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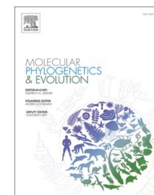

Deconstructing *Difflugia*: The tangled evolution of lobose testate amoebae shells (Amoebozoa: Arcellinida) illustrates the importance of convergent evolution in protist phylogeny

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A B S T R A C T

Protists, the micro-eukaryotes that are neither plants, animals nor fungi build up the greatest part of eukaryotic diversity on Earth. Yet, their evolutionary histories and patterns are still mostly ignored, and their complexity overlooked. Protists are often assumed to keep stable morphologies for long periods of time (morphological stasis). In this work, we test this paradigm taking Arcellinida testate amoebae as a model. We build a taxon-rich phylogeny based on two mitochondrial (COI and NADH) and one nuclear (SSU) gene, and reconstruct morphological evolution among clades. In addition, we prove the existence of mitochondrial mRNA editing for the COI gene. The trees show a lack of conservatism of shell outlines within the main clades, as well as a widespread occurrence of morphological convergences between far-related taxa. Our results refute, therefore, a widespread morphological stasis, which may be an artefact resulting from low taxon coverage. As a corollary, we also revise the groups systematics, notably by emending the large and highly polyphyletic genus *Difflugia*. These results lead, amongst others, to the erection of a new infraorder Cylindrothecina, as well as two new genera *Cylindrifflugia* and *Golemanskia*.

1. Introduction

Dobzhansky's famous sentence (Dobzhansky, 1973) "Nothing in Biology Makes Sense Except in the Light of Evolution" reflects the importance of understanding the evolutionary history of organisms to make inferences on their biology. These evolutionary patterns can be inferred by unravelling the phylogenetic relationships between groups, based on the comparative analysis of independent homologous characters (De Luca et al., 2019; Gorospe et al., 2020; Peters et al., 2017; Zumel et al., 2021). Furthermore, the effect of selective pressures may lead to predictable patterns along these phylogenies. For instance, positive selection may favour a particular trait in a specific environment, typically leading to homoplastic evolution of similar morphological adaptations in far-related organisms across the phylogenetic tree (Losos et al., 1998; Ruedi and Mayer, 2001). On the other hand, stabilizing selection may promote the conservation of a morphological phenotype for long periods of evolutionary time (stasis) (Muñoz et al., 2014; Szudarek-Trepto et al., 2021). The effects of these evolutionary forces has been largely observed in the field and experimentally tested in the laboratory, mostly on large organisms such as plants and animals (Blount et al., 2018). However, most of eukaryotic diversity is of microscopic size and classified as protists (Adl et al., 2019); for this reason, protists must imperatively be taken into account in any generalization patterns about the evolution of eukaryotic life.

Abbreviations: COI, Cytochrome Oxidase subunit I; NADH, nicotinamide adenine dinucleotide dehydrogenase; SSU, SSU rRNA gene.

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One of the most conspicuous protist groups are Arcellinida, a group of Amoebozoa characterized by a self-constructed shell or test which shape, size and composition has been used to build up the systematic classification of the group (Fig. 1); while shape and size were used for species delimitation, shell composition was proposed as a criterion for deep taxonomy. Species were classified into different infra-orders depending on if their shell was organic or included mineral elements (Anderson, 1988; Meisterfeld, 2002). This initial attempt of systematic classification was proved inaccurate by molecular phylogenetics, as organisms with similar shell composition were shown to belong to different evolutionary lineages (Lara et al., 2008). Phylogenies based on single genes (Dumack et al., 2020, 2019; Gomaa et al., 2017, 2012; Soler-Zamora et al., 2021) and transcriptomic data (Lahr et al., 2019), show that the general shape of the shell seemed more in agreement with molecular results to construct the Arcellinida systematics.

To trace the evolutionary patterns of Arcellinida, their general shell shape was compared with the Neoproterozoic vase shaped microfossils (Lahr et al., 2019), which are considered as the oldest fully reliable eukaryotic fossils (Porter and Knoll, 2000). These comparisons seemed to confirm the hypothesis of phenotypic continuity between Precambrian fossils and extant forms, which was formulated based on the striking resemblance between fossils and extant species (Lahr, 2021; Porter et al., 2003). More recent fossils were also attributed to extant genera, species and even subspecies (Farooqui et al., 2014; Singh et al.,

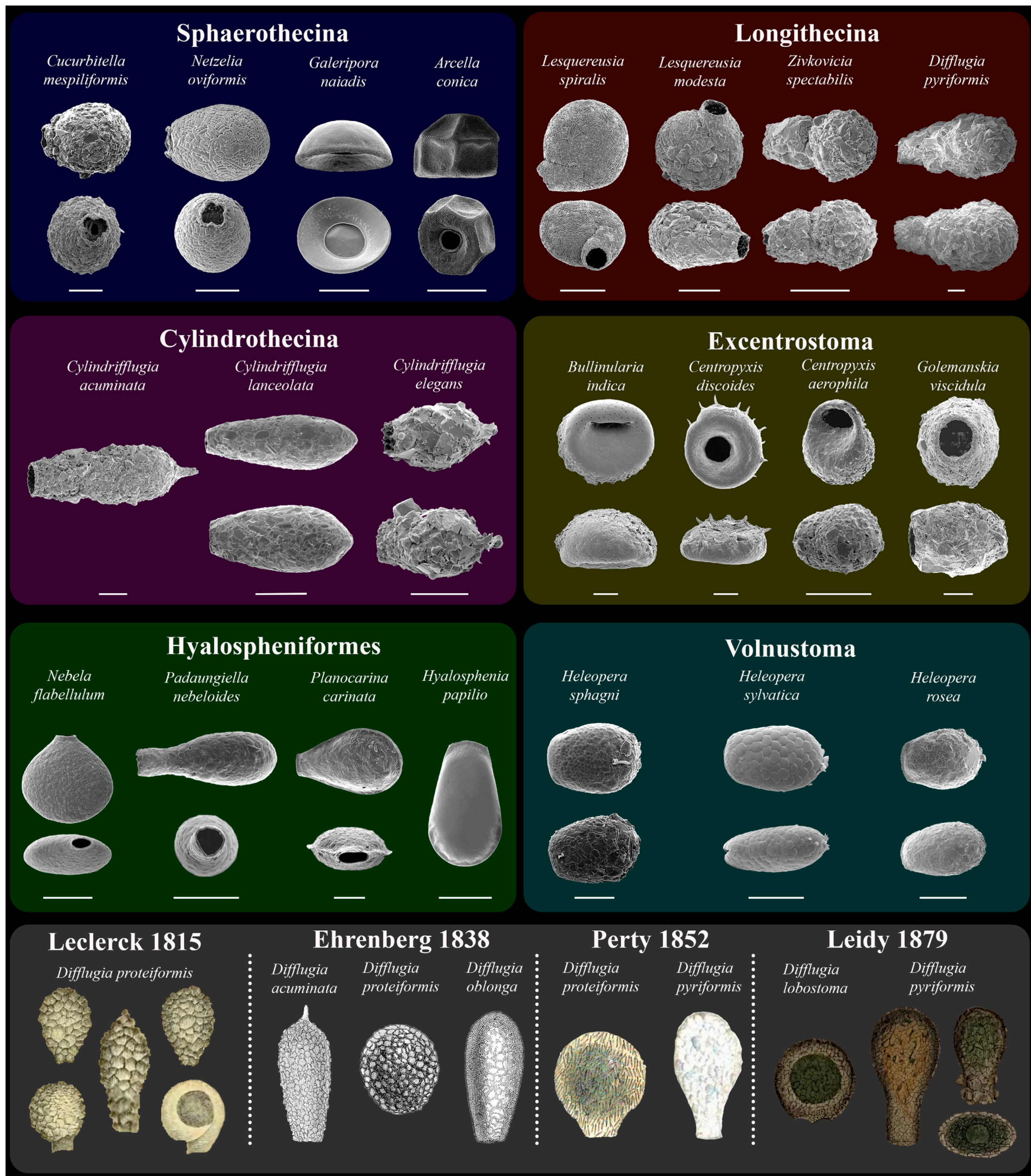

Fig. 1. Scanning electron images of the different infraorders described in Glutinoconcha (Arcellinida). The white bars represent a scale of 50 µm for each species. Bottom part of the figure: original drawings where *Difflugia proteiformis* and its allies are represented.

2015). These taxonomic assignations were based on the assumption that resembling organisms would be likely close related, allowing the description and systematic classification of new genera based solely on shell morphology (Bobrov and Mazei, 2020; Nasser et al., 2021). From an evolutionary point of view, this implies that each lineage became stabilized through selection into a given morphology that lasted for hundreds of million years (=morphological stasis hypothesis) because of long lasting and constant selective pressure or morphological constraints on the shell morphologies.

Alternatively, non-linear evolutionary patterns including convergences and extinctions imply, among others, the intervention of positive natural selection along with adaptive processes (tangled evolution hypothesis). The non-linear morphological diversification in Arcellinida has been recently proposed in family Arcellidae, where similar morphotypes occur in similar habitats, although species are genetically relatively far related (González-Miguéns et al., 2021). Such patterns suggest similar adaptations to similar environmental pressures, in other words, convergent evolution as a product of positive natural selection acting on shell diversification. Shell traits are well characterized, and have been show to correlate well with habitat types and ecosystem conditions, thus suggesting an adaptive value of the shell (Fournier et al., 2015; Marcisz et al., 2020). For instance, compressed shapes, lateral apertures and smaller biovolumes are correlated with drier microhabitats as found in the *Galeripora arenaria* species complex (Koenig et al., 2018). In another species complex, *Hyalosphenia papilio*, shell size and pore numbers are correlated with moisture and climate (Mulot et al., 2017).

Nevertheless, drawing conclusions on the evolutionary history of clades and characters can be perilous if the taxa included are insufficiently sampled. In fact, more than half of all Arcellinida genera lack molecular information; furthermore, the infraorder Hyalospheniformes is overrepresented within barcoded genera (14/27 genera; Lahr et al., 2019). Among all genera, *Difflugia* is particularly species rich and has been largely undersampled. Indeed, there are over 300 species described (Mazei and Warren, 2012). These species were grouped only based on their agglutinated shell and the lack of other remarkable traits. An investigation of the diversity of genus *Difflugia* together with other agglutinating genera should be instrumental in stabilizing the phylogeny of the Arcellinida as a whole (Gomaa et al., 2017) and establishing the background of its evolutionary history.

In this work, we aim at inferring patterns of deep evolution in Arcellinida, and more concretely testing how widespread instances of homoplastic shells are ("tangled evolution hypothesis") *versus* the "morphological stasis hypothesis". In that purpose, we focus on genus *Difflugia* and other agglutinating genera using the nuclear *SSU rRNA* (SSU), backed with the mitochondrial markers *cytochrome oxidase* (COI) and *nicotinamide adenine dinucleotide dehydrogenase* (NADH), and expanding the taxon sampling to genera that have never been molecularly characterized. Also, we investigated how conserved shell morphologies remain stable through evolutionary times within deep lineages (infaorders) by computing the phylo-morphospaces and phylogenetic signals of the different infraorders classified as *Difflugia*. Based on the obtained phylogenetic reconstructions, we tested the importance of partial taxon sampling on ancestral state reconstructions of habitat and shell composition.

2. Materials and methods

2.1. Bibliographic search and taxonomic decisions

We collected the original references for all Arcellinida genera compiled from Lahr et al. (2019) (see taxonomic actions), gathering all the references to facilitate further search into Arcellinida systematics. Also, we added all the genera names not included as well as the newly described ones in the official registry of zoological nomenclature public database "zoobank" (https://zoobank.org/), providing the names to avoid confusions and errors in further nomenclature acts.

Taxonomic identification of the cells collected were carried comparing with the original drawings, the atlas of testate amoebae in Bulgaria (Todorov and Bankov, 2019), the Sphaerothecina revision of González-Miguéns et al., (2021), Eugène Penard's collection (accessible online at https://commons.wikimedia.org/wiki/Commons:P%C3%A9nard_project/taxon_without_categories, accessed 5 September 2021).

Taxonomic and systematic decisions taken in this study were based on congruent phylogenetic signals between nuclear and mitochondrial molecular markers. We took nomenclature acts only when the three molecular markers supported the monophyly of the clades; here, we chose not to focus on species level. The nomenclatural acts were taken based on the International Code of Zoological Nomenclature (ICZN, 1999) rules and recommendations, as they apply to Arcellinida (Lahr et al., 2012).

2.2. Sampling and specimen preparation

Sampled substrates included soil, dry mosses, *Sphagnum* and freshwater sediments. We aimed at recovering the most common Arcellinida genera still lacking molecular data (Table 1). Testate amoebae were concentrated by filtering the samples, transferring living cells to a petri dish to be observed under inverted light microscopy. We used a Leica DMI8 inverted microscope with up to 200 × magnification DIC for observing specimens. We isolated only active cells showing pseudopodia mobility. Organisms were documented with a Leica MC170 HD camera using the software Leica application suite v.4.12.0 (Fig. S1 and S2). We used the software ImageJ v.1.52 (Schneider et al., 2012) to take measurements from the shells directly from the images. These active cells were isolated individually and washed several times with sterile water to remove as much as possible other microorganisms associated with the shells. We deposited then the specimens into Eppendorf tubes, in order to proceed to single cell DNA extraction and amplification, following different procedures: 1) 100 μL of guanidine thiocyanate buffer (Chomczynski and Sacchi, 1987) for total DNA extraction (to amplify several genes from the same cell), 2) 10 μL sterile water, for direct DNA amplification without extraction (to amplify only a single gene from single cell), and 3) 2.3 μL cell lysis mix, following the procedure of Lahr et al. (2019) (for RNA extraction from an active specimen of *Difflugia* cf. *acuminata*). Scanning electron microscope images were taken from Todorov & Bankov (2019). Sample preparation followed the procedure described in González-Miguéns et al. (2021). These experimental procedures are described in detail in the next paragraph.

2.3. DNA extraction, amplification, and sequencing

Total DNA from of isolated cells conserved in guanidine thiocyanate buffer (1) was extracted following the procedure described in (Duckert et al., 2018). This protocol includes an isopropanol precipitation for 12 h at 4 °C, followed by de-salting steps using 70% and 96% ethanol solutions. Pelleted DNA was resuspended into 20 μL of sterile water and stored at 4 °C prior to downstream applications. Amplifications by polymerase chain reaction (PCR) were performed in the following mix: 6 μL of distilled water, 12 μL MyTaq Red DNA polymerase Mix (BioLine), 1 μL of each primer (10 μmol) and 3 μL of DNA template, resulting in a final reaction volume of 23 μL. We then aimed at amplifying three marker genes per DNA extraction whenever possible (Table S1): (1) *cytochrome oxydase COI* (COI) using the universal primer pair LCO 1490 (5′ GGTCAACAAATCATAAAGATATTGG 3′) and HCO 2198 (5′ TAAACTTCAGGGTGACCAAAAAATCA 3′) (Folmer et al., 1994), using the following PCR cycling profile: initial denaturation at 96 °C for 5 min, then 40 cycles at 94 °C for 15 s, 40 °C for 15 s and 72 °C for 90 s, finished with a final extension step at 72 °C for 10 min; (2) *nicotinamide adenine dinucleotide dehydrogenase* (NADH) using the primers NAD9 386F (5′ TGGTTAGAACGAGAAGTTTGGGATATGT 3′) and NAD7 67R (5′ GTGCGCAGCAGGRTGTTGWGGWCC 3′) developed by (Blandenier

Table 1
Sequenced organism in this study, with information of the locality and habitat.

Species	locality	coordinates	Habitat	GenBank COI	GenBank NADH	GenBank SSU
Suborder **Glutinoconcha** Infraorder **Sphaerothecina** Family **Arcellidae**						
Arcella conica	**Bulgaria**: Sofia, Sofia Southern Park	42°39′N 23°18′E	**Freshwater**: submerged vegetation	–	OL549119	OL677467
Family **Netzeliidae**						
Netzelia lithophila	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549143	–	–
Infraorder **Cylindrothecina** Family **Cylindrifflugidae**						
Cylindrifflugia acuminata	**Spain**: Madrid, Parla	40°14′ N, 3°46′ W	**Freshwater**: submerged vegetation	OL549144	–	–
Cylindrifflugia acuminata	**Spain**: Madrid, Parla	40°14′ N, 3°46′ W	**Freshwater**: submerged vegetation	OL549145	–	–
Cylindrifflugia acuminata	**Spain**: Madrid, Parla	40°14′ N, 3°46′ W	**Freshwater**: submerged vegetation	OL549146	–	–
Cylindrifflugia acuminata **(mRNA)**	**Spain**: Madrid, Parla	40°14′ N, 3°46′ W	**Freshwater**: submerged vegetation	OL549124	–	OL677466
Cylindrifflugia acuminata	**Bulgaria:** Rhodopes Mountains	41°44′ N, 24°08′ E	**Freshwater**: *Sphagnum subsecundum*	OL549149	–	–
Cylindrifflugia elegans	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549138	–	–
Cylindrifflugia elegans	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549137	–	–
Cylindrifflugia elegans	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549136	–	–
Cylindrifflugia lanceolata	**Bulgaria:** Rhodopes Mountains	41°44′ N, 24°08′ E	**Freshwater**: *Sphagnum*	OL549150	–	–
Cylindrifflugia lanceolata	**Bulgaria**: Rhodopes Mountains	41°44′ N, 24°08′ E	**Freshwater**: *Sphagnum* teres	–	–	OL677469
Cylindrifflugia lanceolata	**Bulgaria**: Rhodopes Mountains	41°44′ N, 24°08′ E	**Freshwater**: *Sphagnum* teres	–	–	OL677470
Cylindrifflugia lanceolata	**Bulgaria**: Rhodopes Mountains	41°44′ N, 24°08′ E	**Freshwater**: *Sphagnum* teres	–	–	OL677471
Infraorder **Excentrostoma** Family **Centropyxidae**						
Awerintzewia sp.	**Spain**: Pontevedra, Catoira	42°41′ N, 8°43′ W	**Terrestrial**: wet mosses near the sea	OL549135	–	–
Bullinularia gracilis	**Bulgaria**: Stara Planina Mountain, Ljulyaka hut	42°53′ N, 23°10′ E	**Terrestrial**: Rock mosses	OL549122	–	–
Bullinularia gracilis	**Bulgaria**: Stara Planina Mountain, Ljulyaka hut	42°53′ N, 23°10′ E	**Terrestrial**: Rock mosses	OL549123	–	–
Centropyxis aculeata	**Spain**: Madrid, Aldea del Fresno	40°19′N 4°12′W	**Freshwater**: submerged vegetation	OL549142	–	–
Centropyxis aerophila	**Spain**: Madrid, San Lorenzo de El Escorial	40°34′N 4°09′W	**Terrestrial**: granite, wet moss	–	–	OL677465
Golemanskia viscidula	**Bulgaria**: Rhodopes Mountains	41°44′ N, 24°08′ E	**Freshwater**: *Sphagnum teres*	OL549127	OL549114	OL677472
Golemanskia viscidula	**Bulgaria**: Rhodopes Mountains	41°44′ N, 24°08′ E	**Freshwater**: *Sphagnum teres*	OL549128	OL549113	OL677473
Plagiopyxis callida	**Bulgaria**: Rila Mountains	42°14′ N, 23°28′ E	**Terrestrial**: above "Mechit" Hut *Picea abies*	OL549126	–	–
Infraorder **Longithecina** Family **Difflugiidae**						
Difflugia bryophila	**Spain**: Madrid, Navacerrada	40°46′ N, 4°00′ W	**Freshwater**: submerged vegetation in a spring	OL549140	–	–
Difflugia bryophila	**Spain**: Madrid, Navacerrada	40°46′ N, 4°00′ W	**Freshwater**: submerged vegetation in a spring	OL549141	–	–
Difflugia oblonga	**Bulgaria**: Rhodopes Mountains	41°44′ N, 24°08′ E	**Freshwater**: *Sphagnum teres*	OL549147	OL549117	–
Difflugia oblonga	**Bulgaria**: Rhodopes Mountains	41°44′ N, 24°08′ E	**Freshwater**: *Sphagnum teres*	OL549148	OL549118	–
Difflugia nodosa	**Bulgaria**: Sofia, Sofia Southern Park	42°39′N 23°18′E	**Freshwater**: submerged vegetation	–	OL549115	–
Difflugia pyriformis	**Bulgaria**: Sofia, Sofia Southern Park	42°39′N 23°18′E	**Freshwater**: submerged vegetation	OL549151	OL549116	–
Difflugia pyriformis	**Bulgaria**: Sofia, Sofia Southern Park	42°39′N 23°18′E	**Freshwater**: submerged vegetation	–	OL549120	–
Difflugia pyriformis	**Bulgaria**: Sofia, Sofia Southern Park	42°39′N 23°18′E	**Freshwater**: submerged vegetation	–	OL549121	–
Zivkovicia compressa	**Bulgaria**: Rhodopes Mountains	42°39′N 23°18′E	**Freshwater**: submerged vegetation	OL549134	–	–
Zivkovicia sp.	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549139	–	–

(*continued on next page*)

Table 1 (*continued*)

Species	locality	coordinates	Habitat	GenBank COI	GenBank NADH	GenBank SSU
Zivkovicia sp.	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549133	–	–
Zivkovicia sp.	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549132	–	–
Zivkovicia sp.	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549131	–	–
Zivkovicia sp.	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549130	–	–
Zivkovicia sp.	**Spain**: Madrid, Hoyo de Manzanares	40°35′N 4°55′W	**Freshwater**: submerged vegetation	OL549129	–	–
Incertae sedis						
Trigonopyxis arcula	**Bulgaria**: Rila Mountains, Vada hut	42°13′ N, 23°20′ E	**Terrestrial**: Soil from a spruce forest	OL549125	OL549111	–
Trigonopyxis arcula	**Bulgaria**: Rila Mountains, Vada hut	42°13′ N, 23°20′ E	**Terrestrial**: Soil from a spruce forest	–	OL549110	–
Trigonopyxis arcula	**Bulgaria**: Rila Mountains, Vada hut	42°13′ N, 23°20′ E	**Terrestrial**: Soil from a spruce forest	–	OL549112	–

et al., 2017), using the following PCR cycling profile: initial denaturation at 94 °C for 3 min, then 38 cycles at 94 °C for 30 s, 61 °C for 30 s and 72 °C for 60 s, finished with a final extension step at 72 °C for 10 min; and (3) *SSU rRNA* (SSU) using the universal eukaryotic primers EK555F (5′ AGTCTGGTGCCAGCAGCCGC 3′) and EK1498R (5′ CACCTACGGAAACCTTGTTA 3′) with the following PCR cycling profile: initial denaturation at 96 °C for 5 min, then 40 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s, finished with a final extension step at 72 °C for 10 min.

For the cells deposited in 10 µL sterile water (2), direct PCR was performed, adding 8 µL of Phire Green Hot Start II PCR Master Mix (ThermoFisher) and 1 µL of each primer (10 µmol), using only one single cell in each PCR. As the cells were sequenced directly, without any prior DNA extraction, only one gene was sequenced in this case. We used the universal primer described by (Folmer et al., 1994) to amplify the COI, with the following PCR cycling profile: initial denaturation at 98 °C for 5 min, then 40 cycles at 98 °C for 5 s, 40 °C for 5 s and 72 °C for 30 s, finished with a final extension step at 72 °C for 10 min.

All the PCR products were checked by electrophoresis on a 1% agarose gel using 3 µL of the reaction to confirm fragment size and check for contaminations. Electrophoresis bands with an expected size were cut from the gel and stored at 4 °C. These bands were sequenced with Sanger dideoxy-technology, in both directions, by the company Macrogen Inc. (Macrogen Europe, Madrid, Spain). The resulting sequences were checked and assembling both directions with the software Geneious Prime (v.2019.0.4). To ensure that our sequences were the closest related to Arcellinida we performed blastn analysis (Altschul et al., 1990) against the GenBank and our databases.

2.4. Single cell RNA extraction and sequencing

Mitochondrial mRNA editing has been identified in several eukaryotes (Bondarenko et al., 2019; Yang et al., 2017), usually consisting in indels that change the reading frame, and suspected in Arcellinida (Kosakyan et al., 2012). This editing might, potentially, influence phylogenetic analyses. To test this, we compared the mRNA and DNA sequences for the COI gene (see "Data preparation and phylogenetic analyses"). For RNA extraction and amplification, we followed the protocol described in Lahr et al. (2019), modified from Picelli et al. (2014). First, we placed a single *Difflugia* cf. *acuminata* cell in a cell lysis mix which contained Rnase-Inhibitors, 0.2%-TritonX-100 and Diethyl pyrocarbonate (DEPC) H_2O. Then, we performed a thermal shock by alternating cycles with Liquid nitrogen and warm water. We performed a reverse transcriptase PCR using SuperScript™ IV Reverse Transcriptase (ThermoFisher) and LNA using the enhanced oligonucleotide TSO (5'AAGCAGTGGTATCAACGCAGAGTACATrGrG + G 3') (Picelli et al. 2014) with the following PCR profile: 1 cycle 50 °C during 90 min (switching between the reverse transcriptase and template), then 10 cycles at 55 °C for 2 min (unfolding of RNA secondary structures) and 50 °C for 2 min (completion and continuation of switching between reverse transcriptase and template), finished with 1 cycle at 70 °C during 15 min (enzyme inactivation). Finally, we synthesized complementary DNA (cDNA) using KAPA HiFi HotStart ReadyMix (Roche) and IS PCR primers (10 µM) with the following PCR profile: 1 cycle 98 °C during 3 min, then 20 cycles at 98 °C for 20 s, 67 °C for 15 s and 72 °C for 6 min, finished with 1 cycle at 72 °C for 10 min. PCR products were stored at −80 °C. We used a Qubit 3 Fluorometer with dsDNA high sensitivity (HS) assay kits (ThermoFisher) for cDNA quantification.

We sequenced the obtained cDNA with the protocol developed by Oxford Nanopore Technologies (ONT) and prepared the library following the protocol Direct RNA sequencing (SQK-RNA002). Resulting Nanopore libraries were sequenced using a MinION Mk1b with a R9.4.1 flow cell left running for 19 h. Data were generated using the software MinKNOW UI 4.2.8 and basecalled with Guppy 4.5.2. The output was checked in real time with MinKNOW UI 4.2.8 and plotted using the R package NanoR (Bolognini et al., 2019) (Fig. S3).

2.5. Data preparation and phylogenetic analyses

COI, NADH and SSU sequences were aligned using the MAFFT (Katoh et al., 2002) auto algorithm as implemented in Geneious Prime. After the removal of primers and unalignable regions, we obtained alignments of, respectively 643 (COI), 263 (NADH) and 1426 (SSU) bp. The number of taxa and detailed information are in Table S1. Tree topologies and node supports were evaluated with Bayesian inferences (BI), with the following procedure:

First, we checked the best substitution model and among-site rate variation using "ModelFinder" (Kalyaanamoorthy et al., 2017), implemented in IQ-TREE ver. 2.0 (Nguyen et al., 2015), under the Bayesian information criterion (BIC), for each gene (COI, NADH and SSU). In all three genes, we employed the among-site rate variations with an estimated proportion of invariable sites and a gamma shaped distribution of variable sites (I + G). Then, to perform the Bayesian inference (BI) analyses, we used MrBayes 3.2.7a (Ronquist et al., 2012), implementing two independent runs, with four chains for each run and 20* 10^6 generations for the Markov chain Monte Carlo (MCMC) setting; trees were sampled every 1000 generations. Substitution models were selected with the reversible-jump MCMC method (Huelsenbeck et al., 2004). Finally, we discard 25% if the sampled trees, and evaluated the convergence of the different runs with TRACER v.1.7.1, with all the effective sample sizes (ESSs) values over 200. The resulting trees for each gene were summarized in a 50% majority rule consensus tree.

For quality control and taxonomic assignation of ONT reads, we used the cloud based What's In My Pot? WIMP via EPI2ME (v 3.2.1), with a quality average of 9.37 (Fig. S4). We first discarded human and bacterial reads. We used two approaches in order to gather all transcriptomic information related to *Difflugia* cf. *acuminata* (1) we aligned our obtained nanopore FASTQ sequences with the database generated Lahr et al. (2019) using the software MinKNOW UI 4.2.8, to find homologous genes that can be used in the phylogenetic analysis, and (2) we follow the pipeline of isONcorrect (Sahlin et al., 2021); first we use pychopper v2 to orient and trim the reads (https://github.com/nanoporetech/pychopper, RRID:SCR_018966). Then we clustered the reads using isONclust (Sahlin and Medvedev, 2020), and finally we corrected the errors using isONcorrect (Sahlin et al., 2021) merging the reads, which were compared with the data from Lahr et al. (2019) with blastx in BLAST+ (Altschul et al., 1997; Camacho et al., 2009). Outputs of both procedures were processed in Geneious Prime (v.2019.0.4). We obtained 192 COI and 258,889 SSU good quality reads, respectively, which were assembled using MinKNOW UI 4.2.8.

To characterize the mitochondrial editing in Arcellinida, we compared COI mitochondrial DNA and cDNA (from mRNA) sequences as obtained with our transcriptome of *Difflugia* cf. *acuminata*. The comparisons were performed in the software Geneious Prime (ver. 019.0.4), aligning the sequences using the MAFFT auto algorithm (Katoh et al., 2002), and translating the sequences into amino acids to compare the reading frames between the sequences. We also performed this comparison between native DNA sequence and cDNA on the SSU gene in order to formally confirm the presence of introns in ribosomal sequences (Lara et al.,2008; Gomaa et al., 2012), based on the published sequence JQ366064. We tested for saturation in all datasets by plotting the raw or uncorrected pairwise genetic distances in an alignment against model-corrected genetic distances, using the R package "ape" (ver. 5.5) (Paradis and Schliep, 2019) as in Philippe et al. (1994) and (https://www.kmeverson.org/blog/simple-dna-saturation-plots-in-r), taking this as an exploratory analysis.

2.6. Morphometric analyses (Hyper-phylomorphospace) and phylogenetic signal

To test whether the morphological diversification is consistent with molecular diversification within *Difflugia* ("mineral agglutinated"), we followed the procedure described in (González-Miguéns et al., 2022). First, we pruned the SSU tree leaving only the species historically classified as *Difflugia* (*Difflugia*, *Netzelia* and *Phryganella*), with the function "drop.tip" (Fig. 6a), using the package Ape ver. 5.5 (Paradis and Schliep, 2019). We retrieved morphological data of each organism from the original articles where SSU sequences were obtained, as well as in Todorov & Bankov (2019). Our recompiled morphological database contains then five continuous traits: (1) shell width, (2) shell length, (3) aperture width on the largest axis (4) shell length/aperture width ratio and (5) shell length/shell width ratio. We obtained data for a total of 309 cells (available in supplementary material).

We performed an exploratory Principal Components Analysis (PCA), using the R package stats and plotting the results with the R package ggplot2 (Wickham, 2016) (Fig. 6b). We used the R package factoextra ver. 1.0.7 (Kassambara and Mundt, 2020) to evaluate the contribution of each morphological variable measured in the analysis (Fig. S5).

To visualize the morphospace of the continuous characters described above, we performed a hypervolume analysis. First, we computed a PCA using only data from Todorov & Bankov (2019), which contain the full set of characters for each species. We excluded from the analysis all species where only one single character was measured. Then, using the first three principal components (PC) obtained in the PCA, we computed hypervolumes using the R packaged hypervolume ver. 2.0.12 (Blonder et al., 2014), with a quantile threshold of 0.05 (Fig. 6c). We then computed phylomorphospaces using the centroids generated in the hypervolume of each specie in the pruned SSU tree (Fig. 6a). For those species not included in the hypervolume, in which only a single character has been measured, we take the morphological data from the original description; in the case of the species *Difflugia acuminata* and *D. lanceolata* which seem to represent species complexes rather than single species, we inferred values directly from the barcoded individuals. Then we reconstruct the ancestral states along the morphospace (Sidlauskas, 2008), determined by the three PC (Table S2), using the function "phylomorphospace" in the R package phytools (Revell, 2012). Finally, using the same phylogenetic and morphological data as for phylomorphospaces (Table S2), we measured the strength of the phylogenetic signal (Blomberg and Garland, 2002) using the package "phylosignal" (Keck et al., 2016). First, we created three databases: (1) morphological data of the PC1, which explain the majority of the total variation of the morphological data, (2) data generated under Brownian motion model with the function "rTraitCont" of the package Ape ver. 5.2 (Paradis and Schliep, 2019) and (3) random data. Then, we compute Local Indicators of Phylogenetic Association (LIPA), a local Moran's I (Anselin, 1995), for each species to detect hotspots of autocorrelation in each PC of the "phylomorphospace".

2.7. Ancestral state reconstruction

To test the effect of taxon sampling on inferences on Arcellinda evolutionary patterns, we performed ancestral state reconstruction (ASR) with two phylogenetic trees: (1) including all the Arcellinida sequences (Fig. 7, nodes in green squares) and (2) removing the species considered as *incertae sedis* (*Argynnia dentistoma* and *Physochila griseola*) from the last dataset (Fig. 7, nodes in red squares). We based our ancestral state reconstruction on the SSU tree, as this marker is the one that includes most molecular data on Arcellinida. Another reason for choosing this marker is that the latest SSU tree (Soler-Zamora et al., 2021) is congruent with the latest phylotranscriptomic tree (Lahr et al., 2019).

First, we removed outgroups with the function "drop.tip" in R package ape. Then, we constructed two databases, for the two phylogenetic trees, to infer 1) ancestral habitats, considering only aquatic and terrestrial environments, considering as aquatic ("freshwater") habitats with permanent high humidity (such as *Sphagnum*) or submerged and terrestrial ("soil") habitats with varying humidity (dry mosses e.g. *Grimmia*, forest litter) and 2) ancestral shell composition, considering "organic" organisms that form a proteinaceous shell (such as *Galeripora*), "self-secreted" organism that construct an agglutinated shell with self-secreted mineral elements (like *Lesquereusia*), "prey agglutinated" (kleptosquamic) organisms that take the inorganic plates from the preys (as *Nebela*) and "mineral agglutinated", organism that construct the shell agglutinating mineral particles acquired from the environment (as *Difflugia*). The ancestral habitat and shell composition assignation may be subjective, as the same species can inhabit several environments or construct shells with different components in function to the materials present in the environment. Therefore, we selected the characters based on habitat and shell composition based on the original article from which sequences derive. To perform the ASR we used the software "MrBayes Ancestral States with R" (MBASR) (Heritage, 2021), using 50,000 generations (500 samples), that used the native continuous-time Markov modelling implemented in MrBayes ver. 3.2 (Ronquist et al., 2012) to provide the likelihood for the discrete character states described above at ancestral tree nodes.

3. Results

3.1. Phylogenetic results

We used 63 sequences to build the *mitochondrial cytochrome oxidase subunit I* (COI) tree, which includes 4 sequences of Tubulinea as outgroups (Fig. 2; Table 1 and S1). Our phylogenetic analyses recovered the monophyly of Arcellinida with a Bayesian posterior probability (PP) of

1, and the monophyly of the different infraorders characterized in Lahr et al. (2019): Sphaerothecina (blue color in Fig. 2, PP = 0.99), Excentrostoma (yellow color in Fig. 2, PP = 0.89), and Hyalospheniformes (green color in Fig. 2, PP = 1). Like in previous phylogenies of the Arcellinida, Longithecina appears paraphyletic (Gomaa et al., 2017; Macumber et al., 2020); it contains the two directly unrelated groups Longithecina I (red color in Fig. 2, PP = 0.99) and Longithecina II (pink color in Fig. 2, PP = 0.60). Genus *Trigonopyxis* did not appear unambiguously related to any surveyed infraorder and still needs to be considered *incertae sedis*. The relationships between these infraorders differ with respect to the phylotransciptomic tree of Lahr et al. (2019), which can be explained in part by the saturation level reached by COI when resolving the deepest nodes (Fig. 5). This is reflected by the low PP values that support basal nodes as well as some infra-orders (Longithecina II, Excentrostoma). Higher PP values are nevertheless obtained within infraorders, and morphologically close-related species are well differentiated. *Plagiopyxis callida* and *Bullinularia gracilis* do not form a monophyletic clade, even though they have a similar aperture hidden behind a "visor" (cryptostomy) which placed them together formally into the family Plagiopyxidae. *Zivkovicia compressa* and an unidentified member from the same genus branch together within Longithecina I; this genus had not been formerly placed within a known infraorder. The species *Difflugia viscidula* branches also within Excentrostoma, an unexpected result given the terminal position of the aperture. Likewise, an unidentified species from genus *Awerintzewia* branches also robustly within Excentrostoma; genus *Awerintzewia* was formerly considered as *incertae sedis* (Lahr et al., 2019).

We used 29 sequences for the mitochondrial *nicotinamide adenine dinucleotide dehydrogenase* (NADH), including 27 Arcellinida and 2 Centramoebida species as outgroups (Fig. 3; Table 1 and S1). Our phylogenetic analyses recovered the monophyly of Arcellinida with a Bayesian posterior probability (PP) of 0.99. We also obtained the monophyly of Sphaerothecina (blue colour in Fig. 3, PP = 0.98); here again, Longithecina I (red colour in Fig. 3, PP = 0.99) and Longithecina II (pink colour in Fig. 3, PP = 0.1) appear as separated groups, giving further support to the paraphyly of Longithecina. Here also, there is no good support for any close relationship between *Trigonopyxis* and other infra-orders and remain as *incertae sedis*; *Difflugia viscidula* is not related

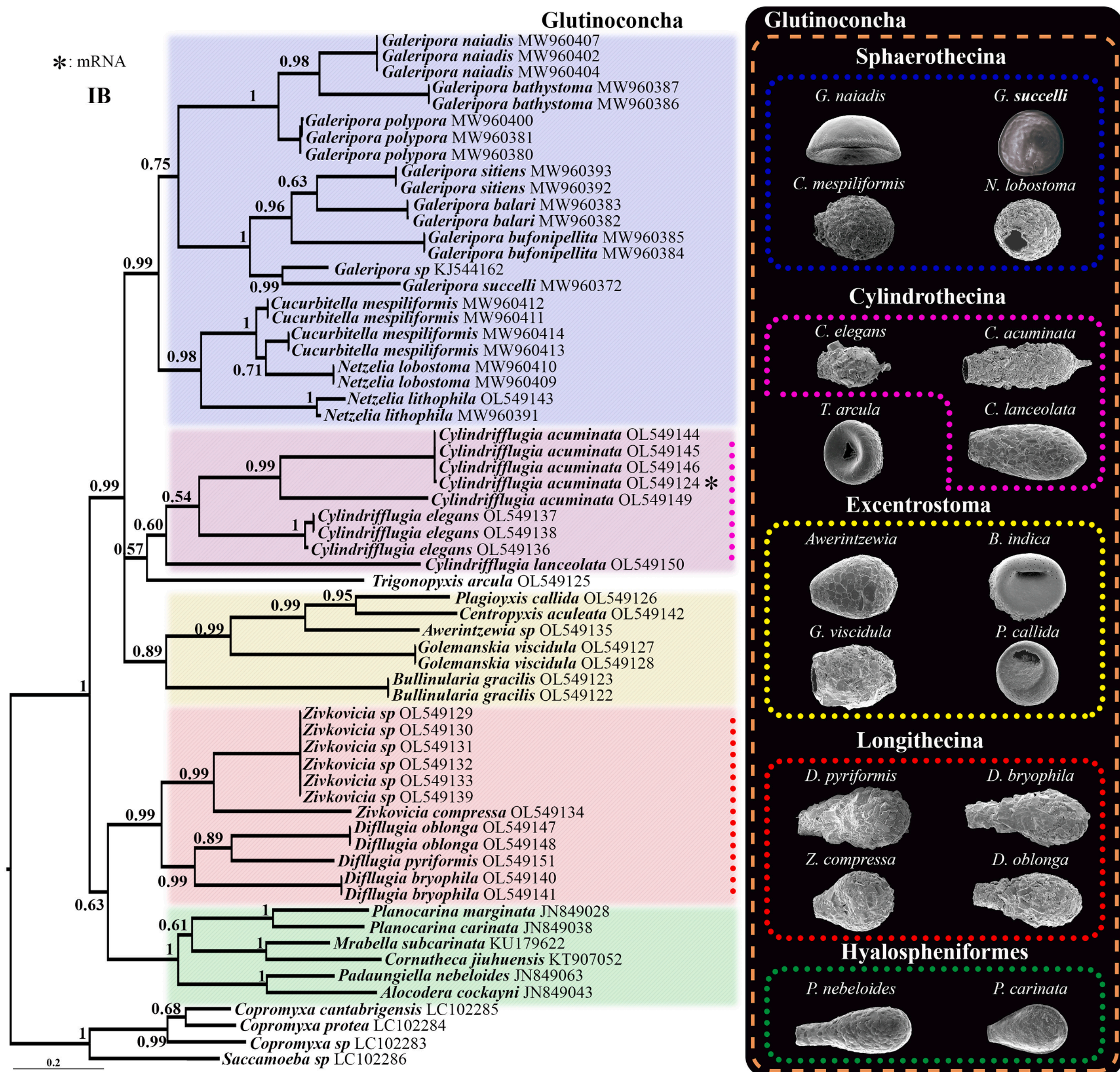

Fig. 2. Bayesian phylogenetic tree based on COI sequences of the suborder Glutinoconcha (Arcellinida). Posterior probability values are represented at each node. The colours represent the different infraorders in Glutinoconcha. The images of the right are scanning electron microscopy photographs showing typical shell morphologies for each infraorder.

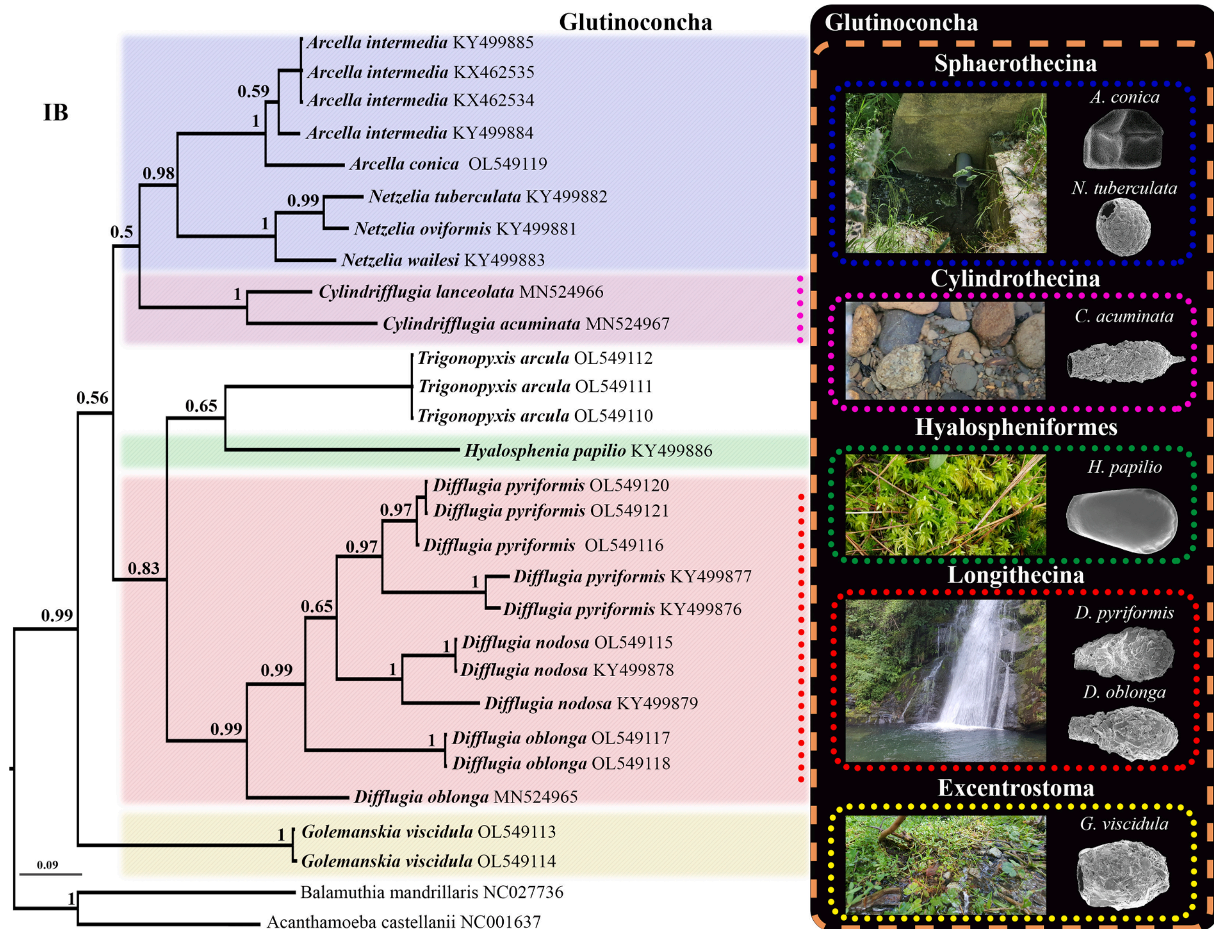

Fig. 3. Bayesian phylogenetic tree based on NADH sequences of the suborder Glutinoconcha (Arcellinida). The colours represent the different infraorders in Glutinoconcha. The images of the right are scanning electron microscopy photographs showing typical shell morphologies for each infraorder and an illustration of a typical habitat.

neither to Longithecina I or II, nor to Sphaerothecina. Like in COI, NADH seems to reach saturation in deep nodes, which results in a lack of support at the base of the tree.

The *SSU rRNA* (SSU) tree was performed with a total of 59 sequences, including 4 outgroup sequences (Tubulinea) and 55 Arcellinida (Fig. 4; Table 1 and S1). Our phylogenetic analyses recovered the monophyly of Arcellinida (PP = 1). In average, infraorders were better supported than with the two former molecular markers; Sphaerothecina (blue colour in Fig. 4), Hyalospheniformes (green colour in Fig. 4) and Volnustoma (Turquoise colour in Fig. 4) obtained total support (PP = 1); Excentrostoma (yellow colour in Fig. 4) received a low support (PP = 0.72). Deeper nodes were better supported than with mitochondrial markers. For instance, the relationship between Sphaerothecina and Longithecina I (PP = 1), or the suborder supports like for Glutinoconcha (PP = 0.72), Organoconcha (PP = 0.92) and Phryganellina (PP = 1), were recovered with similar support than in the transcriptomic analyses (Lahr et al., 2019). Here again, Longithecina is divided between Longithecina I (red colour in Fig. 4, PP = 0.98), Longithecina II (pink colour in Fig. 4, PP = 1) and its paraphyly is supported. Single sequences of *Argynnia* and *Physochila* are not clearly related to any infraorder and remain as *incertae sedis*. Here again, *Difflugia viscidula* is robustly rooted within Excentrostoma, thus confirming its position as inferred with mitochondrial data. The species *Difflugia lanceolata* and *D. acuminata* appear paraphyletic, which reveals the existence of a hidden diversity also within these groups.

The comparison between the native and cDNA COI sequences of *Difflugia acuminata* (Longithecina II) shows a characteristic pattern of mitochondrial editing (Fig. 5a). Indeed, it presents deletions or insertions of single nucleotides which influence the reading frame in the messenger RNA, reminding patterns observed in deep-branching Heterolobosea (Discoba) (Yang et al., 2017). Thus, Arcellinida mitochondrial DNA sequences cannot be translated into proteins as such without knowing the correct reading frame. Within COI, these deletions only affect a few nucleotides (three in Fig. 5a), which still allow comparisons between native mitochondrial sequences; it is still a good marker for Arcellinida systematics. Likewise, regions of the nuclear gene SSU are found in native sequences and not in the cDNA; we conclude that these regions are introns (Fig. 5b). These regions are best discarded from phylogenetic analyses, as the homology between sequences needs stills to be characterized.

The saturation plots reach a plateau first for NADH, then for COI (Fig. 5c); the SSU, in turn, does not stabilize totally. This suggests that the phylogenetic information is less saturated than for the mitochondrial markers, even though all Arcellinida suborders are covered with SSU.

3.2. Morphometrics and Hyper-PhyloSignalMorphospace

We used 17 species barcoded with SSU (Fig. 7a) to create the morphogroups which include the full set of measurements for each organism. These organisms originally classified as *Difflugia* are currently divided in 5 groups: suborder Glutinoconcha, including Sphaerothecina, Excentrostoma, Longithecina I and II (blue, yellow, red and pink colour, respectively in Fig. 6) and suborder Phryganellina (black colour in Fig. 6).

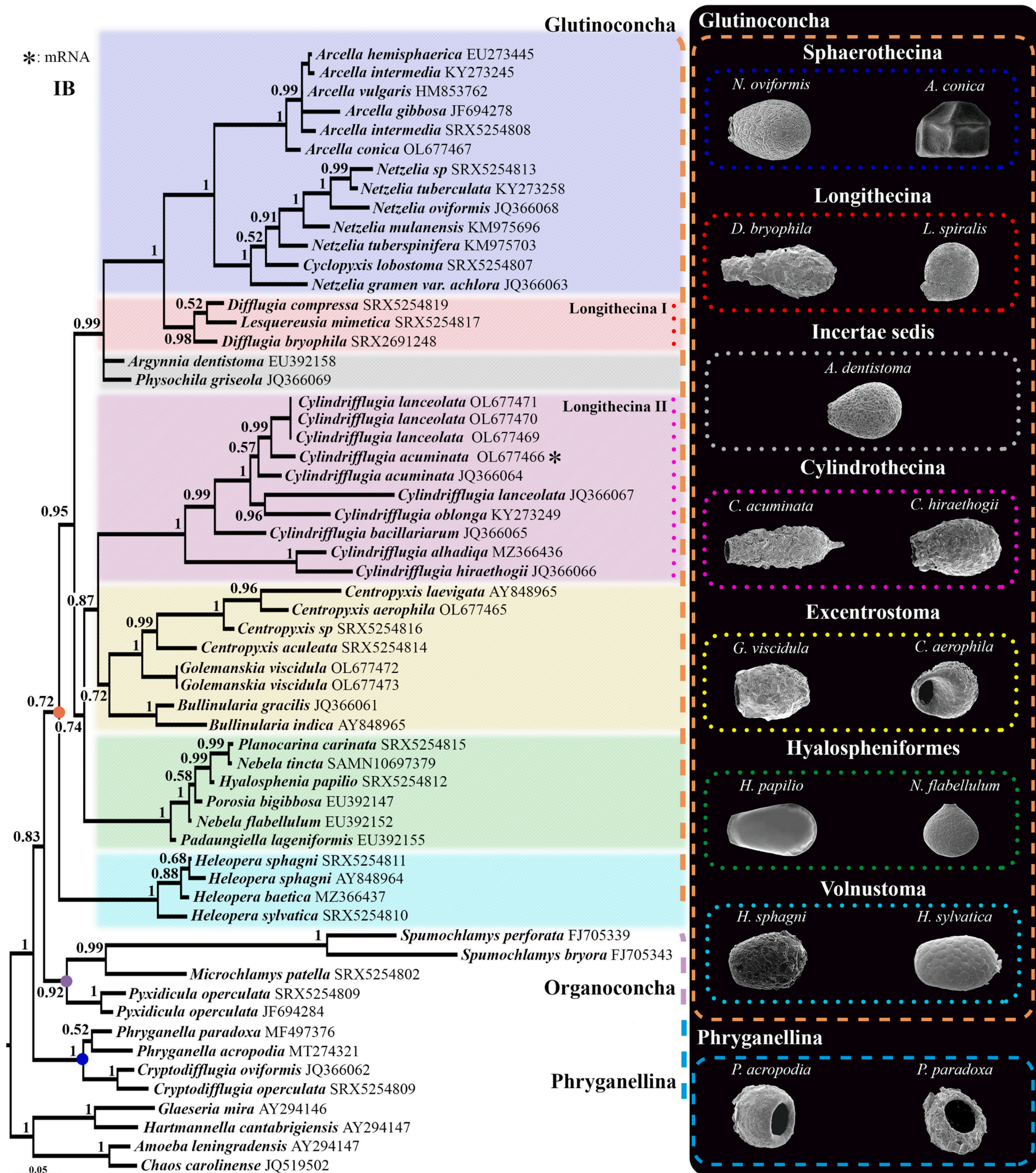

Fig. 4. Bayesian phylogenetic tree based on SSU sequences of the order Arcellinida. Posterior probability values are represented at each node. The colours represent the different suborders and infraorders in Glutinoconcha. The circles in the nodes represents the most recent common ancestor for each suborder. The images of the right are scanning electron microscopy photographs showing typical shell morphologies for each infraorder of Glutinoconcha and the suborder Phryganellina.

The first two axes in the Principal Component Analysis (PCA) explain 98.5% (PC1 = 87.4%; PC2 = 11.1%) of the morphological variables (Fig. 6b). The variables with the highest contributions are length and the width of the shell (Fig. S5). The PCA and the "Hyper-phylomorphospace" do not show a clear morphological pattern in the different higher taxonomic groups. For instance, Longithecina I and Longithecina II show some degree of overlap, sharing thus the same morphospace (Fig. 6b and c) (Table S3). Sphaerothecina has its own morphospace not shared with other taxonomic groups. Some morphologically defined species (*Difflugia acuminata*, *D. lanceolata*) do not appear monophyletic in the phylogeny and cover large morphospaces (Fig. 6), which suggests the existence of complexes of (pseudo)cryptic species.

The principal component 1 does not show phylogenetic signal K = 0.4537697 (P-value = 0.105 Table S5), K = 0.5329809 under Brownian Motion (P-value = 0.062). The clade of *P. hemisphaerica* and *P. acropodia* shows autocorrelation with positive values in local Moran's I; species like *D. viscidula* and *D. alhadiqa* shows the opposite pattern with negative values.

3.3. Ancestral state reconstruction (ASR)

The ancestral state reconstructions of the habitat reveal the

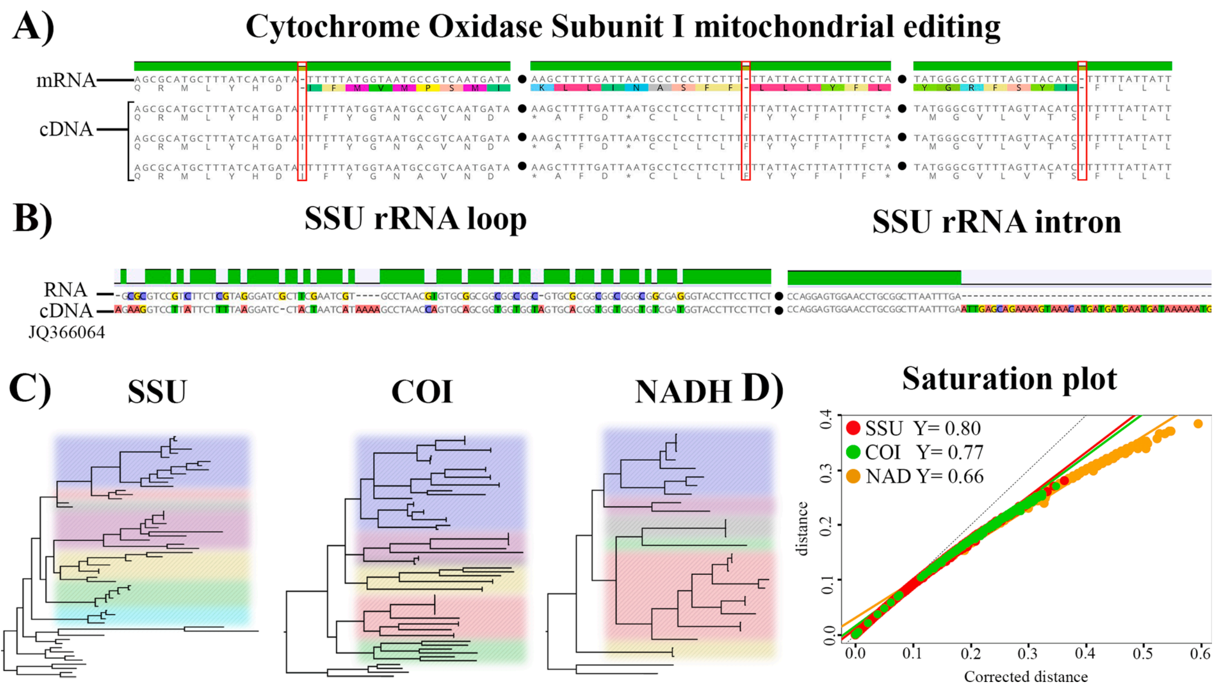

Fig. 5. (A) comparison between cDNA and mRNA sequences of COI gene in *Cylindrifflugia* cf. *acuminata*, showing mitochondrial editing. The nucleotides in a red square are present in the native mitochondrial sequence but are removed by editing, changing the reading frame. (B) comparison between cDNA and mRNA of SSU gene in close related *C. acuminata* showing the presence of introns and secondary structures. (C) comparison of Bayesian trees of COI, NADH and SSU genes respectively. Colours represent the different infraorders of Glutinoconcha. (D) Saturation plot between COI, NADH and SSU genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

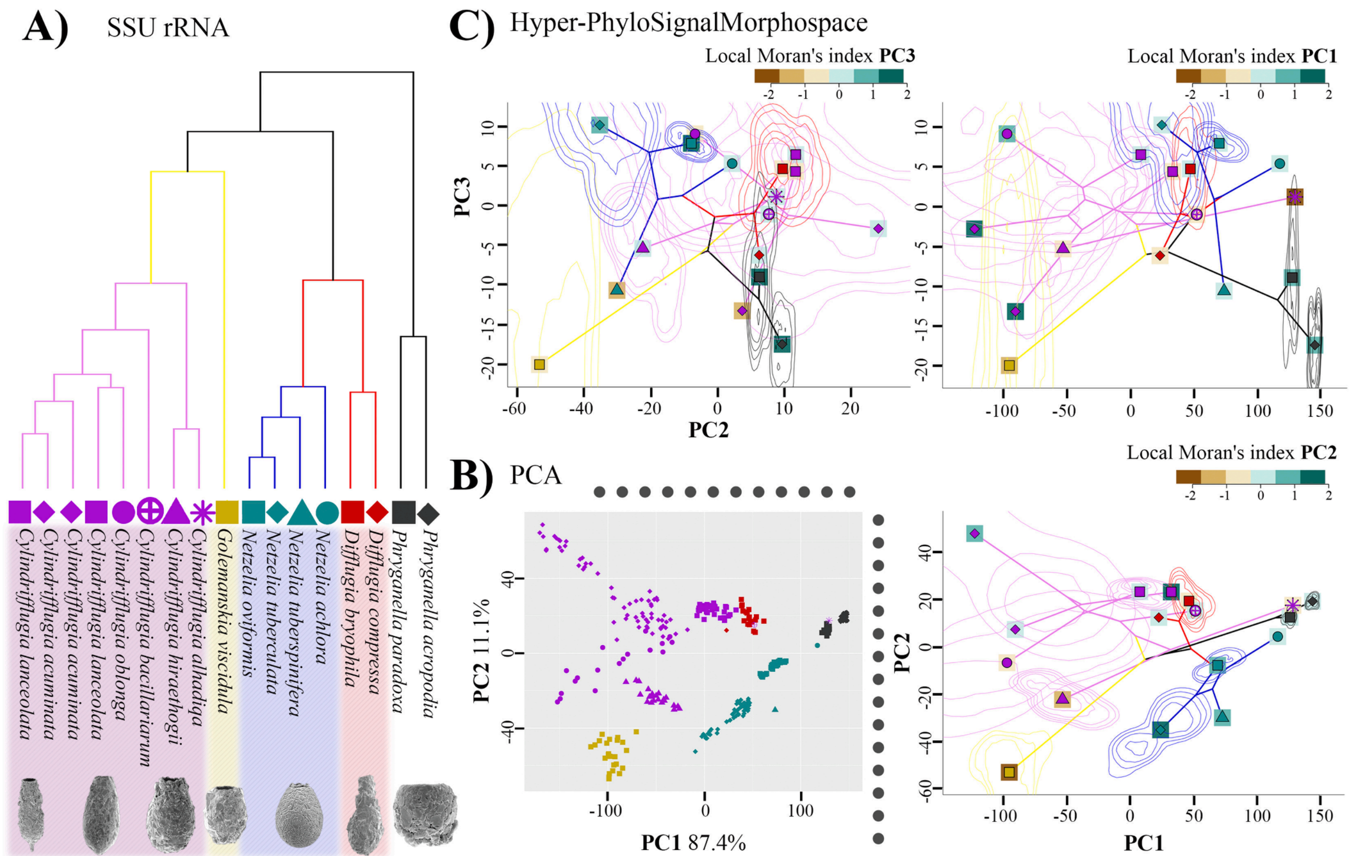

Fig. 6. (A) Bayesian phylogenetic tree based on SSU gene, pruned leaving one tip per species considered *Difflugia*; branch colours represent the infraorder and the symbols the different morphospecies. (B) Principal component analysis (PCA). (C) "Hyper-PhylosingalMorphospace" of the phylogeny (A); lines represent the Kernel density of the random points generated in the hypervolume and colour squares represents the Local Morn's Index for each PC.

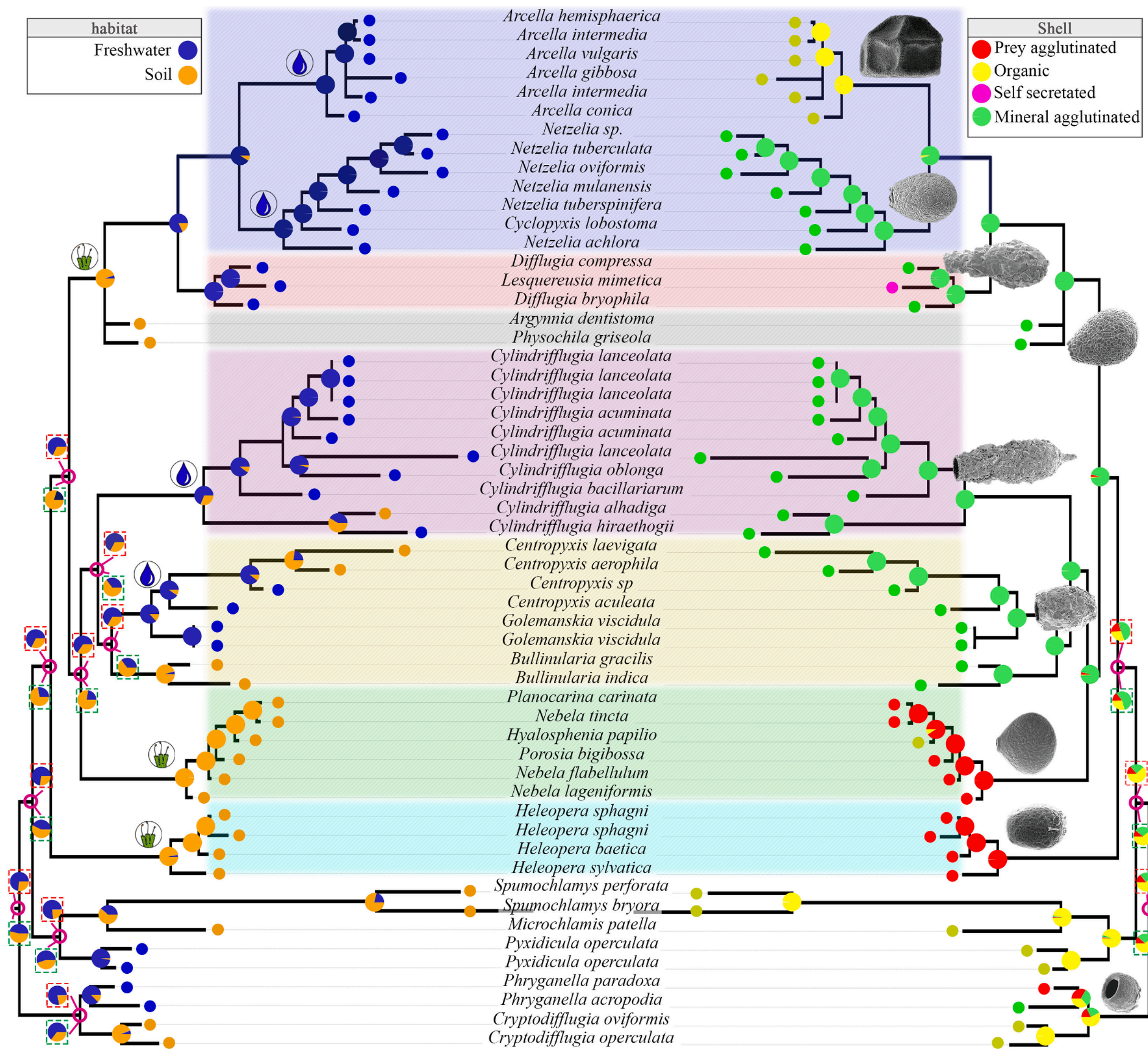

Fig. 7. Ancestral state reconstruction of habitat and shell construction over the SSU tree. The nodes inside red squares represent probabilities without *Argynnia dentistoma* and *Physochila griseola* (*incertae sedis*); and the green squares represent the probability considering these group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

considerable influence of taxon sampling on the reconstructions. The incorporation of the *incertae sedis* taxa *Argynnia* and *Physochila* showed the character state soil as the most likely habitat for the common ancestor of Arcellinida (Fig. 7). In turn, if these species are not considered, then the most probable habitat becomes “freshwater”. The origin of the whole order Arcellinida is, therefore, still unclear. Nevertheless, some preliminary patterns can be guessed from the best sampled families and infraorders; the ancestors of the infraorders Sphaerothecina, Longithecina I and II (blue, red and pink, respectively in Fig. 7) lived most probably in aquatic environments; the ancestors of the infraorders Hyalospheniformes and Volnustoma (green and turquoise colour respectively in Fig. 7) were probably terrestrial. Given the sensitivity of the results of ASR to taxon addition/removal, and the fact that over 1300 species have been described overall in Arcellinida, these results must be taken nevertheless with caution.

The shell construction ASR shows constant results with or without the *incertae sedis* genera *Argynnia* and *Physochila*, supporting the idea that the last common ancestor of all Arcellinida had an organic shell (Fig. 7). The ancestor of suborder Glutinoconcha seems to have had a mineral agglutinated shell. The infraorders in Glutinoconcha shows clear patterns: the ancestors of, respectively, Sphaerothecina, Longithecina I, Longithecina II and Excentrostoma had a mineral agglutinated shell. Hyalospheniformes and Volnustoma ancestors had most probably an ancestor with a xenosomic agglutinated shell. The Arcellidae organic shell construction seems therefore to be a derived character which appeared within a group with mineral agglutinated shells. Despite the apparent consistency of the results, more data are still necessary to obtain a clear and complete picture of the evolutionary patterns in Arcellinida.

A visible pattern that emerges from all analyses is a correlation between habitat and shell type. The mineral agglutinated shells appear related to aquatic habitats, with independent transitions to the soil environment (Fig. 7). Xenosomic shells are correlated principally with terrestrial environment. Organic shells seem to be less specialized and can be found in aquatic and terrestrial environments.

4. Discussion

4.1. More genes and more species to de(re)construct the systematics of Arcellinida

The search for neutral, homologous characters is a prerequisite for building phylogenies on which systematics can be based. Molecular markers used for reconstructing Arcellinida phylogenies (SSU rRNA, COI and NADH genes) have been applied successfully in other micro and "macro-" eukaryotes (Heger et al., 2011; Rach et al., 2008; Sánchez-Vialas et al., 2020). Especially in protists, for which the number of morphological traits is limited, molecular data are the objective background against which evolutionary hypotheses can be tested. Accordingly, molecular studies have challenged Arcellinida traditional systematics, using the SSU rRNA as marker (Gomaa et al., 2015; Kudryavtsev et al., 2009; Nikolaev et al., 2005), NADH (Blandenier et al., 2017; Macumber et al., 2020) or COI (González-Miguéns et al., 2021; Kosakyan et al., 2016b, 2013). However, our analyses also show that each of these markers saturates at different taxonomic depths, first NADH, then COI and finally SSU (Fig. 5). The consequence is that the deepest nodes appear unstable in single gene phylogenies (see references above). A phylotranscriptomic approach, however, has been carried out, providing internal nodes with a robustness never reached before (Lahr et al., 2019). The groupings that emerged from this analysis allowed the erection of four new infraorders within suborder Glutinoconcha: Hyalospheniformes, Longithecina, Excentrostoma and Volnustoma. However, this approach for single cell organisms is still challenging, definitively costly, and computation intensive when performed at large scales, which prevents, for the moment, the expansion of these analyses to a wide number of taxa. Low taxon coverage can bias the systematics and its posterior interpretations (Heath et al., 2008; Sanderson et al., 2010). Therefore, single cell barcoding remains very useful as a quick way to retrieve data from a larger number of taxa to resolve the phylogeny and systematics of Arcellinida, to characterize the biodiversity of the different infraorders.

Amongst these molecules, SSU rRNA gene has been, until now, the most frequently used single marker to reconstruct Arcellinida phylogenies, as well as for barcoding and metabarcoding (Ruggiero et al., 2020). Obtaining SSU sequences from Arcellinida is often a difficult task, as other eukaryotes (symbionts, undigested preys) are often preferably amplified in the PCR process (Gomaa et al., 2012). The presence of introns and secondary structures, such as the evidenced in this work in *Cylindrifflugia acuminata sensu lato* (Fig. 5) difficult amplifications by expanding fragments size, as well as alignment. Mitochondrial markers, besides being easier to amplify, are well suited to build intra-infraorder phylogenies and to differentiate organisms at the species level (Kosakyan et al., 2013; Blandenier et al., 2017; González-Miguéns et al., 2021). As shown in this study, mRNA COI undergoes mitochondrial editing, which consists in removing single nucleotides (usually a thymine) with respect the native DNA sequence. This thus changes the reading frame as documented in the far-related group Heterolobosea (Yang et al., 2017). However, when performing the phylogenies directly on nucleotide sequences, only a few individual nucleotides are concerned (three in all COI alignment Fig. 5) which does not affect tree topology, although negatively influences node support values.

In this work, we reconstructed Arcellinida phylogeny by increasing the number of taxa and the number of markers, an approach that has not been followed before in this group. Our phylogenetic reconstructions based on two mitochondrial and one nuclear marker (Figs. 2, 3 and 4) are congruent among themselves, and with previous works on Arcellinida systematics, which validates our approach. The SSU tree (Fig. 4) recovers the same topology as the phylogenomic tree in Lahr et al., 2019; all infraorders receive total support (PP = 1) except Excentrostoma, in which the position of genus *Bullinularia* remains weakly supported (but confirmed with COI, Fig. 2). It is likely that further sampling of related genera (*Hoogenraadia*, *Planhoogenraadia*) will increase the support for this node. Likewise, suborder Organoconcha receives a moderate support with SSU (PP = 0.92), resulting from the highly diverging branch lengths encountered; further organism sampling will likely reduce long branch attraction and increase node support, even if only a few more species are added (Wiens, 2005).

Our ancestral state reconstructions illustrate the important influence of taxon sampling on the inferences that can be drawn from our analyses (Fig. 7). Given our datasets, patterns emerge at the infra-ordinal level; for instance, the ancestral Hyalospheniformes were probably terrestrial, and built their tests out of their preys' mineral scales. Longithecina I, Longithecina II and Netzeliidae (ex-*Difflugia*) had all aquatic ancestors and built their shells by agglutinating particles found in the environment. However, our sampling remains biased as many taxa still need to be barcoded and may modify our conclusions. Some taxa, despite having been barcoded, have still an uncertain position in the tree, like *Argynnia* and *Physochila* (Fig. 5); adding more species can change their position, and thus affect conclusions on general evolutionary patterns. Many other genera are still awaiting to be barcoded, and their position can change topologies as well. In addition, the existence of extinct groups has not been considered.

In Arcellinida, there is still many genera that have not been genetically characterized and remain considered *incertae sedis* as their systematic position remains unresolved (Adl et al., 2019; Lahr et al., 2019). The addition of these taxa to SSU trees will probably help stabilizing weakly supported nodes between infraorders. Moreover, expanding taxon sampling can possibly add new major subdivisions within Arcellinida. There is evidence from previous works that genus *Difflugia* is paraphyletic (Macumber et al., 2020) even after all *Netzelia* species have been included within Sphaerothecina (Kosakyan et al., 2016a). This pattern is recovered in our SSU tree as well as with the other molecular markers (Figs. 2, 3 and 4). This implies the erection of a new infraorder to designate all members of Longithecina II, which we name Cylindrothecina. To resolve further the paraphyly of *Difflugia*, we erected the new genus *Cylindrifflugia* to group all members of *Difflugia* belonging to Cylindrothecina (see **Taxonomic actions**). Our SSU tree shows even a weakly supported relationship between Cylindrothecina and Excentrostoma (PP = 0.87; Fig. 5); further taxon sampling may also confirm the existing of this deep clade. Furthermore, genus *Trigonopyxis* does not seem to be related to any known infraorder, and more genes/taxa will be instrumental in associating it to a known group or erecting another new infraorder.

4.2. Homoplasic morphological evolution in Arcellinida

An examination of the SSU, NADH and COI gene trees (Figs. 2, 3 and 4) shows different instances in which morphology changes quickly within infraorders. Excentrostoma included genera with a lateral aperture like *Bullinularia*, *Centropyxis* and *Plagiopyxis*. Here, we show that species with a terminal aperture such as *Awerintzewia* sp. and *Difflugia viscidula* also belong to Excentrostoma. Moreover, a lateral aperture seems to have evolved independently at least twice in the infraorder, as *Bullinularia* spp. and *Centropyxis* spp. belong to two well separated clades (Figs. 2 and 4). The inclusion of *D. viscidula* within Excentrostoma makes *Difflugia* paraphyletic again, which calls for a taxonomic action; we erect here the new genus *Golemanskia* (see **Taxonomical actions**). Intra-infraorder variations in general test outline occur also within Longithecina where complex two-chamber tests appear (genus *Zivkovicia*; Fig. 2) as well as convoluted, spiral-shaped tests like in *Lesquereusia* (Fig. 4). The bicameral organization of genus *Apodera* within the otherwise pyriform compressed Hyalospheniformes has already been documented (Duckert et al., 2021). Other infraorders (e.g., Cylindrothecina, Volnustoma) appear to have more conserved morphologies, but this picture may well change when new species will be barcoded. All these instances show that morphologies may vary quicker than expected within infraorders.

Arcellinida shells shape seems to have a non-linear evolution,

following the "tangled evolution" model, in which similar morphologies appear in different parts of the tree. Within Arcellidae, far related species with similar shells have been shown to occupy similar ecosystems (González-Miguéns et al., 2021). Here, we show that similar conclusions can be drawn at the scale of the whole order Arcellinida. Species with a mineral agglutinated shell construction and cylindrical morphology can be found both in Longithecina and Cylindrothecina. These species can be found in lotic systems, and no single morphological trait has been found to discriminate members from both infraorders (Fig. 6); as an example, individuals classified as *Difflugia oblonga* have been placed phylogenetically in both groups (Figs. 2 and 4). Netzeliidae have a unique morphospace (Fig. 6), reflected in their (sub-)spherical shape which may be correlated with their unique temporally planktonic lifestyle (Han et al., 2011). Terrestrial genera *Bullinularia* and *Plagiopyxis,* formerly classified within a single family Plagiopyxidae (Bonnet, 1959a) based on their slit-like aperture with a protective "visor" have converged towards a similar morphology, most probably to fight desiccation (Fig. 4). Genera *Argynnia* and *Physochila*, previously classified within Hyalospheniformes because of their similar general test outline appear unrelated in molecular phylogenies (Lara et al., 2008); both share similar sub-aquatic environments with many Hyalospheniformes species. In sum, shells are likely to undergo changes in the evolutionary history whenever new environments are colonized and selective pressures are modified. Likewise, Excentrostoma, Longithecina, Cylindrothecina and *Phryganella* share the same morphospace (Fig. 6) which means it is currently impossible to classify species within one of these clades based solely on morphology. These examples advocate for the "tangled evolution" hypothesis rather than for the "morphological stasis" along lineages.

What is true for extant species verifies also for assigning taxonomically fossils, as ancient shells have been placed into extant genera and even subspecies based on their shell morphology (Farooqui et al., 2014; van Hengstrum et al., 2007). We have shown that morphologies can change fast in evolutionary times. On the other hand, evolutionary convergences can cause the pooling of far-related taxa under the same species. Therefore, we recommend the highest caution with the taxonomical assignation of Arcellinida fossils. Like in macroscopic organisms, let aside a few notable exceptions improperly called "living fossils" organisms that have been considered as unchanged for long periods of time (Mathers et al., 2013) morphologies change fast in evolutionary times. Under our "tangled evolution" scenario and the lack of morphological stability in most lineages, linking extant Arcellinida with the Precambrian Vase Shaped Microfossils appears premature. Even if we admit that current lineages actually descend from VSM, it is not clear if they derive from one or more lineages (Lahr, 2021), as there could be extinctions events eliminating the Precambrian lineages. These questions may only be answered based on a continuous fossil record including many Phanerozoic fossils and, also, more genetic data on missing genera.

Evolutionary convergences in shells is not a particular case in Arcellinida, but seems to be widespread amongst protists (Leander, 2008). In particular, correlations between morphotypes and habitat have been found in Foraminifera (Coxall et al., 2007), in diatoms (Pinseel et al., 2019), Euglyphida testate amoebae (González-Miguéns et al. 2022) and radiolarians (Kachovich et al., 2019). Likewise, some foraminiferan shells (*Lagenammina*) resemble strongly genus *Difflugia* (Stefanoudis et al., 2016), making it difficult it differentiate only with general shape of the shell. Beyond the microbial world, similar evolutionary patterns appear in caddisflies (Trichoptera), where shell shape is correlated with water oxygenation rather than with phylogenetic position (Williams et al., 1987). Convergent evolution in shell or test morphology is therefore most probably a widespread phenomenon in Eukaryotes, which matches these general patterns of 'rampant homoplastic morphological evolution' found in other groups as well (e.g., Wake et al., 2011).

4.3. Taxonomic decisions: The history of *Difflugia proteiformis*

The current taxonomic and systematic situation in Arcellinida is analogousto the first name ever given to a *Difflugia* (and Arcellinida) species. This genus was originally erected by Leclerc (1815). Lamarck, based in his illustrations placed the newly described organisms amongst the "*polyps",* under the name *Difflugia proteiformis*. This first description included several Arcellinida morphotypes (Fig. 1), which later made Ehrenberg (1838) split the species into several taxonomic units. Amongst these new species was *Difflugia acuminata*, which he had found previously in Berlin (Ehrenberg, 1830) and distinguished from other species by a tip on the shell fundus (Fig. 1). Those shells without tip were designated *D. oblonga*, and the smallest morphotypes remained *D. proteiformis*. Given the high morphological diversity of testate amoebae with agglutinated shells, several authors started to describe newly encountered organisms as *D. proteiformis*, describing several variants of the same species and leaving it polymorphic. For instance, Perty (1849) described new morphologies in his illustrations which did not match with Ehrenberg's interpretations. During the next twenty years, the number of species and of *Difflugia* and variants of *D. proteiformis* increased to more than 100. This led Leidy (1877) to point out that most *Difflugia* were badly characterized, thus inflating species numbers as the "same things having been described over and over again under different names". The problem of *D. proteiformis* was eventually pointed out by Leidy (1879), and Penard (1902) finally invalidated this species to avoid more taxonomic confusions, including this species within *Difflugia pyriformis*.

This historical chaos is the result of the difficulty in setting boundaries between *Difflugia* species as well as the lack of synapomorphic characters to separate genera. Therefore, grouping species by shell similarity leads often to mistakes, as exemplified here by the polyphyletic phylogenetic position of *Difflugia*. Nowadays, molecular techniques and integrative approaches are available, using the history of independent characters to see if they coalesce into concordant results. As a result, the classification of genera into higher taxonomic classification levels based only on shell morphology is virtually impossible. For these reasons, we recommend an integrative taxonomy approach to (re)classify the many orphan Arcellinida genera and species, leaving the genera not correctly classified as *Incertae sedis* (see **Summarized Classification of the Arcellinida**).

This integrative approach is also applied to family Plagiopyxidae, which was described by Bonnet & Thomas (1960) based on the aperture shape and position "Lobose testate amoebae with bilaterally symmetrical organic test covered with fragments of mineral grains, diatoms, and organic debris; aperture a more or less invaginated slit on the ventral side, eccentric, overhung by an anterior lip, in ventral view often difficult to observe (cryptostome)". Under this definition, *Plagiopyxis* and *Bullinularia* should branch together excluding *Centropyxis*, a topology which is not supported neither by SSU nor by COI. Therefore, we invalidate the family Plagiopyxidae, grouping all the genera in the family Centropyxidae.

In line with this, the three molecular markers result in the monophyly of Longithecina I and II, which should be then formally described. We erect Longithecina II as a new infraorder, Cylindrothecina, which hosts for now genus *Cylindrifflugia.* Genus *Difflugia* will remain within Longithecina (I) as its type species is *D. pyriformis*.

New infraorder **Cylindrothecina** González-Miguéns, Todorov, Porfirio-Sousa, Ribeiro, Ramos, Lahr, Buckley & Lara 2022.

Type family: Cylindrodifflugiidae.

Diagnosis: can be diagnosed by its specific sequences of the mitochondrial and nuclear DNA markers (COI, NADH and SSU) and by its phylogenetic placement.

New family **Cylindriflugiidae** González-Miguéns, Todorov, Porfirio-Sousa, Ribeiro, Ramos, Lahr, Buckley & Lara 2022.

Type genus: *Cylindrifflugia*.

Diagnosis: can be diagnosed by its specific sequences of the

mitochondrial and nuclear DNA markers (COI, NADH and SSU) and by its phylogenetic placement.

New genus ***Cylindrifflugia*** González-Miguéns, Todorov, Porfirio-Sousa, Ribeiro, Ramos, Lahr, Buckley & Lara 2022.

Type species: *Cylindrifflugia acuminata* (Ehrenberg, 1838).

Penard collection: https://commons.wikimedia.org/wiki/Category:Difflugia_acuminata.

New species combinations in *Cylindrifflugia*:

Cylindrifflugia acuminata (Ehrenberg, 1838) comb. nov.

Zoobank registration: lsid:zoobank.org:act:09799CDB-9F1F-4E3D-B880-02237BA49D73.

Cylindrifflugia elegans (Penard, 1890) comb. nov.

Zoobank registration: lsid:zoobank.org:act:B8C232C6-E51D-4C29-BE40-2ABFE1D71B89.

Cylindrifflugia lanceolata (Penard, 1890) comb. nov.

Zoobank registration: lsid:zoobank.org:act:59893EE0-F131-4598-9A95-BC8F88BE3128.

Cylindrifflugia bacillariarum (Perty, 1849) comb. nov.

Zoobank registration: lsid:zoobank.org:act:64D55BAC-19DC-46D1-A13D-1AEEAADB0CA9.

Cylindrifflugia hiraethogii (Ogden, 1983) comb. nov.

Zoobank registration: lsid:zoobank.org:act:BC13C9A1-7AFB-438A-A110-B0C0AADFE081.

New genus ***Golemanskia*** González-Miguéns, Todorov, Blandenier, Porfirio-Sousa, Ribeiro, Ramos, Lahr & Lara 2022.

Type species: *Golemanskia viscidula* (Penard, 1902).

New species combinations in *Golemanskia*:

Golemanskia viscidula (Penard, 1902) comb. nov.

Zoobank registration: lsid:zoobank.org:act:5FCEB4DB-9AF9-4699-90CA-FDF437070214.

As demonstrated with the molecular results, the diversity of Arcellinida is far from being characterized. Quoting Leclerc (1815); "Besides, I am far from believing that my task is finished, and having drawn the attention of naturalists to this singular animal which, without doubt, has passed many times unnoticed under the lens of the observer, I do not believe that I am exempt from devoting mine to it once again, and I shall endeavour to arrive at some results that are more satisfactory than those which I am reduced to presenting to you today".

4.4. Summarized classification of the Arcellinida:

Suborder **Glutinoconcha** Lahr et al., 2019.

Infraorder **Sphaerothecina** Kosakyan et al., 2016a.

Family **Arcellidae** Ehrenberg, 1843.

Genera: ***Arcella*** Ehrenberg, 1830 (urn:lsid:zoobank.org:act:D31423F5-E6E6-4E54-B404-37241F932F99), ***Galeripora*** González-Miguéns et al., 2021 (urn:lsid:zoobank.org:act:73C0B8C8-FFA4-48CC-8AE5-B0266D983995).

Family **Netzeliidae** Kosakyan et al., 2016a.

Genera: ***Cucurbitella*** Penard, 1902 (urn:lsid:zoobank.org:act:2BD82251-DD63-405E-8921-1D8483E15044), ***Cyclopyxis*** Deflandre, 1929 (urn:lsid:zoobank.org:act:2C3A445F-6B5E-44B5-AC63-5922F52FB8B5), ***Netzelia*** Ogden, 1979 (rn:lsid:zoobank.org:act:1F34795D-5774-46A6-A4FA-5CF44E58A56C).

Infraorder **Longithecina** Lahr et al., 2019.

Family **Difflugiidae** Wallich, 1864.

Genera: ***Difflugia*** Leclerc, 1815 (urn:lsid:zoobank.org:act:642F3774-1053–4549-949E-5157434B4BCD), ***Zivkovicia*** Ogden, 1983 (urn:lsid:zoobank.org:act:A1F9C197-AB87-43E7-B82A-F02CDF0244C0).

Family **Lesquereusiidae** Jung, 1942.

Genera: ***Lesquereusia*** Schlumberger, 1845 (urn:lsid:zoobank.org:act:89A05EC5-1B4E-4F66-94E7-99C844A333F0).

Infraorder **Cylindrothecina** González-Miguéns, Todorov, Blandenier, Porfirio-Sousa, Ribeiro, Ramos, Lahr & Lara **new infraorder**.

Family **Cylindrifflugiidae** González-Miguéns, Todorov, Blandenier, Porfirio-Sousa, Ribeiro, Ramos, Lahr & Lara **new family**.

Genera: ***Cylindrifflugia* new genus** González-Miguéns, Todorov, Blandenier, Porfirio-Sousa, Ribeiro, Ramos, Lahr & Lara (urn:lsid:zoobank.org:act:62C7FAA1-CD5D-4865-BB4E-46CC77ED5C2E).

Infraorder **Excentrostoma** Lahr et al., 2019.

Family **Centropyxidae** Jung, 1942

Genera: ***Awerintzewia*** Schouteden, 1906 (urn:lsid:zoobank.org:act:E0F308AB-8FA3-459D-9493-7C32B53B3657), ***Bullinularia*** Deflandre, 1953 (urn:lsid:zoobank.org:act:06366D36-DD64-4C0C-8C02-FC26EB7D61E6), ***Centropyxis*** Stein, 1859 (urn:lsid:zoobank.org:act:2F7FCC8B-1A4C-41C9-8CB1-5697B80E92B6), ***Golemanskia*** González-Miguéns, Todorov, Blandenier, Porfirio-Sousa, Ribeiro, Ramos, Lahr & Lara (urn:lsid:zoobank.org:act:DF9D1D11-06FA-4F24-8362-17132CB23FBE), ***Plagiopyxis*** Penard, 1910 (urn:lsid:zoobank.org:act:CEBB0164-90B6-4137-AC5F-7FD328FC60DA).

Infraorder **Hyalospheniformes** Lahr et al., 2019.

Family **Hyalospheniidae** Schultze, 1877 emend. Kosakyan et al., 2012.

Genera: ***Alabasta*** Duckert et al., 2018 (urn:lsid:zoobank.org:act:7408C1E9-2DD6-4098-97DA-3CF743954B5F), ***Alocodera*** Jung, 1942 (urn:lsid:zoobank.org:act:38495728-64C4-4B53-BE70-D6FD189AA555), ***Apodera*** Loeblich and Tappan, 1961 (urn:lsid:zoobank.org:act:2A253E61-6C8B-4324-9E61-9A2D304BD848), ***Certesella*** Loeblich and Tappan, 1961 (urn:lsid:zoobank.org:act:CBA76680-88D8-40F4-A5A3-5EEFF8B38F0A), ***Cornutheca*** Kosakyan et al., 2016b (urn:lsid:zoobank.org:act:C2B0C4FF-4BAE-46ED-BC9C-CC7B77510389), ***Gibbocarina*** Kosakyan et al., 2016b (urn:lsid:zoobank.org:act:76D63465-04BC-4A6C-9FB8-CB148B787DC0), ***Hyalosphenia*** Stein, 1859 (urn:lsid:zoobank.org:act:C1CB7196-8C45-4076-975A-1053018DFAB4), ***Longinebela*** Kosakyan et al., 2016b (urn:lsid:zoobank.org:act:6A69AF6F-60B8-4383-95FA-05911D806FE4), ***Mrabella*** Kosakyan et al., 2016b (urn:lsid:zoobank.org:act:E9D5B101-EB13-436D-8B10-0E43C00DEDB6), ***Nebela*** Leidy, 1874 (urn:lsid:zoobank.org:act:26F54823-8DA3-4395-8EE9-EBA0845E1CA8), ***Padaungiella*** Lara et Todorov, 2012 (urn:lsid:zoobank.org:act:7050AE36-A879-46 EB-A3C2-051C8241234F), ***Planocarina*** Kosakyan et al., 2016b (urn:lsid:zoobank.org:act:0706752B-4BAD-4FF5-9DE6-D044A6CDB72B), ***Porosia*** Jung, 1942 (urn:lsid:zoobank.org:act:D8A54F6C-5751-4C2E-BFB0-57E7F547071F), ***Quadrulella*** Cockerell, 1909 (urn:lsid:zoobank.org:act:54DFC2D4-AEDC-44F3-819C-F20651B2A3AB).

Infraorder **Volnustoma** Lahr et al., 2019.

Family **Heleoperidae** Jung, 1942.

Genus ***Heleopera*** Leidy, 1879 (urn:lsid:zoobank.org:act:3AA5030B-BF41-43AD-958B-00FF70FFCA3C).

Suborder **Organoconcha** Lahr et al., 2019.

Family **Microchlamyiidae** Ogden, 1985.

Genera: ***Microchlamys*** Cockerell, 1911 (urn:lsid:zoobank.org:act:7-D3AFE77-D4D3-4927-A971-4C8D0BB3C3CC), ***Spumochlamys*** Kudryavtsev & Hausmann, 2007 (urn:lsid:zoobank.org:act:CABAC4CB-0453-46F2-AA33-3E95DB46FBC1), ***Pyxidicula*** Ehrenberg, 1838 (urn:lsid:zoobank.org:act:3E1971A0-0637-426C-98CB-D6CFD2B31090).

Suborder **Phryganellina** Bovee, 1985.

Family **Phryganellidae** Jung, 1942.

Genus ***Phryganella*** Penard, 1902 (urn:lsid:zoobank.org:act:E7D4C306-B826-4B5A-BD68-08992AD0B71D).

Family **Cryptodiflugiidae** Jung, 1942.

Genus ***Cryptodifflugia*** Penard, 1890 (urn:lsid:zoobank.org:act:1E182D4B-74C8-4790–9454-4C2E460DF85C).

Incertae Sedis (*genus barcoded):

Genera: ***Acipyxis*** Jung, 1942 (urn:lsid:zoobank.org:act:6811D231-6DA0-4D28-BD6F-AFB8C4EDF61C), ***Apolimia*** Korganova, 1987 (urn:lsid:zoobank.org:act:CEA558F4-49DF-47A9-A6D4-A92E5797DB9E), ***Argynnia**** Vucetich, 1974 (urn:lsid:zoobank.org:act:BCB3A3BE-355D-4E65-8AC0-521501DA3831), ***Armipyxis*** Dekhtjar, 2009 (urn:lsid:zoobank.org:act:A5E855AD-B404-4F69-9B10-E577FC07CC60), ***Conicocassis*** Nasser & Patterson, 2015 (urn:lsid:zoobank.org:act:CF0D9768-05CE-4703-9A7F-3B43521C2217), ***Cornuapyxis*** Côûteaux & Chardez, 1981 (urn:lsid:zoo

bank.org:act:2CA46A68-90C2-4218-B53A-9C75B1E56C82), ***Ellipsopyxella*** Bonnet, 1975a (urn:lsid:zoobank.org:act:726EDDA0-98B3-4C63-8829-8DC5DADF475D), ***Ellipsopyxis*** Bonnet, 1965 (urn:lsid:zoobank.org:act:56D4AE49-1F38-4260-8F11-F933FD438861), ***Erugomicula*** Nasser et al., 2021(urn:lsid:zoobank.org:act:A42A7EB9-B8CC-45E6-867E-2CDBCA038B23) ***Frenopyxis*** Bobrov & Mazei, 2020 (urn:lsid:zoobank.org:act:63C16117-92E0-47F8-91A2-3CD5D3FDA221), ***Geamphorella*** Bonnet, 1959b (urn:lsid:zoobank.org:act:405A3645-1162-4BC9-BDAD-C031A37DC458), ***Geoplagiopyxis*** Chardez, 1960 (urn:lsid:zoobank.org:act:9BF9B9F9-4C5E-462F-8533-AC2EB6CF3B04), ***Geopyxella*** Bonnet & Thomas, 1955 (urn:lsid:zoobank.org:act:4A7A0CB7-20D5-4E67-8F18-46EA047C8D65), ***Hoogenraadia*** Gauthier-Liévre & Thomas, 1958 (urn:lsid:zoobank.org:act:F1B3B043-2BF7-458E-9551-18167E35C6D8), ***Jungia*** Loeblich and Tappan, 1961 (urn:lsid:zoobank.org:act:EE13920C-FB94-4ED6-A8E9-359EF5548B8D), ***Lagenodifflugia*** Medioli & Scott, 1983 (urn:lsid:zoobank.org:act:255C48A1-9425–4647-B4A4-9EE979B4286E), ***Lamptopyxis*** Bonnet, 1974 (urn:lsid:zoobank.org:act:07556E10-9CC9-4C04-953B-FB00A9AD512D), ***Lamtoquadrula*** Bonnet, 1975b (urn:lsid:zoobank.org:act:C9D9F207-CBC8-4C5A-AD18-0881D93E9539), ***Leptochlamys*** West, 1901 (urn:lsid:zoobank.org:act:FB2129A3-9C44-4379-86D1-CFFCAFFBDAF8), ***Maghrebia*** Gauthier-Liévre & Thomas, 1958 (urn:lsid:zoobank.org:act:88C90F6F-FCB9-49E8-A909-3D977704A0D8), ***Meisterfeldia*** Bobrov, 2016 (urn:lsid:zoobank.org:act:378A8BE0-F641-42E7-9D45-BD0A83900E49), ***Microquadrula*** Golemansky, 1968 (urn:lsid:zoobank.org:act:E005C63B-6E52-45BB-84E8-C73B8FED660A), ***Nabranella*** Snegovaya & Alekperov, 2009 (urn:lsid:zoobank.org:act:F5391958-704A-4B80-9E11-9BFFD7E9DE45), ***Oopyxis*** Jung, 1942 (urn:lsid:zoobank.org:act:670A7D1B-1A03-49C6-BF03-CC344C15A7C9), ***Paracentropyxis*** Bonnet, 1960 (urn:lsid:zoobank.org:act:9790F11C-BEB5-4373-ABB9-D74B299549C3), ***Paraquadrula*** Deflandre, 1932 (urn:lsid:zoobank.org:act:2041AE2D-4D49-4D48-A795-0F095A49F60B), ***Pentagonia*** Gauthier-Liévre & Thomas, 1958 (urn:lsid:zoobank.org:act:3A99093A-D4DD-4299-B963-9C75EA65126A), ***Physochila**** Jung, 1942 (urn:lsid:zoobank.org:act:3BDD5D3C-548D-41A1-96EA-8D14970EB4CF), ***Planhoogenraadia*** Bonnet, 1977 (urn:lsid:zoobank.org:act:CCEFFB8F-6E49-4372-9E10-C1E29627005C), ***Pomoriella*** Golemansky, 1970 (urn:lsid:zoobank.org:act:EC215477-3616-4D69-B0F8-485F1B9395A6), ***Pontigulasia*** Rhumbler, 1895 (urn:lsid:zoobank.org:act:2A1965E3-1822-49AD-973A-4049392799E2), ***Proplagiopyxis*** Schönborn, 1964 (urn:lsid:zoobank.org:act:1CB758D2-71A4-4A71-8709-3AAAD5E79A3A), ***Protoplagiopyxis*** Bonnet, 1962 (urn:lsid:zoobank.org:act:157F1593-1095–4610-92BF-B0AD6FE2BCF0), ***Protocucurbitella*** Gauthier-Liévre & Thomas, 1960 (urn:lsid:zoobank.org:act:9138B2DC-C53C-4D2D-A68F-8665141D3E24), ***Pseudawerintzewia*** Bonnet, 1959b (urn:lsid:zoobank.org:act:F8A7DE7B-54B0-47A5-8C3A-095E6A28575F), ***Pseudonebela*** Gauthier-Liévre & Thomas, 1953 (urn:lsid:zoobank.org:act:BA3A05F0-9AC0-471E-8BD2-95AA890ADE90), ***Schoenbornia*** Decloître, 1964 (urn:lsid:zoobank.org:act:FEB00E4B-2065-4B1B-8E91-170DBEDEA8C9), ***Sexangularia*** Awerintzew, 1906 (urn:lsid:zoobank.org:act:093805BA-54BF-4CBD-96BF-EF2A005AFB67), ***Suiadifflugia*** Green, 1975 (urn:lsid:zoobank.org:act:B18FFF6A-3EE8-4C23-9F23-E8CD13256702), ***Trigonopyxis**** Penard, 1912 (urn:lsid:zoobank.org:act:0F46EE16-5C36-4B4D-B957-D18C94B867CA), ***Wailesella*** Deflandre, 1928 (urn:lsid:zoobank.org:act:3D0EE5DE-E390-41D6-9106-F5C5C9038CAA).

5. Conclusion

In this study, we established the systematics of Arcellinida by increasing the taxon sampling, and using three genes (COI, NADH and SSU). These topology resulting from SSU gene is equivalent to that of the phylotranscriptomic study of Lahr et al. (2019). All genes recovered the monophyly of the new infraorder Cylindrothecina and the inclusion of genus *Awerintzewia* (only with COI) and new genus *Golemanskia* within Excentrostoma, despite of their *Difflugia*-like morphology. They also supported the idea that the similar aperture found in genera *Plagiopyxis* and *Bullinularia* is the result of a convergence, possibly since that these organisms inhabit similar environments and undergo similar selective pressures and constrains. These examples illustrate the versatility of morphological evolution in Arcellinida clades, which follow then a "tangled evolution" model instead of a "morphological stasis". Convergent shell evolution has not only been documented in Arcellinida but happens also in diatoms, foraminifera and in animals such as caddisflies (Trichoptera). We speculate that such convergences could be due to positive (directional) selection acting on shell evolution, whose effects can be witnessed in microbial as well as in multicellular eukaryotes alike. The entirety of convergence patterns can only be revealed once a robust phylogeny for the group is thoroughly completed. To paraphrase Dobzhansky's famous quote, "Nothing in evolution makes sense except in the light of systematics".

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CRediT authorship contribution statement

Rubén González-Miguéns: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Milcho Todorov:** Investigation, Resources, Writing – review & editing. **Quentin Blandenier:** Resources, Writing – review & editing. **Clément Duckert:** Resources. **Alfredo L. Porfirio-Sousa:** Writing – review & editing. **Giulia M. Ribeiro:** Writing – review & editing. **Diana Ramos:** Resources, Writing – review & editing. **Daniel J.G. Lahr:** Writing – review & editing. **David Buckley:** Investigation, Writing – review & editing. **Enrique Lara:** Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition.

Declaration of Competing Interest

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Appendix A. Supplementary material

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