three obtained sequences with NAD sequences of Blandenier et al. (2017) and sequences from other Amoebozoan mitochondrial genomes retrieved on GenBank. In order to obtain a better resolution, the nucleotide sequences were converted into amino acid sequences and the intergenic region was removed (see Appendix B). The root was placed on the Discosea based on the phylogenetic reconstruction of Kang et al. (2017). Phylogenetic reconstructions were conducted using the CIPRES Portal (Miller et al., 2010). We used three distinct phylogenetic reconstruction methods: (1) we built a Maximum Likelihood phylogenetic tree using the RaxML v.8.2.10 algorithm (Stamatakis, 2014) with the JTT + Γ model.

206 Bootstrapping was stopped after 150 replicates based on MRE bootstrapping criterion; (2)
207 we built a Bayesian phylogenetic tree using MrBayes (Ronquist and Huelsenbeck, 2003)
208 with a GTR+GAMMA+INVARIANT model ran on two independent chains for 200,000
209 generations sampled every 100 generations resulting in 4,000 trees, of which 25% were
210 discarded as the burn-in; and (3) parsimony analysis was performed using TNT version
211 1.1. (Goloboff et al., 2008), running 1,000 bootstrap replicates using the PAM 250 step
212 matrix in a New Technology search allowing sectorial search, ratchet, drift and tree fusing
213 parameters.

214 215 216 Geometric morphometric analyses

217
218 To quantify test size and shape variation we followed a modified geometric morphometric
219 protocol based on MacLeod (2008) and Zelditch et al. (2012). Appendix C Table C.1
220 provides detailed information on the source of the images used for geometric
221 morphometric analysis. Images were digitised using the “tps” series of software (Rohlf,
222 2015). To digitise an image, a landmark was placed on the left and right edge of the test
223 aperture and a set of 28 equally spaced semilandmarks were used to outline the tests
224 between landmarks 1 and 2 (Fig. 1). Landmark and semilandmark coordinates underwent
225 a Procrustes superimposition using sliders to align all specimens and isolate size variation
226 as an independent variable (Rohlf, 2015; Zelditch et al., 2012). Size variation is quantified
227 by centroid size, defined as the square root of the sum of squared distances of all the
228 landmarks of an object to their centroid (Fig. 1; MacLeod 2008). The centroid is the
229 center of gravity of an object obtained by averaging the x and y coordinates of all
230 landmarks (Fig. 1). We modeled the Arcellinida test shape space using a principal
231 components analysis (PCA) of the Procrustes superimposed landmarks using the
232 “geomorph” R-package (Adams and Otárola-Castillo, 2013). This package was also used
233 to create five shape models along the first principal component (PC1). We used the FIJI
234 distribution of “ImageJ” with the “ObjectJ” plugin to measure test length (mm), width
235 (mm) and aperture width (mm) from scaled Arcellinida test images. Surface area was
236 estimated using the equation for the surface area of an ellipse.

237 238 239 Results

240 241 NADH phylogenetic tree

242
243 We obtained sequences (254-257 bp) of the NAD9-NAD7 genes of *D. oblonga*, *D.*
244 *acuminata* and *D. lanceolata* (Fig. 1 and Table 1). The sequence of *D. oblonga* possesses
245 a short intergenic region (indel of 6 bp), whereas the sequences of *D. acuminata* and *D.*
246 *lanceolata* possess an overlap of 1 bp as described for *Arcella* sequences in Blandenier et
247 al. (2017). The phylogenetic reconstruction (Fig. 2) places the Arcellinida in a single
248 clade (98 bootstrap support (bs)/1.00 posterior probability (pp)). Within Arcellinida,
249 infraorder Sphaerothecina represents a robust clade (93 bs/0.99 pp) and our new
250 sequences plot on two distinct clades. *Diffflugia lanceolata* and *D. acuminata* form the
251 “Lanceolate” clade (95 bs/0.99 pp) and *D. oblonga* along with *D. nodosa* and *D.*
252 *pyriformis* comprise the “Pyriform” clade (95 bs/0.99 pp). The node supports for these

three clades within Arcellinida are too low to make any inferences on their placement and relationship to one another (Fig. 2).

Geometric morphometric analysis

The first two Procrustes PCA components explained 82.9% of test morphological variance (Table 3), with the first principal component (PC1) explaining 72.5%. We display geometric morphometric analysis results as a biplot of PC1 scores and centroid size (Fig. 3; see Appendix C Table C.1 for all results). We grouped data points by phylogenetic clade (symbol colour) and by taxa (symbol shape; Fig. 3). Specimens that have been sequenced are represented by larger symbols.

Centroid size has a positive linear to superlinear relationship with test length (mm), test width (mm) and test surface area (mm²) (Appendix D Fig. D.1). Thus, centroid size serves as a proxy for test surface area and test volume for radially symmetrical tests such as *D. lanceolata*, *D. acuminata*, *D. pyriformis* and *D. oblonga*. Centroid size can be used to distinguish the “Pyriform” clade (centroid size > 486.5; Appendix C Table C.1) from the “Lanceolate” clade with minimal (n = 1) overlap (Fig. 3). Centroid size can also be used to characterise members of the “Lanceolate” clade as *D. lanceolata* has a smaller average centroid size (centroid size < 300; Appendix C Table C.1; Fig. 3). We note that the sequenced specimen of *D. oblonga* (small morphotype) from Gomaa et al. (2017) and Lahr et al. (2019) falls within the centroid size and PC1 score ranges of the “Lanceolate” clade rather than the “Pyriform” clade (Fig. 3).

In Fig. 3, we show PC1 shape models ranging from negative to positive PC1 scores. Negative PC1 scores reflect columnar morphologies and acuminate bases, while positive PC1 scores reflect pyriform morphologies and wide flat bases. *Diffflugia oblonga* has similar PC1 scores as members of the “Lanceolate” clade, making PC1 scores an unreliable metric to distinguish between the “Pyriform” and “Lanceolate” clades. However, test shape can be used, with minimal overlap, to distinguish between members of the “Pyriform” clade. Taxa known to house endosymbiotic algae (i.e., mixotrophic taxa) have positive PC1 scores, reflective of wide flat bases and inflation of the lower two thirds of the test.

Discussion

Previous phylogenetic reconstructions of the Arcellinida have either lacked “Pyriform” taxa (Gomaa et al., 2017; Lahr et al., 2019) or “Lanceolate” taxa (Blandenier et al., 2017). The addition of two “Lanceolate” taxa and a third “Pyriform” taxon to the NADH phylogenetic tree allows the molecular comparison of these two morphotypes and reveals significant diverging between them (Fig. 2). Importantly, the test morphologies of both the “Lanceolate” and “Pyriform” taxa are relatively similar, having elongate tests with terminal apertures (Fig. 1). It is thus appropriate to investigate the evolutionary significance of morphological traits other than an elongate test with a terminal rounded aperture.

NADH phylogenetic tree

Our NADH phylogenetic tree replicates the global topology of the 18S rRNA phylogenetic reconstruction of Gomaa et al. (2017) and Lahr et al. (2019) and the NADH phylogenetic reconstruction of Blandenier et al. (2017). They all show Arcellinida as monophyletic containing three well resolved infraorders: Sphaerothecina, Hyalospheniiformes and Longithecina. Our tree suggests distinct diverging within the Longithecina between a “Lanceolate” clade consisting of *D. lanceolata* and *D. acuminata*, and a “Pyriform” clade that includes *D. oblonga*, *D. nodosa*, and *D. pyriformis*.

Our phylogenetic placement of *D. oblonga* contradicts that of the 18S rRNA reconstructions of Gomaa et al. (2017) and Lahr et al. (2019) that placed it amongst the “Lanceolate” group. It should be noted that the same sequence for *D. oblonga* was used by both Gomaa et al. (2017) and Lahr et al. (2019). Fig. 3 demonstrates that their specimen of *D. oblonga* is morphologically similar, in both shape and centroid size, to other members of the “Lanceolate” group. Gomaa et al. (2017) first described this specimen as *Diffflugia parva* (Thomas, 1954) Ogden, 1983 but reclassified it as *D. oblonga* (small morphotype) following the recommendations of Mazei and Warren (2014), who indicated *D. parva* as a junior synonym of *D. oblonga*. The similarity in shape and centroid size of *D. lanceolata*, *D. acuminata* and *D. parva* could explain why they group together in the 18s rRNA tree of Gomaa et al. (2017). Thus, we recommend that because of its distinct size, *D. parva* should not be synonymized with *D. oblonga* sensu Mazei and Warren (2014).

If the *D. oblonga* (small morphotype) sequenced in the 18S rRNA phylogenetic reconstructions is in fact *D. parva*, a member of the “Lanceolate” group and a distinct species from *D. oblonga*, this leaves no barcoded taxa from the “Pyriform” clade with the 18S rRNA marker. Mazei and Warren (2014) define *D. oblonga* as being of similar shape to *D. pyriformis* but being relatively narrower in breadth. Our NADH phylogenetic reconstruction further demonstrates that within the lanceolate to oblong shape range, centroid size may be a more reliable morphological trait than PC1 scores (i.e., shape) in predicting genetic relatedness. Our specimens of *D. oblonga* are distinct in centroid size from members of the “Lanceolate” group and represent an intermediate shape between the “Lanceolate” group and *D. pyriformis* (Fig. 3). Centroid size is also an important characteristic in distinguishing other members of the “Pyriform” clade from the “Lanceolate” clade (Fig. 3). We propose that centroid size is a defining feature in the classification of *D. oblonga* sensu Mazei and Warren (2014) and differentiating it from other members of the “Lanceolate” group.

There are four remaining difflogiids with 18S rRNA sequences, listed in decreasing PC1 score order (Fig. 3): *Diffflugia compressa* Carter, 1864, *Diffflugia hiraethogii* Ogden, 1983, *Diffflugia bryophila* (Penard, 1902) Jung, 1942 and *Diffflugia bacilliarum* Perty, 1849. *Diffflugia bacilliarum* plots within the “Lanceolate” group on Fig. 3 and with the “Lanceolate” group in the 18S rRNA reconstructions (Gomaa et al., 2017; Lahr et al., 2019). *Diffflugia bryophila* has the smallest centroid size and PC1 scores that fall within the oblong shape range, but it is not found within the “Lanceolate” group in the 18S rRNA reconstruction (Lahr et al., 2019). *Diffflugia hiraethogii* has a compressed and pyriform shape but a centroid size that falls within “Lanceolate” range and groups along

with *D. bacillariarum* with the “Lanceolate” group in the 18S rRNA reconstructions (Gomaa et al., 2017; Lahr et al., 2019). *Diffflugia compressa* has a centroid size at the lower end of the “Pyriform” range and a shape like that of *D. nodosa*. *Diffflugia compressa* groups distinctly, along with *D. bryophila*, from the “Lanceolate” group in the 18S rRNA reconstruction (Lahr et al., 2019). Although *D. compressa* seems like a candidate for the “Pyriform” clade and thus could place this clade within the 18S rRNA reconstructions; grouping of *D. compressa* with *D. bryophila* in the 18S rRNA reconstructions, who do not share similar morphological characteristics, complicates the situation. It might be that other features unite these two taxa. The position of the “Pyriform” clade on 18S rRNA phylogenetic reconstructions remains uncertain and thus members of this clade should be the target of future molecular phylogenies.

Test centroid size and its ecological significance

Based on the data presented, test centroid size is a more reliable metric in differentiating between the “Lanceolate” and “Pyriform” clades than test shape (Fig. 3). Unlike estimates of biovolume that reduce test shape to an idealized shape (i.e., ellipse; Fournier et al., 2012), centroid size is based on the actual dimensions of the test (i.e., distance of each (semi)landmark from the centroid). Thus, centroid size is likely to be the most precise and accurate method for estimating test surface area and estimating test volume for radially symmetrical tests (i.e., *D. lanceolata*, *D. acuminata*, *D. pyriformis* and *D. oblonga*; see Appendix D Fig. D.1).

The different test centroid size ranges of the “Lanceolate” and “Pyriform” clades might be related to ecological differences between these clades. Jassey et al. (2013) in their study of the microbial food web from a *Sphagnum*-peatland demonstrated that taxa with large body sizes, as estimated by test length and biovolume, occupy higher trophic levels with more varied food sources as compared to smaller taxa that occupy lower trophic levels, and have more specific feeding strategies. Coeval changes in test size of multiple taxa have been shown to occur in response to environmental stresses: (1) smaller biovolumes associated with higher temperatures could reflect cellular respiration increasing at a greater rate than oxygen diffusion (Wanner, 1999); (2) reduction in test size in response to warmer climates could be indirectly beneficial, by increasing CO₂ access for the *Chlorella* symbionts in mixotrophic taxa, whose metabolism increases in warmer temperatures (Mulot et al., 2017); (3) Marcisz et al. (2016) observed a decrease in average Arcellinida test size in response to multiple disturbances (e.g., fire and peat farming) in their study of *Sphagnum* peatlands in Poland. Arcellinida test size is thus a trait of major ecological importance and it is imperative to consider it in future studies of Arcellinida communities. We propose that test centroid size to be the most precise and accurate method (to date) of estimating test size and recommend its utilization.

Mixotrophy

Mixotrophic taxa represent valuable ecological indicators as they signal nutrient poor conditions in peatlands (Jassey et al., 2013) and perhaps in other environments such as lakes but this has yet to be tested. Penard (1902) observed that mixotrophic specimens could survive dysoxic conditions and lack of nutrition longer than non-mixotrophic specimens and hypothesized that it was due to the inclusions of zoochlorellae. Leidy

(1879) characterised *D. pyriformis* and *D. nodosa* as being bright green and full of “chlorophyll grains” and stated that the cells of *D. acuminata* and *D. lanceolata* are colourless. The absence of mixotrophy in the “Lanceolate” clade might partly explain the genetic divergence from the “Pyriform” clade.

All members of the “Pyriform” clade share a similar centroid size range (Fig. 3) but, unlike the other members, *D. oblonga* does not house endosymbionts (unpublished observation) making centroid size alone an unreliable metric to define mixotrophic taxa. Within the “Pyriform” clade, the combination of centroid size and test shape (i.e., PC1 scores) can characterise mixotrophic taxa as *D. pyriformis* and *D. nodosa* tend to have positive PC1 scores reflecting broader shapes with flatter bases and have greater centroid sizes on average as compared to *D. oblonga* (Fig. 3). In addition, *D. nodosa* is compressed lateral further helping to distinguish it from both *D. oblonga* and *D. nodosa*. Other mixotrophic taxa such as *Diffflugia capreolata* Penard, 1902 and *Diffflugia bidens* Penard, 1902 are also characterised by larger average sizes and broad morphologies, with *D. bidens* being compressed as well (Penard, 1902). There are several small ovoid arcellinid taxa that have been observed to house symbiotic algae: *Netzelia gramen* (Penard, 1902) Gomaa et al., 2017, *Diffflugia lobostoma* Leidy, 1879, *Diffflugia labiosa* Wailes, 1919, *Diffflugia urceolata* Carter, 1864 and *Cucurbitella mespiliformis* Penard, 1902. However, the 18S rRNA phylogenetic reconstruction shows that these taxa, apart from *D. urceolata* and *C. mespiliformis* that have yet to be phylogenetically placed, belong to a separate infraorder, namely Sphaerothecina, characterised by ovoid to spherical and hemispherical tests (Gomaa et al., 2017). Based on our results, mixotrophic taxa with elongate tests and terminal apertures (i.e., Longithecina) are characterised by both larger average centroid sizes and broader morphologies, especially in the lower portion of the test. This hypothesis requires further testing through the sequencing of missing members of Longithecina and confirmation with other molecular markers (e.g., SSU, COI). The larger average size of the tests could be the result of the greater amounts of energy provided by the endosymbionts, facilitating increases in test size and growth rate. The broader shapes could provide additional space to house endosymbionts in the cell body. In addition, broadening of the test increases surface area and could also allow for greater transmission of light, with the extreme being compression of the test as seen in *D. nodosa* and *D. bidens*.

Conclusions

Our approach of applying fast evolving genetic markers and advanced morphometric analysis to Arcellinida with comparable test morphologies was successful in highlighting ecologically relevant morphological traits, namely centroid size and characterising test morphology for mixotrophic taxa. Moreover, it represents a valuable tool for future taxonomic studies in creating detailed morphological descriptions of the phenotypic range of taxa. We did not erect new taxa names as the inability to anchor the clades within the Arcellinida makes inferences on their taxonomic rank (e.g., genus) uncertain. Our results highlight the importance of test centroid size when studying elongate difflogiids and the need for continued sequencing. A large test size range could be indicative of an organism’s ability to exploit several trophic levels or perhaps a range of different species. Mixotrophic elongate difflogiids are characterised by the inflation of the lower portion of

the test, larger centroid size and in some cases lateral compression. Continued research into the ecological relevance of morphological traits will serve to strengthen ecological inferences, increasing the importance of Arcellinida as environmental proxies.

Author Contributions

Research design (ALM, QB, CD, EL, DL, HR); Provided samples (MT); Provided research space (EM, HR); Laboratory analyses (ALM, QB); Data analyses (ALM, QB, CD, DL); Wrote manuscript (ALM); All authors contributed to revision.

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Figure captions/legends

Fig. 1. A-D. Scanning electron micrographs of taxa studied: **(A)** *Diffflugia oblonga*; **(B)** *D. acuminata*; **(C)** *D. lanceolata*. All images are to scale. **(D)** Schematic diagram showing geometric morphometric digitization: numbers represent landmarks; small black circles semilandmarks; the white circle is the centroid from which centroid size is derived.

Fig. 2. Maximum likelihood phylogenetic tree of Arcellinida based on NAD9-NAD7 gene sequences. Numbers at the nodes correspond respectively to bootstrap values (bs), posterior probabilities (p.p.), as calculated with Bayesian inference, and parsimony scores (ps) obtained by maximum parsimony analysis. NA values correspond to cases where the topology was diverging between analysis. The tree was rooted with Discosea, as shown by Kang et al. (2017), and new sequences are highlighted in bold.

Fig. 3. A biplot of principal component 1 (PC-1) scores (x-axis) and centroid sizes (y-axis) from geometric morphometric analysis. Specimens are illustrated as coloured (phylogenetic clade) symbols (taxa). PC-1 scores are translated into shape models illustrated along the top of the figure. Larger symbols represent specimens that have been sequenced and the letter reflects where the results were published: B - (Blandenier et al. 2017); G - (Gomaa et al. 2012, 2017); L - (Lahr et al. 2019); M - present study.

Table 1. List of sequenced species, sampling locations and sequence details.

Table 2. Nested PCR protocol.

Table 3. Summary of Procrustes Principal Component Analysis (first 7 of 60 components).

Appendices

Appendix A. Taxonomic details for Arcellinida in this study (**Table A.1**).

Appendix B. Alignment after converting nucleotide sequences to amino acid sequences and the removal of the intergenic region (**Table B.1**).

Appendix C. Details of the images used for morphometric analysis, including results from geometric morphometric analysis and ImageJ analysis (**Table C.1**).

Appendix D. Biplots demonstrating the relationship between test centroid size (x-axis) and test length (mm), test width (mm) and test aperture size (mm) (**Fig. D.1**).