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The devil is in the details: *Cylindrifflugia luciferina* spec. nov. reveals the phylogenetic backbone of the infraorder Cylindrothecina (Amoebozoa, Arcellinida)

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ABSTRACT

The application of molecular approaches has revealed an immense biodiversity of lobose testate amoebae (Amoebozoa, Tubulinea, Elardia, Arcellinida). However, most newly discovered species are difficult or impossible to differentiate morphologically from already described forms, even though they are genetically distinct. Consequently, while diversity assessments based on sequence data overtake classical estimations of Arcellinida diversity, only a handful of morphologically divergent species have been discovered recently. These species are of key importance for reconstructing the evolutionary history of Arcellinida, including both morphology and morphogenesis. Here, we describe a large and conspicuous new species, *Cylindrifflugia luciferina*, found in an urban pond located in the Royal Botanical Garden of Madrid, Spain. We characterize it morphologically and molecularly, together with the closely related species *Cylindrifflugia elegans*. Our phylogenetic reconstruction based on the SSU rRNA gene brings evidence for three main well supported and morphologically consistent clades within the infraorder Cylindrothecina. This example illustrates that the quest for new Arcellinida diversity is far from being over and even the less expected ecosystems can bring new discoveries.

1. Introduction

The quest for novel biodiversity has always been one of the major pursuits of biologists and naturalists. Nowadays, as the current biodiversity crisis implies a huge threat to the stability of ecosystems, studying biodiversity has become a priority for the scientific community. Protists have been shown to be much more diverse than plants and fungi (De Vargas et al., 2015; Mahé et al., 2017). They represent, thus, the last frontier in our knowledge of eukaryotes. Testate amoebae from the order Arcellinida Kent, 1880 have been considered as a counterexample as their self-built shells are large (for protist standards) and have a typical shape and composition that can be potentially used to identify them. Before the application of molecular tools to species identification, several works (e.g., Ogden, 1979; Penard, 1890, 1902) aimed to establish the systematic classification of the group. However, early authors were already aware that, although test shape was inherited, variations could be observed even within clonal lines under experimental conditions (Jennings, 1916, 1937). Certain environmental factors, e.g. food and temperature, have been shown to influence shell traits (Wanner, 1999; Wanner and Meisterfeld, 1994). Climate can also influence shell shape and size (Mulot et al., 2017). Morphologies, therefore, appear in certain groups at least as a continuum of shapes where species discrimination through traits appears often arbitrary (Foissner and Korganova, 2000). These factors hampered considerably the Arcellinida biodiversity research as many published descriptions were later invalidated, causing confusion in systematics (Kosakyan et al., 2016).

The application of molecular techniques impacted Arcellinida systematics at the deepest levels (Gomaa et al., 2012; González-Miguéns et al., 2022; Kosakyan et al., 2016; Lahr et al., 2019; Lara et al., 2008), evidencing synapomorphies for large groups called “infraorders”. Molecular techniques also revealed a wealth of diversity at fine taxonomic scales, like species rank and below. Very often, described “morphospecies” were split into many “cryptic” and “pseudocryptic” species (González-Miguéns et al., 2022; Kosakyan et al., 2012, 2013) that are often difficult or impossible to identify morphologically. These lineages differed nevertheless in their ecology (Singer et al., 2018) and/or their spatial distributions (Singer et al., 2019). A consequence of these results is that, most probably, the estimation of 1100 species by Kosakyan et al.

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(2016) needs to be revised by one or more orders of magnitude. These new species, however, are closely related to known morphs to the point that molecular approaches are required to discriminate them (González-Miguéns et al., 2022; Ribeiro et al., 2023; Useros et al., 2023). The finding of species whose morphology diverges unmistakably from known species is limited to a few cases (Féres et al., 2016; Nasser and Patterson, 2015; Nasser et al., 2021). Usually, new species with diverging morphology are found when undersampled regions such as the Neotropics are visited (Bobrov et al., 2022; Burdman et al., 2021; Féres et al., 2016; Reczuga et al., 2015). Karstic caves have also revealed morphologies that had never been registered before (Baković et al., 2019). Urban habitats, represented, for instance, by artificial ponds, may seem less attractive for bioprospection at first sight, though they typically harbor a variety of plants whose presence is correlated with Arcellinida diversity (Tsyganov et al., 2023). Here, we describe a new species of Arcellinida found for the first time in a pond in the Royal Botanical Garden in Madrid, Spain. This large-sized species (>200 μm), with rugged elongated test and two "horn"-like protuberances, was characterized genetically based on the SSU rRNA gene sequences, often used to explore the deepest levels of the phylogenetic tree of Arcellinida (González-Miguéns et al., 2022). We also documented another closely related species, *Cylindrifflugia elegans*, and discussed the phylogenetic implications for the Arcellinida evolutionary history.

2. Material and methods

2.1. Sampling

Samples were collected from an artificial pond located in the Royal Botanical Garden in Madrid, Spain (40°24′36.1″N, 3°41′22.1″W). This habitat (Fig. 1) comprises several aquatic plants such as *Hydrocharis morsus-ranae* L., *Scirpoides holoschoenus* (L.) Soják, *Typha domingensis* Pers., *Nymphaea mexicana* Zucc. and *Pontederia cordata* L. The bottom of this pond is composed of sandy sediments. Additionally, there are numerous individuals of the Iberian pond frog *Pelophylax perezi* (Seoane, 1885) and their tadpoles during specific seasons of the year.

Sampling was repeated at different locations in the pond: two samples were taken from zone 1 in early February 2023 (Fig. 1B) and other samples were taken from zones 1 and 2 in early March 2023 (Fig. 1C). A throw-away Pasteur pipette was used to collect the samples from the upper layer of the sediments and subsequently transferred to a Falcon tube, which was maintained at 4 °C prior to microscopic observations and cells isolation.

2.2. Microscopic observation and cell isolation

All samples were observed using a Leica DM18 inverted microscope and documented with a Leica MC70 HD camera using the software Leica suite ver. 4.12.0. Some tests were deposited on stubs for observation under a scanning electron microscope (SEM) Hitachi S-3000N after being covered with gold (8 nm) with a sputter coater Q150R S plus.

Only testate amoebae showing active mobility were isolated using a narrow-diameter glass pipette and deposited in distilled water for further washing. Finally, each cell was placed individually in 0.2 mL Eppendorf tubes containing 100 μL of guanidine thiocyanate buffer for DNA extraction (Chomczynski and Sacchi, 1987).

2.3. DNA extraction, PCR amplification and sequencing

DNA of washed cells was extracted following the protocol described by Duckert et al. (2018). This method includes an isopropanol precipitation and several washes with ethanol at different concentrations (70 % and 96 %). Finally, the resulting pelleted DNA was resuspended into 6 μL of sterile water and stored at 4 °C until amplification.

Fig. 1. Sampling site. (**A**) Panoramic view of the pond in the Royal Botanical Garden in Madrid, Spain. (**B**) Sampling zone 1. (**C**) Sampling zone 2.

PCRs were aimed at amplifying the gene coding for the SSU rRNA molecule and performed in the following mix: 6 μL of filtered water Mili-Q, 12 μL MyTaq Red DNA polymerase Mix (BioLine), 1 μL of each primer (10 μmol) and 1 μL of template DNA, resulting in a final reaction volume of 20 μL. SSU rRNA gene sequences were obtained in three steps through nested PCRs and the procedures described by Soler-Zamora et al. (2021). A first PCR (1) was performed using the universal eukaryotic primers: EK42F (5′-CAC CTA CGG AAA CCT TGT TA-3′) as forward primer and EK1498R (5′-TAA CAA GGT TTC CGT AGG TG-3′) as reverse primer (Marande et al., 2009). The products obtained from PCR (1) were used as templates for a nested PCR (2) using the universal eukaryotic primers: EK555F (5′-AGT CTG GTG CCA GCA GCC GC-3′) as forward primer and EK1498R as reverse primer (López-García et al., 2001). The PCR cycling profile for both PCRs was: 95 °C for 3 min, followed by 37 cycles of 94 °C for 30 s, an annealing temperature of 58 °C for 30 s, 72 °C for 90 s and a final extension at 72 °C for 10 min. The obtained products from PCR (2) were used for the next nested PCR (3) with an Arcellinida-specific primer Arcell 1F (5′-GAA AGT GGT GCA TGG CCG TTT-3′) (Lara et al., 2008) as forward primer and the universal eukaryotic primer EK1498R as reverse primer. The PCR cycling profile was: 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, an annealing temperature of 60 °C for 30 s, 72 °C for 90 s and a final extension at 72 °C for 10 min.

All PCR products were observed by electrophoresis on a 1 % agarose gel to examine the presence of amplicons with the expected molecular size and check for contaminations. Electrophoresis bands with the expected size were purified through extraction from the gel. Purified samples were sequenced by the company Secugen S.L. (Madrid, Spain) through Sanger dideoxy technology. The resulting sequences were visualized with the software MEGA X ver. 11 (Tamura et al., 2021) and those exhibiting high quality were analysed following the blastn method (Altschul et al., 1990) to determine their taxonomic classification.

2.4. Alignment and phylogenetic analysis

New SSU rRNA gene sequences were manually aligned against a 1426 base pair (bp) alignment of other Arcellinida sequences published as Supplementary data 5 by González-Miguéns et al. (2022). The sequence of *Heleopera steppica* (OQ646056) from Ribeiro et al. (2023) was also included in the alignment. This alignment was constructed using the MAFFT algorithm in the software Geneious Prime ver. 2019.0.4 and visually checked to remove introns.

First, the best substitution model and rate variation among sites, according to the Bayesian Information Criterion (BIC), were found using ModelFinder (Kalyaanamoorthy et al., 2017) implemented in IQ-TREE ver. 2.1.2 (Nguyen et al., 2015). A phylogeny using the Maximum Likelihood (ML) algorithm was built in the same software. Node robustness was assessed with 10,000 replicates of ultrafast bootstrapping. Then, we built a phylogeny using Bayesian Inference (BI) in MrBayes ver. 3.2.7a (Ronquist and Huelsenbeck, 2003), implementing two independent runs with four chains for each run and 20×10^6 generations for the Markov chain Monte Carlo (MCMC) simulations. Trees were sampled every 1000 generations. Substitution models were selected with ModelFinder as mentioned above. Finally, we discarded 25 % of sampled trees and evaluated the convergence of the two runs with Tracer ver. 1.7.1 (Rambaut et al., 2018). All effective sample sizes (ESSs) values were over 200. The resulting trees for each gene were summarized in a 50 % majority rule consensus tree. Sequences from the sister order Euamoebida Lepşi, 1960 were used to root the tree.

2.5. Morphological analysis

Morphological parameters shown in Figure 2 were measured (Table 1) from the images obtained through optical microscopy using the software ImageJ ver. 1.52 (Schneider et al., 2012).

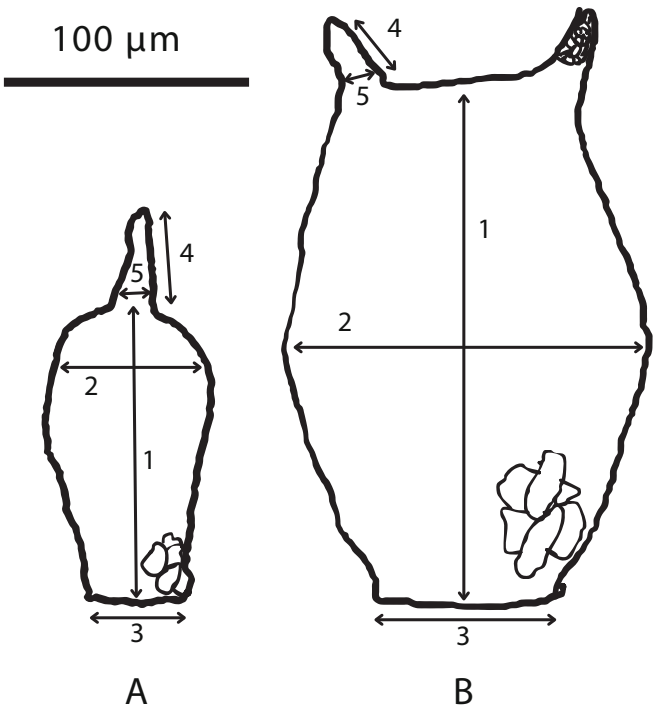

Fig. 2. Outline of shells and parameters measured. (**A**) *Cylindrifflugia elegans*. (**B**) *Cylindrifflugia luciferina* (SP2). Measurements: 1, shell length; 2, shell width; 3, aperture diameter; 4, horns length; 5, horns width.

2.6. Organism identification

We looked exhaustively into the relevant literature to compare the new morphotype with the species previously reported in the literature (for example, Ehrenberg, 1838; Nasser et al., 2021; Ogden, 1980; Ogden and Fairman, 1979; Penard, 1890, 1902, 1904; Perty, 1852; Vucetich, 1978). Following an integrative taxonomic approach, morphological, molecular and ecological data were used to describe the new species: test shape, dimensions and composition, SSU rRNA gene sequence and habitat conditions, respectively. We deposited one SEM stub with the new species at the Royal Botanical Garden of Madrid; this preparation is available under the name MA-Algae 11275.

3. Results

3.1. Observations on the morphology

We observed two morphotypes of Arcellinida, which we identified and analysed morphologically and molecularly. The first morphotype (Fig. 3) corresponds well to *Difflugia elegans* (Penard, 1890), renamed as *Cylindrifflugia elegans* by González-Miguéns et al. (2022). This species presents a short, slightly pyriform shell with a straight aboral horn and a circular aperture with a small neck. The second morphotype, SP2 (Fig. 4), exhibits a larger size compared to *C. elegans*, 205–241 μm vs. 79–115 μm (Table 1), and possesses an oval shell with two horns. The aperture is also circular but with a less pronounced neck.

Both morphotypes have an agglutinated test built through the incorporation of environmental materials, which provides them with a rugged outlook. The shells appear greyish with brown, yellow, and/or green tones. No biological elements, such as diatom frustules or Euglyphida scales were observed on any of the shells.

In addition, measurements provided in Table 1 have been collected to determine test dimensions and compare them with previous descriptions of related species.

Table 1
Biometric characterization of studied morphotypes.

	Cylindrifflugia elegans ($n = 14$)					*Cylindrifflugia luciferina* (SP2) ($n = 3$)				
Measure	(1)	(2)	(3)	(4)	(5)	(1)	(2)	(3)	(4)	(5)
$\bar{x}$	97.18	57.64	37.49	19.52	8.06	219.13	134.48	70.80	35.07	13.34
Min.	78.87	49.22	26.71	11.43	5.15	205.80	121.74	65.68	32.20	12.71
Max.	117.65	69.19	56.74	29.90	16.2	241.51	140.16	78.68	37.94	13.97

Measurements (μm) are indicated in Figure 2.
Min., minimum; Max., maximum; *n*, number of measured tests; $\bar{x}$, arithmetic mean.

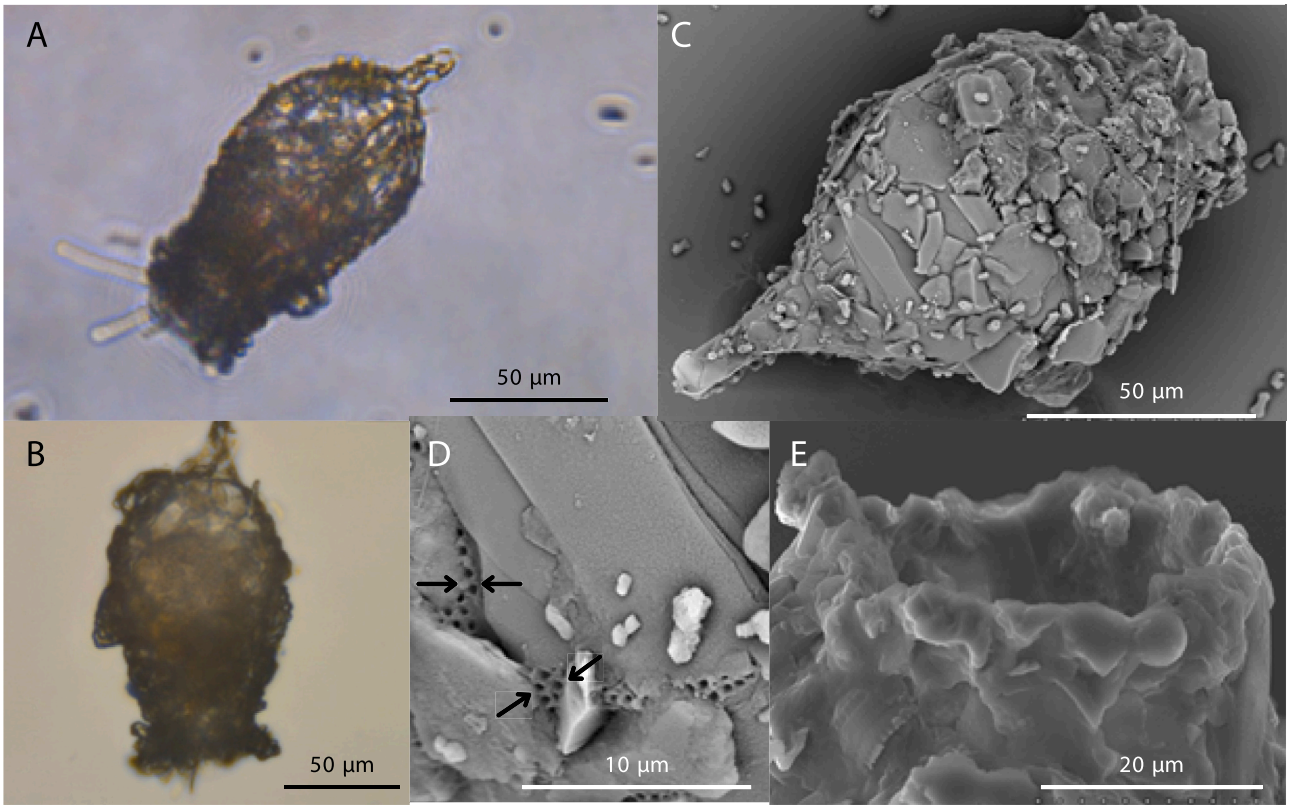

Fig. 3. *Cylindrifflugia elegans*, optical microscope (A, B) and SEM (C–E). (**A–C**) Lateral views. (**D**) Surface view showing the test ornamentation. Arrows mark pores in the organic cement matrix. (**E**) Aperture. Images in (A) and (B) were taken with a Leica DMi8 inverted microscope and a NPlan L 20× objective with phase contrast.

3.2. Results of phylogenetic analyses

Altogether, we obtained six sequences of the SSU rRNA gene from four *Cylindrifflugia elegans* specimens and two morphotype SP2 individuals. To build the phylogenetic tree, we used 60 sequences of the SSU rRNA gene: 56 sequences from the order Arcellinida and four from the sister order Euamoebida, which served as an outgroup (Fig. 5). Sequences are available in GenBank under the following accession numbers: PP989312, PP989313, PP981362, PP981367, PP981372 and PP981373.

The topology of the ML and BI trees is consistent with the taxonomy presented in other previous studies (González-Miguéns et al., 2022). The majority of infraorders are monophyletic and supported with a bootstrap value (BB) higher than 70 % and a posterior probability (PP) higher than 0.96. *Argynnia dentistoma* Penard, 1890 and *Physochila griseola* (Penard, 1911) do not appear to be closely related to any infraorder, therefore classified as *incertae sedis*.

Newly obtained sequences fall within the infraorder Cylindrothecina, which confirms inferences based on morphological observations. Sequences of both *C. elegans* and morphotype SP2 were identical with their respective conspecifics. The six new sequences form a robust clade (BB = 92 %, PP = 0.98), which we name here Clade A, together with *Cylindrifflugia bacillariarum* (Perty, 1852). This clade includes forms with an ovoid shape and at least one horn on the aboral side of the shell (or two in the case of SP2). The sister group (Clade B), also well-supported (BB = 98 %, PP = 1.00), is composed of species with more elongated shells: *Cylindrifflugia acuminata* (Ehrenberg, 1838), *Cylindrifflugia lanceolata* (Penard, 1890), *Cylindrifflugia oblonga* (Ehrenberg, 1838) and *Cylindrifflugia periurbana* Ribeiro et al., 2023. A single horn is present in some species only. Clades A and B branch together in a well-supported cluster (BB = 93 %, PP = 0.99). Finally, a third clade (Clade C) branches as sister to all other Cylindrothecina, composed of the species *Cylindrifflugia hiraethogii* (Ogden, 1983) and *Cylindrifflugia alhadiqa* (Soler-Zamora et al., 2021) (BB = 100 %, PP = 1.00).

3.3. Taxonomic account

- ● Arcellinida Kent, 1880
- ●● Cylindrothecina González-Miguéns et al., 2022
- ●●● Cylindriflugiidae González-Miguéns et al., 2022
- ●●●● *Cylindrifflugia* González-Miguéns et al., 2022

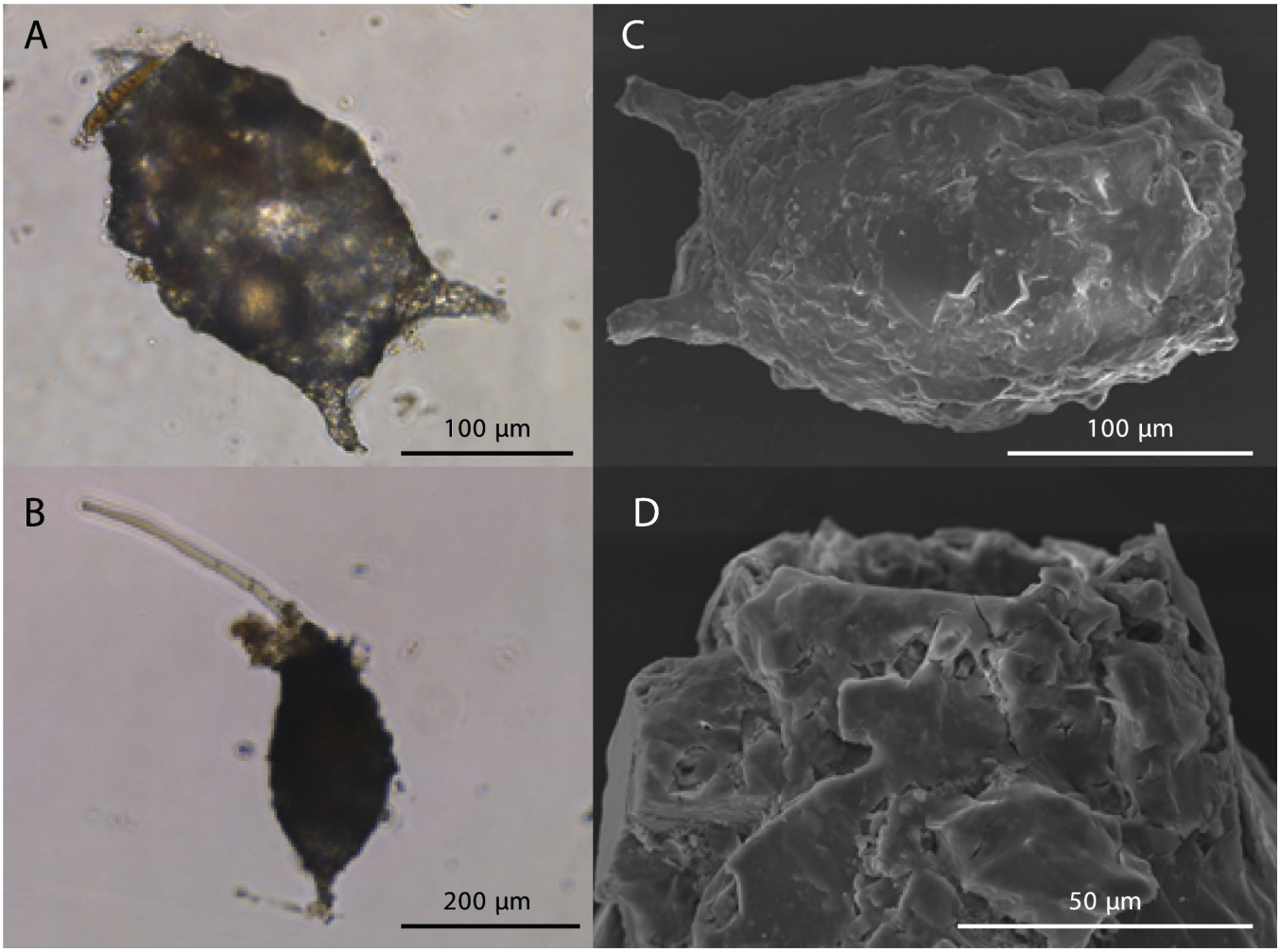

Fig. 4. *Cylindrifflugia luciferina* (SP2), optical microscope (A, B) and SEM (C, D). (**A, C**) Frontal views. (**B**) Lateral view, showing a long pseudopod. (**D**) Aperture. Images in (A) and (B) were taken with a Leica DMi8 inverted microscope and a NPlan L 20× objective with phase contrast.

***Cylindrifflugia elegans* (Penard, 1890) population (Fig. 3)**

Description. Ovoid test with semi-transparent appearance and dark brown tones and a neck at the base of the aperture. The aperture is circular and surrounded by agglutinated particles. The test ends with a long straight horn that appears more transparent than the rest of the structure. The test is constructed from mineral particles integrated into an organic cement matrix whose structure can be observed under electron microscopy. Matrix has a network-like appearance formed by small pores arranged in a fashion typical for the species; Ogden (1983) highlights the importance of this structure as a diagnostic character for species identification.

Dimensions. 79–118 µm length, 49–69 µm breadth, 27–57 µm aperture diameter. Horns measurements: 12–30 µm length, 5–16 µm breadth ($n = 14$) (Table 1). In the original description of Penard (1890), test lengths between 80 and 100 µm were reported, which may seem to be a slightly narrower range than our population. However, this difference may well be due to the accuracy reached by measuring instruments in the early twentieth century.

Intraspecific variability. Some individuals have the aboral horn slightly tilted towards one side. The constriction at the base of the aperture can be less noticeable sometimes and there can be variations in the breadth and roundness of the aboral part of the test.

Habitat. Sand and sediments in a pond with vegetation. Corresponds with the type habitat ("swamps").

Locality. Artificial pond in the Royal Botanical Garden of Madrid, Spain (40°24′36.1″N, 3°41′22.1″W) (Fig. 1).

***Cylindrifflugia luciferina* Mauricio-Sánchez, Soler-Zamora and Lara sp. nov. (Fig. 4)**

Zoobank registration. This publication was registered in ZooBank with the following code: urn:lsid:zoobank.org:pub:103B9E85-D7C1-4605-A80F-60D9DEA66D58. The new species-group name *Cylindrifflugia luciferina* was registered in ZooBank under the code: urn:lsid:zoobank.org:act:6E1CC206-D9B7-4CA4-8B59-EE0665584074.

Description. Opaque and robust test with shades of brown, grey and yellow, oval in shape, and with a neck and circular aperture. It has two aboral horns with a smoother and more transparent appearance than the rest of the test. The test is formed by minerals and environmental materials. The pseudopodia can reach a greater length than the test itself.

Dimensions. 206–242 µm length, 122–140 µm breadth, 66–79 µm aperture diameter. Horns measurements: 32–38 µm length, 13–14 µm breadth ($n = 3$) (Table 1).

Intraspecific variability. Some individuals can have their horns more separated from each other than others. There can be variations in the depth of the constriction at the base of the aperture.

Differential diagnosis. Test morphology and the presence of two aboral horns resemble *Difflugia bicornis* Penard, 1890 (Fig. 8D) but its test size (50–60 µm) rules out any confusion between both species.

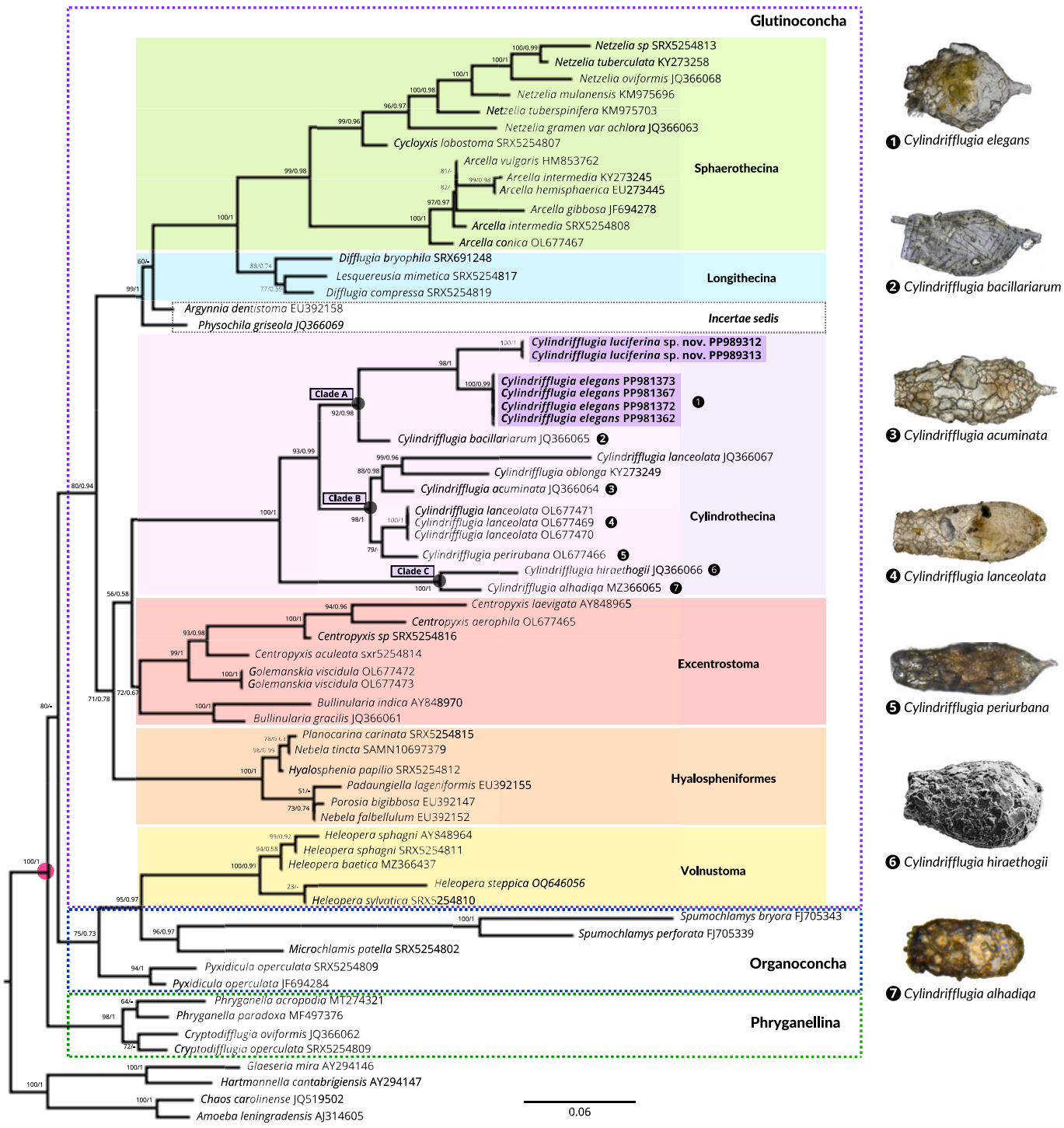

Fig. 5. Maximum Likelihood phylogenetic tree based on Arcellinida SSU rRNA gene sequences. Bootstrap values and Bayesian posterior probabilities are indicated for each node. The pink circle represents the most recent common ancestor of the order Arcellinida; black circles indicate ancestors of clades A, B and C. The sequences obtained in this work are highlighted in purple. Images were obtained from arcella.nl. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Difflugia diafana (Vucetich, 1978), a South American species, has a similar test length but a much narrower test, and hence a smaller test breadth (108–114 μm). *Erugomicula bidens* (Penard, 1902) (Fig. 8A) has a more regular shape, the texture of the test is less rough and the horns are relatively shorter. The dimensions of the test could fit with those described in the species *Cylindrifflugia acuminata* (Fig. 8C) but that species has a more elongated test, a single aboral horn, and does not have a constriction at the base of the aperture. Compared to the rest of the species of the genus *Cylindrifflugia*, it shares neither size nor test morphology similarities (see discussion below).

Habitat. Sandy sediment in a pond with abundant aquatic vegetation.

Type locality. Artificial pond in the Royal Botanical Garden in Madrid, Spain (40°24′36.1″N, 3°41′22.1″W) (Fig. 1).

Etymology. The specific epithet refers to the resemblance to a small devil due to its two horns. According to the Christian faith, Lucifer is one

of the names given to the Fallen Angel who fell from Heaven after trying to reign instead of God. He is often associated with Satan, and is traditionally represented with two horns on his head. Therefore, this name has been chosen to designate this new species as *Cylindrifflugia luciferina*.

Derived from the Latin noun *lūx* (genitive *lūcis*) [f] (light), the Latin verb *ferre* (bear, carry), and the Latin feminine suffix *-īna*. The species epithet is to be treated as a composite noun in the nominative singular standing in apposition to the generic name (Article 11.9.1.1 of the ICZN, 1999). A species-group name that is a noun in apposition needs not agree in gender with the generic name with which it is combined (the original spelling is to be retained, with gender ending unchanged) according to Article 31.2.1 of the ICZN (1999).

Holotype. The type material is placed on a metallized scanning electron microscopy stub that has been deposited at the Royal Botanical Garden in Madrid (Spain) and registered under the code MA-Algae 11275.

4. Discussion

The discovery of such a peculiar species as *Cylindrifflugia luciferina* in a heavily managed environment, a pond in a public garden, may seem surprising at first sight. Still, the Iberian Peninsula, as opposed to other regions in temperate and boreal Europe, has been left practically unexplored for new Arcellinida species except in the last few years (González-Miguéns et al., 2022; Soler-Zamora et al., 2021). The few classical works that investigated testate amoebae diversity concerned terrestrial habitats such as forest litter (Bonnet and Gomez-Sanchez, 1984; Gracia-Royo, 1978) or peatlands (Gracia-Royo, 1972, 1976). The rivers of the Iberian Peninsula are isolated from European basins, and it is therefore likely that freshwater systems have high endemism rates as evidenced by fishes (Filipe et al., 2009). It is not unlikely that *C. luciferina* might be an Iberian endemism. However, it must be noticed also that several allochthonous plants, which are native to the American continent (e.g., *Pontederia cordata* and *Nymphea mexicana*), grow in the Botanical Garden pond. These plant species, vastly distributed in North and Central America (and even South America in the case of *P. cordata*), may have been the source of introduction of testate amoebae in the pond, which would then have traveled most likely on the leaves or within the entangled root system. Similar introductions of protists into new continents through host plants have already been documented (Aguilar et al., 2014). In any case, the finding of *C. luciferina* proves that new species are not only to be found in unexplored continents or ecosystems. To the contrary, this suggests that we have barely scratched the surface of Arcellinida diversity.

Our vision of Arcellinida systematics, particularly the Cylindrithecina infraorder, has been influenced by the placement of *C. luciferina*. The erection of Cylindrothecina was motivated by the finding that the genus *Difflugia* Leclerc, 1815 remained paraphyletic even after the rounded test species were transferred to *Netzelia* Ogden, 1979 and *Difflugia viscidula* (Penard, 1902) to *Golemanskia* González-Miguéns et al., 2021 (González-Miguéns et al., 2021, 2022; Kosakyan et al., 2016). It resulted in a group of species whose morphologies and lifestyles remained heterogeneous, ranging from the cave inhabiting *Cylindrifflugia alhadiqa* to the large freshwater predator *Cylindrifflugia acuminata*. The characterization of both *Cylindrifflugia elegans* and *C. luciferina* allowed the recognition of three morphologically and genetically well-defined clades: (1) clade A that includes species with ovoid tests (*Cylindrifflugia bacillariarum*, *C. elegans* and *C. luciferina*), (2) clade B that contains species with elongated tests (*C. acuminata* and other species) and (3) clade C that aggregates *C. alhadiqa* and *C. hiraethogii*, both with moderately elongated tests and without horns. The first two clades include aquatic species, especially members of clade A seem to be linked to the presence of dense aquatic vegetation. Clades A and B appear to be ecologically homogeneous so far. Clade C, in turn, is composed of two species that differ considerably both in their morphology and ecology. Each clade could potentially represent a new genus within Cylindrothecina. This possibility certainly needs to be explored in the future when more species will be described and barcoded.

The classification of *C. luciferina*, *C. elegans* and *C. bacillariarum* as different species within a single clade has been a subject of discussion prior to the introduction of molecular data. While *C. luciferina* clearly differs in size and shape from the two other species (Fig. 4), it branches closely to *C. elegans* (Fig. 5). *Cylindrifflugia elegans* and *C. bacillariarum* can be differentiated mostly by the material used to build tests (Figs. 6 and 7). While *C. elegans* (and, for that matter, *C. luciferina*) uses quartz material gathered in the surroundings, *C. bacillariarum* uses only diatom frustules, presumably taken from its prey. Based on their resemblance, Penard (1902) did not believe that *C. bacillariarum* was a valid species and argued that it was a small variety of *C. elegans*. However, according to the original description by Perty (1852), *C. bacillariarum* could only live in ecosystems with populations of diatoms due to the active selection of the material for the construction of its test. While some Arcellinida species do not show a preference for any material to build their tests (Delaine et al., 2017), the presence of certain prey is indispensable to closing the life cycle of others (Dumack et al., 2019). This may apply also

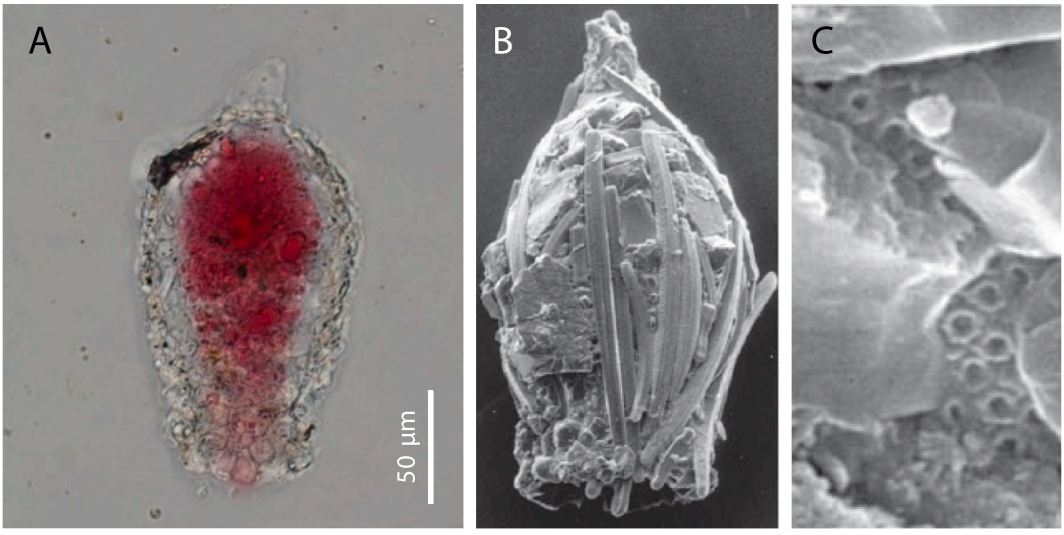

Fig. 6. Micrographs of *Cylindrifflugia elegans*. (**A**) Type material from Penard collection, Natural History Museum of Geneva. (**B, C**) Photographs from the SEM Ogden collection (Mazei and Warren, 2012). The ultrastructure of organic cement is shown in (C).

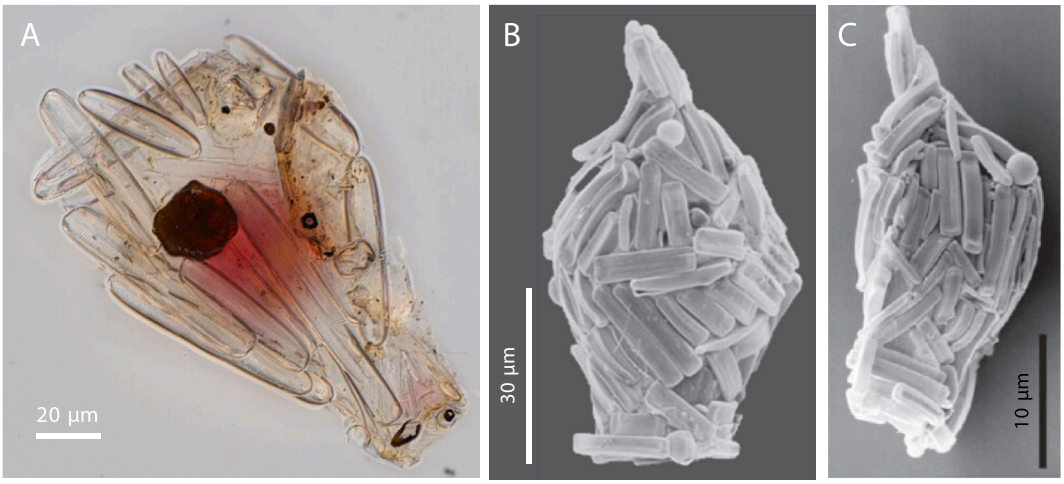

Fig. 7. *Cylindrifflugia bacillariarum*. (**A**) Type material from Penard collection, Natural History Museum of Geneva. (**B, C**) Photographs from the SEM Ogden collection (Mazei and Warren, 2012).

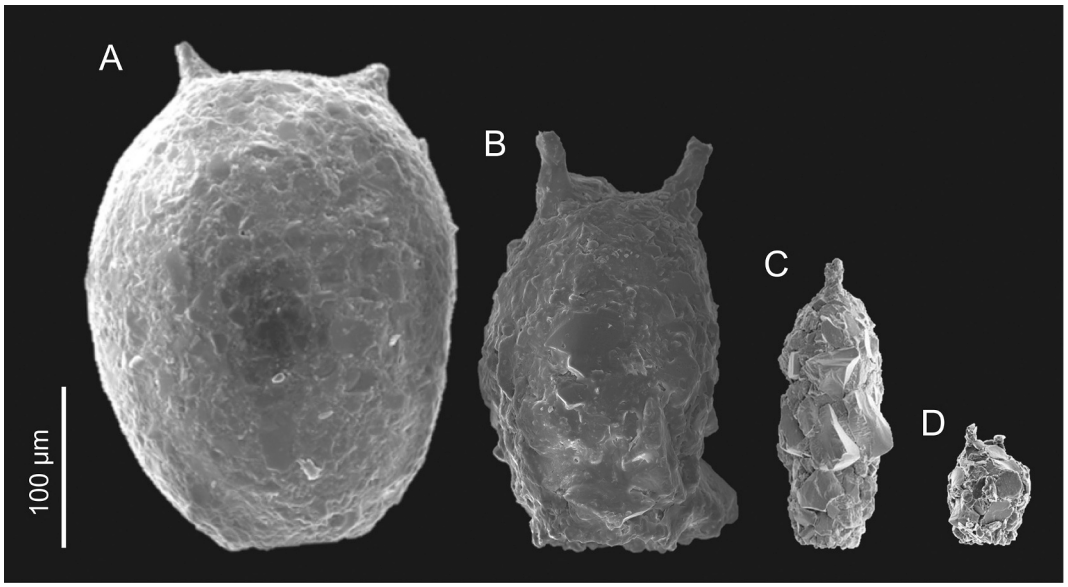

Fig. 8. (**A**) *Erugomicula bidens*, SEM photograph from Nasser et al. (2021). (**B**) *Cylindrifflugia luciferina*. (**C**) *Cylindrifflugia acuminata*. (**D**) *Difflugia bicornis*. Photographs in (C) and (D) from the SEM Ogden collection (Mazei and Warren, 2012).

to *C. bacillariarum*, which could represent another example of extreme resource specialization in Arcellinida.

In addition, a wide variation in shell dimensions has been reported in the literature for *C. elegans*: 57–103 µm (Ogden, 1980) or 117–158 µm in length (Ogden and Fairman, 1979). Several varieties of test lengths that diverge strongly from the original dimensions have been described, for example, *C. elegans* var. *lepida* with 30 µm test length (Schönborn, 1966). Such size range suggests the existence of several different species all designated as *C. elegans*, whose taxonomic position needs to be clarified before taking definitive taxonomic decisions.

In turn, although *C. luciferina* resembles the two other species by its test outline, its morphology may suggest a closer relationship with *Difflugia bicornis* Penard, 1890 (Fig. 8D) due to the oval shape of the test and the two terminal horns. However, *D. bicornis* has a much shorter test, 85–105 µm (Penard, 1890), and its horns are considerably longer in proportion. The test of *Difflugia diafana* also presents two aboral horns and has similar length (210–235 µm) but it is more elongated, and breadth measurements do not overlap (108–114 µm) (Vucetich, 1978). Moreover, its horns are also significantly longer (50–60 µm) (Vucetich, 1978). Given their resemblance to *C. luciferina*, it seems likely that both *D. bicornis* and *D. diafana* may be closely related and would therefore branch within clade A. Lastly, *Difflugia bidens*, recently combined with *Erugomicula* by Nasser et al. (2021) (Fig. 8A), has similar test dimensions as *C. luciferina* and possesses two (or three) horns. But given the much more spherical test outline and the smaller horns, its position remains uncertain.

To summarize, the exploration of Cylindrothecina diversity reveals interesting eco-evolutionary patterns within this newly erected infraorder. There is, apparently, a correspondence between the environment where species are found and their phylogenetic position. The presence of horns, reported in most taxa from clades A and B, has been interpreted as a protection against predators (Penard, 1902). Given the fact that these organisms seem to favour the presence of aquatic vegetation, we speculate that this trait is linked to plants, perhaps to stabilize organisms in the vegetation. More data will be needed to test this hypothesis, and probably also others that may arise later. While many discoveries may wait in extreme ecosystems and/or in underexplored countries, our direct surroundings certainly hold unexpected surprises.

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CRediT authorship contribution statement

Elena Mauricio-Sánchez: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carmen Soler-Zamora:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Enrique Lara:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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