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ORIGINAL ARTICLE

The second most abundant dinophyte in the ponds of a botanical garden is a species new to science

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Abstract

In the microscopy realm, a large body of dark biodiversity still awaits to be uncovered. Unarmoured dinophytes are particularly neglected here, as they only present inconspicuous traits. In a remote German locality, we collected cells, from which a monoclonal strain was established, to study morphology using light and electron microscopy and to gain DNA sequences from the rRNA operon. In parallel, we detected unicellular eukaryotes in ponds of the Botanical Garden Munich-Nymphenburg by DNA-metabarcoding (V4 region of the 18S rRNA gene), weekly sampled over the course of a year. Strain GeoK*077 turned out to be a new species of *Borghiella* with a distinct position in molecular phylogenetics and characteristic coccoid cells of ovoid shape as the most important diagnostic trait. *Borghiella ovum*, sp. nov., was also present in artificial ponds of the Botanical Garden and was the second most abundant dinophyte detected in the samples. More specifically, *Borghiella ovum*, sp. nov., shows a clear seasonality, with high frequency during winter months and complete absence during summer months. The study underlines the necessity to assess the biodiversity, particularly of the microscopy realm more ambitiously, if even common species such as formerly *Borghiella ovum* are yet unknown to science.

KEYWORDS
biodiversity, dinoflagellate, metabarcoding, molecular phylogenetics, morphology, seasonality

INTRODUCTION

THE number of species that populate this planet is currently almost impossible to grasp (Wilson, 2017), and estimates range between 10 and 1000 million species (Locey & Lennon, 2016; Mora et al., 2011; Scheffers et al., 2012). Habitats that are remote and are more difficult to access, such as the deep sea, the glaciated polar regions, or the soil layers of tropical rainforests, certainly harbor numerous hitherto unknown species and have been the subject of intensive research in recent years (Deppeler & Davidson, 2017; Elferink, Wohlrab, et al., 2020; Mahé et al., 2017; Scheckenbach et al., 2010). There is concern that rare species in particular are becoming extinct due to massive environmental degradation before they could be scientifically inventoried (Costello et al., 2013). However, previously unknown species can also be found in places heavily influenced by humans, even among vertebrates (Feinberg et al., 2014) and flowering plants (Suetsugu et al., 2023).

It is primarily the microorganisms whose true extent of biodiversity remains to be uncovered (Pawlowski

et al., 2012; Vargas et al., 2015). In recent years, the sequence-based study of environmental samples, and the high-throughput identification and (semi-) quantification of multiple species (metabarcoding: Hörstmann et al., 2022; Taberlet et al., 2012), have further underlined the magnitude of the task in assessing biodiversity. Numerous studies have generated previously unknown DNA sequences, some of which could not even be assigned to one of the established major phylogenetic lineages (Janouškovec et al., 2017; Massana, 2011; Seenivasan et al., 2013). Even within known lineages, there is a considerable amount of diversity that currently cannot be associated with a scientific name (Jang, 2022), ultimately due to the incompleteness of reference databases ("taxonomic gap") that are currently available (Gottschling et al., 2020; Salmaso et al., 2022). Without the basis of a reliable identification and naming of species, however, further research, for example, on the role of species in the ecosystem, is hardly possible (Hebert et al., 2003; Thomson et al., 2018; Vitorino & Bessa, 2018).

An ecologically and economically important group of unicellular eukaryotes is the dinophytes. With some 2500 species accepted so far, they colonize a wide variety of aquatic habitats from the poles to tropical regions (Gómez, 2012; Ott et al., 2022). They are predominantly distributed in marine environments, but also in freshwater habitats encountering some 350 species (Mertens et al., 2012; Moestrup & Calado, 2018). During their development, many dinophytes form two morphologically and ecologically differentiated stages, namely a flagellated and motile cell of the plankton and a coccoid and immobile cell frequently deposited in the sediment (Dale, 1983; Fensome et al., 1993). However, dormancy is not the only biological function of coccoid cells (Bravo & Figueroa, 2014; Figueroa et al., 2018), and some species show a wide range of different stages, whose cells vary in shape, coloration, or other traits. If the motile stage builds a cell wall, it exhibits a species- and group-specific pattern of cellulose plates (the so-called theca), and those species are distinguished from the unarmoured members of the dinophytes (Fensome et al., 1993; Moestrup & Calado, 2018; Taylor, 1987). The cell surface of the latter might be covered by thin, amphiesmal plates but without a clear pattern, identification of such species is particularly challenging (Escarcega-Bata et al., 2022).

As inferred from metabarcoding, suessialean dinophytes include a reasonable fraction of so far unknown or "dark" diversity (Jang, 2022), also among the freshwater lineages (Annenkova et al., 2011; Gottschling et al., 2021). One of the early branches of the †Suessiales is the Borghelliaceae (Knechtel et al., 2020; Moestrup et al., 2009) currently encountering 11 known species from freshwater habitats. Eight of them are linked to DNA sequence information, which is of crucial importance in a group of species with difficult delimitation due to the poorness of diagnostic traits (Daugbjerg et al., 2014; Knechtel et al., 2020; Moestrup et al., 2008, 2018). Borghelliaceae may segregate into *Baldinia* Gert Hansen & Daugbjerg and *Borghiella* Moestrup, Gert Hansen & Daugbjerg (but the support in molecular phylogenetics is low) and are characterized by the amphiesmal vesicles covering the flagellated cell and containing very thin, plate-like structures (Moestrup & Calado, 2018). In the linear apical complex (LAC) of unknown function, a row of pores extends from a single, linear, amphiesmal vesicle at the apex of the flagellated cell. The LAC is only demonstrated for members of *Borghiella* (and other suessialean and tovellialean dinophytes: Jeong et al., 2014; Pandeirada et al., 2014) but not of *Baldinia* and can only be observed by using advanced techniques such as scanning electron microscopy (SEM). Furthermore, an intraplastidic type B eyespot (Moestrup & Daugbjerg, 2007) has been assigned to the group and at least for some species, spherical through ellipsoid coccoid cells with a smooth surface are reported additionally to the flagellated cells. No fossils have been assigned to Borghiellaceae yet, but the age of the crown group has been dated to the Lower Cretaceous ca 110 mya (Chacón & Gottschling, 2020).

In this study, we present a new species of *Borghiella* from the Bavarian countryside (Germany). It is distinct from all other known species of *Borghiella* as inferred from DNA sequence comparison and exhibits characteristic coccoid cells of ovoid shape. Simultaneously, we have been working on an extensive metabarcoding study focusing on the seasonal dynamics at six artificial sites located in the Botanical Garden of Munich. By evaluating the abundance of amplicon sequence variants (ASVs) throughout sampling, we became aware of a winter-dominant dinophyte sharing the same DNA sequence with the new species. Our results clearly underline how important it still is to inventory biodiversity in places that are supposedly already well understood. The socio-economic value of microorganisms remains largely elusive until they are rigorously explored.

MATERIALS AND METHODS

Strain establishment, microscopy, and molecular phylogenetics

Strain GeoK*077 was established by micropipetting from field material collected at Reut (Germany, Bavaria, Rottal-Inn; 48°18.715′ N, 12°56.484′ E) on Feb 11, 2020. Cultivation using freshwater WC growth medium (Woods Hole Combo, modified after Guillard & Lorenzen, 1972) without silicate took place in climate chambers at 12°C and a 12:12 h light:dark photoperiod. The strain (Table S1) is currently held in the culture collection at the Institute of Systematics, Biodiversity, and Evolution of Plants (University of Munich) and is available upon request. Strain GeoK*077 is additionally

available at the Central Collection of Algal Cultures (University of Duisburg-Essen).

Cells were observed and documented with a CKX41 inverse microscope (Olympus) equipped with a phase-contrast option. Images were taken with a DP73 digital camera (Olympus) and if applicable, samples were covered with a droplet of Protogel (Protist Motility Inhibitor, C340). For nuclear staining, cells were treated with 4′-6-diamidino-2-phenylindole (DAPI, 10 μg ml^{-1} final concentration) for 10 min. For visualizing of the nuclei, and also for observing chloroplasts and presumable eyespots of motile cells applying autofluorescence, a DM1000 light microscope (Leica) equipped with a DAPI filter (Leica; excitation: 350/50, dichroic mirror: 400, emission BP 460/50) and an I3 filter (Leica; excitation: 450/490, dichroic mirror: 510, emission LP 515) was used as described previously (Romeikat et al., 2020). Measurements were made using the programs "cellSens Entry" (Olympus) and "Fiji" (https://imagej.net/software/fiji/).

For the preparation of permanent slides, cells of the strain GeoK*077 were fixed with a 10% formaldehyde (Roth), 5% acetic acid (AppliChem), and 50% ethanol (Roth) formalin-aceto-alcohol solution in cacodylate buffer. Double-staining was performed using 0.5% (water-based) astra blue in 2% tartaric acid (Fluka) in cacodylate buffer and 0.1% (ethanol-based) eosin (Merck) during a graded ethanol (Roth) series. Ethanol-based Technovit 7100 (Heraeus) was used for embedding, following the manufacturer's instructions. For the final specimens, 40 mL aliquots of the Technovit mixture including the embedded samples were transferred to three microscope slides. The specimens are deposited at the Centre of Excellence for Dinophyte Taxonomy (CEDiT), and duplicates are held in Berlin, B and Munich, M.

The preparative techniques for SEM were performed at room temperature and were basically the same as described in Romeikat et al. (2020) and Knechtel et al. (2020). As the dinophytes under study have thin and small amphiesmal vesicles, 1 mL of cells in WC medium were fixed with 1 mL of 1.5% OsO_4 (Science Services) incubated for 1 h. Afterward, the cells were washed three times in cacodylate buffer in 10, 20, and 30 min intervals, respectively. Cells were dehydrated using a graded acetone series (Roth) in 15 min intervals (10%, 30%, 60%, 80%). 100% ace-tone was used for the last dehydration step, repeated three times in 5 min and 2 × 30 min intervals. Each washing and dehydration step was followed by centrifugation (Eppendorf) at 500 *g* for 5 min. After critical point drying and mounting on aluminum stubs, cells were sputter-coated (BAL-TEC SCD 050) with platinum and supplied with Planocarbon (Plano). The material was observed with a LEO438VP SEM (LEO Electron Microscopy) or the Zeiss Auriga Crossbeam workstation (Zeiss). All images were adjusted in Photoshop (Adobe Systems) and arranged with QuarkXPress (Quark Software).

DNA-sequencing

DNA harvest and isolation, as well as PCR amplification and sequencing, are already described previously (Knechtel et al., 2020). To build the alignment, we defined three regions of the rRNA: SSU, ITS, LSU, and studied a systematically representative set of †Suessiales (Table S1, including information of the outgroup comprising Gymnodiniales and Dinophysales). We also performed NCBI Blast Searches (Altschul et al., 1990) and included all sequences associated with *Borghiella*. Phylogenetic analyses were the same standards as applied in Knechtel et al. (2020).

As part of a bigger DNA-metabarcoding study described in more detail elsewhere, freshwater samples were taken in six ponds of the Botanical Garden in Munich-Nymphenburg once a week and throughout an entire year. Environmental DNA was extracted using the Genomic DNA from Soil kit (Machery-Nagel) following the manufacturer's protocol, and PCR amplification and purification followed established methods and protocols as described in Gottschling et al. (2020). Briefly, the SSU V4 region was targeted for amplification using forward and reverse primers (Bradley et al., 2016). The workflow for the preparation of V4 amplicons for the Illumina MiSeq system was adjusted from the "16S Metagenomic Sequencing Library Preparation B" document (part no. 15044223; Rev. B) distributed by Illumina with modifications for preparation of eukaryotic gene amplicons. Paired-end Illumina sequencing (MSC 2.5.0.5/RTA 1.18.54, 2 × 300 bp) of samples was performed on a MiSeq platform (Illumina, United States). The sequence data were processed with the DADA2 pipeline using PR^2 version 4.10.0 (https://github.com/pr2database/pr2database/releases/tag/4.10.0; Guillou et al., 2013) for detecting ASVs in a sample from the library of noisy reads generated by amplicon sequencing (Callahan et al., 2016; Rosen et al., 2012) as described in Elferink, John, et al. (2020). The DinoRef database (Mordret et al., 2018) reliably identifies 89% of the V4 dinophyte ASVs down to the species level and was used for identification.

RESULTS

Morphology of strain GeoK*077

The strain under investigation exhibited flagellated (Figures 1 and 5A) and coccoid cells (Figures 2, 4, and 5B–D), with the coccoid cells being predominant (approximate ratio: 3:1). Flagellated cells were grossly globular in shape and did not show apparent dorso-ventral flattening. The cingulum was descending and was displaced ca one cingulum width. The sulcus extended almost to the antapex. Two types of flagellated cells could be distinguished, varying (though not significantly) in size, shape, and coloration. The bigger flagellated cells

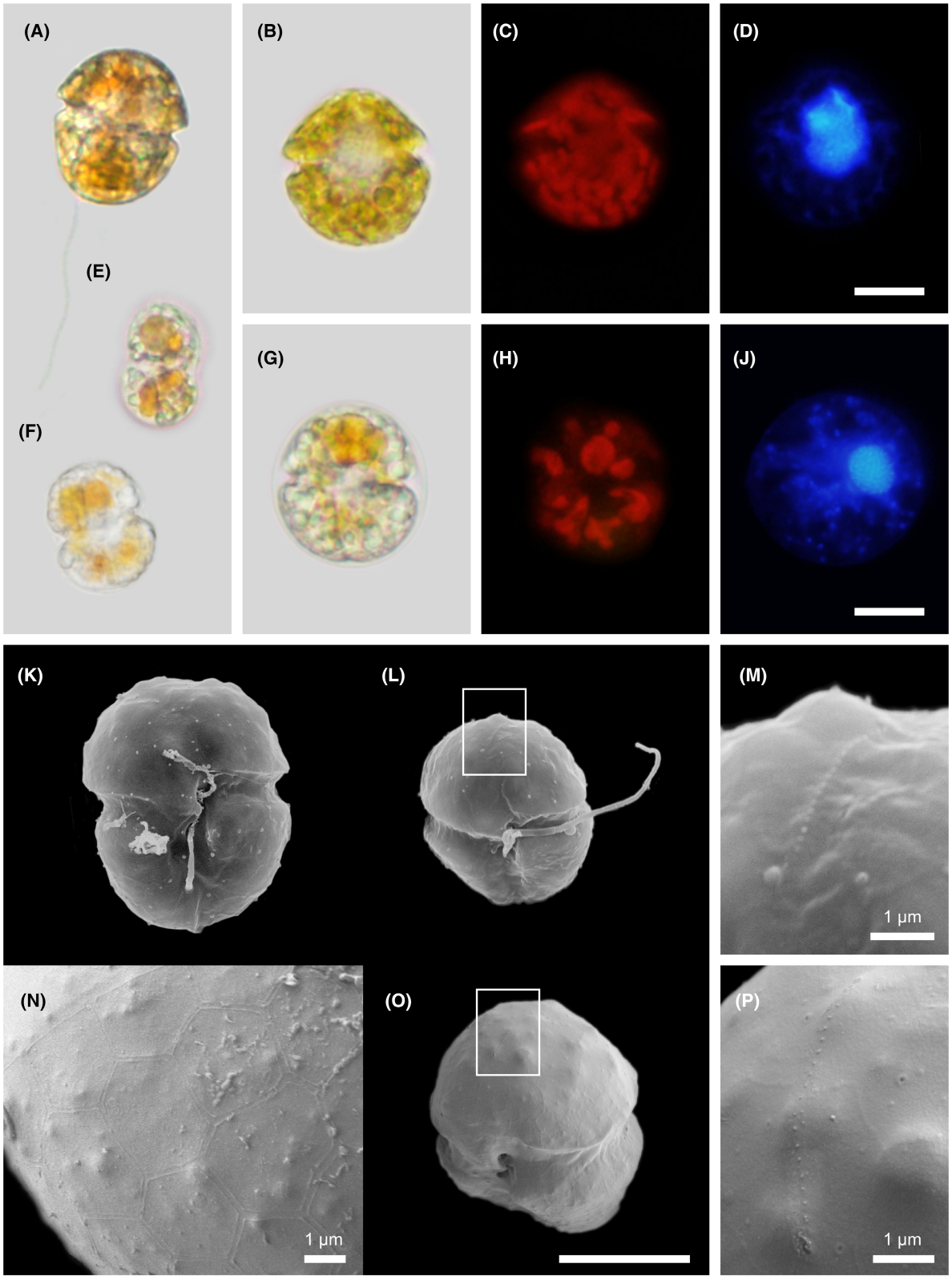

FIGURE 1 Flagellated cells of *Borghiella ovum* (GeoK*077) in LM (A, B, E–G), fluorescence LM (C, D, H–J), and SEM (K–P). (A, B) The overall bigger and more brightly colored phenotype, note the distinct flagellum in (A). The same cell shows the nucleus (B, D DAPI-stained) in a central position and numerous peripheral chloroplasts (C). (E–G) The overall smaller, less brightly colored phenotype, note the considerably varying shape. (G, H) The same cell shows a reduced number of chloroplasts. (J DAPI-stained) Distinct chromosomes. (K) Ventral view, note the remnants of the flagella. (L, M) Apical-ventral view, note the remnants of the flagella and the cut-out of the linear apical complex (magnification in M). (N) Surface shows the periplast of the amphiesmal hexagonal vesicles. (O, P) Apical-ventral view, note the flagellar pores and the cut-out of the linear apical complex (magnification in P). Scale bar: 10 µm if not otherwise stated.

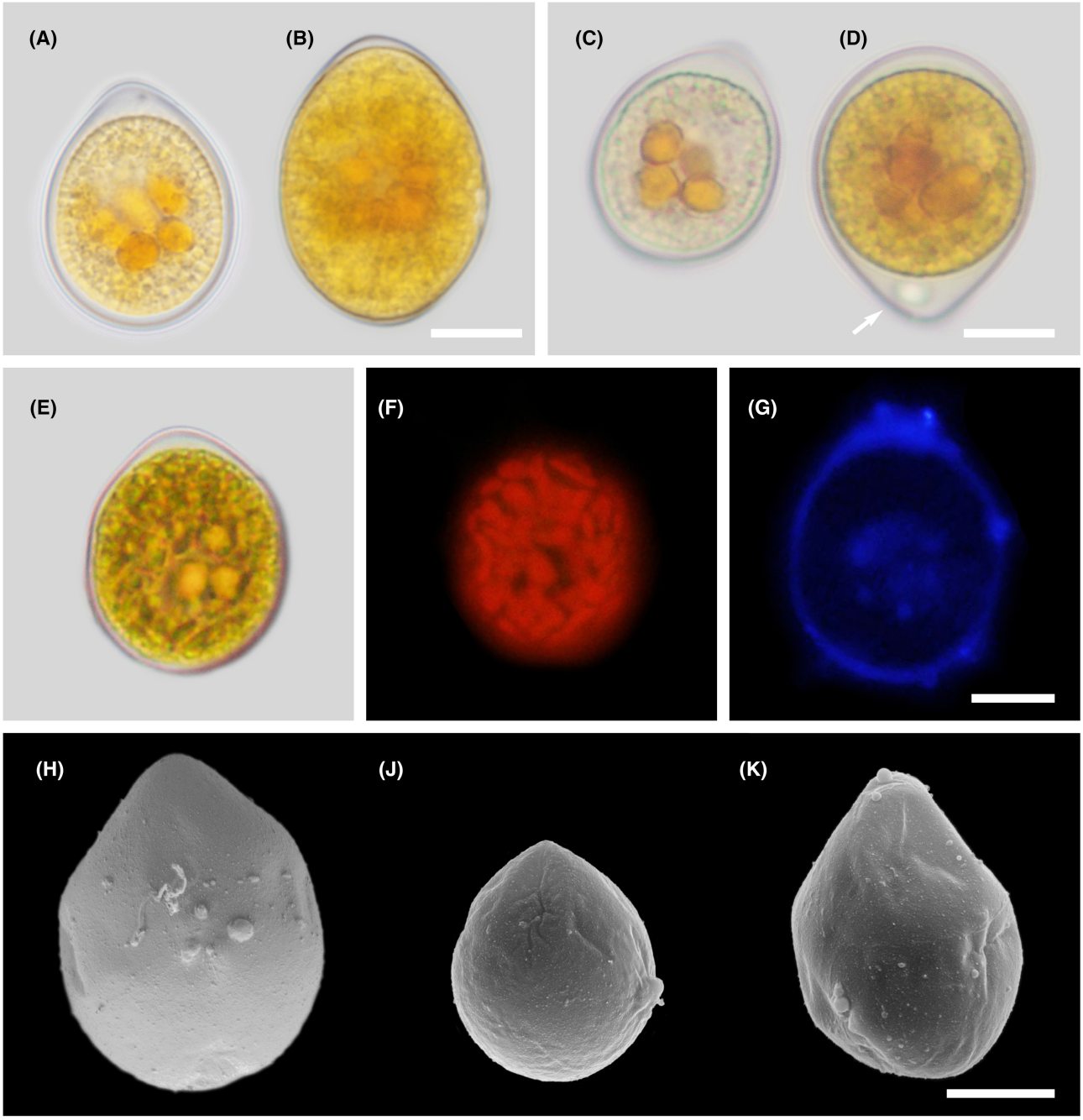

FIGURE 2 Coccoid cells of *Borghiella ovum* (GeoK*077) in LM (A–E), fluorescence LM (F, G), and SEM (H–K). (A–E) Ovoid cells of varying size and coloration, note the hyaline coat of varying thickness, the central position of accumulation bodies, and small inclusions found in some cells (white arrow in D). (E–G) The same cell shows numerous peripheral chloroplasts (F) and the slightly visible nucleus (G DAPI-stained), due to a putatively earlier stage of development. (H–K) Ovoid cells of varying sizes show a smooth surface. Scale bar: 10 μm.

(Figure 1A–D) ranged from 17 to 40 μm (mean: 22 μm; SD: 3.0 μm; $n = 109$) in length and from 12 to 26 μm (mean: 17 μm; SD: 2.7 μm; $n = 109$) in width and were predominant. The shape was not varying remarkably and was widely ovoid, widely ellipsoid, or widely obovoid. The episome ranged from conical to hemispheric. The cells were of brightly yellow-brown color and had a distinct nucleus in the center with approximately 40 identifiable chromosomes (Figures 1B,J, 3A,D, and 5A). The smaller flagellated cells (Figure 1E–H) ranged from 12 to 22 μm in length (mean: 17 μm; SD: 2.0 μm; $n = 176$) with a width of 8–19 μm (mean: 13 μm; SD: 2.1 μm; $n = 176$) and were rare (less than 10% of cells). The shape was varying remarkably from ellipsoid through globular. The cells were less brightly colored, and the nucleus was not distinct. Motility of these cells was higher compared to the bigger cells.

Cells were surrounded by a periplast of many pentagonal or hexagonal vesicles (Figure 1K,N–P). The sutures between the vesicles were more or less distinct and of

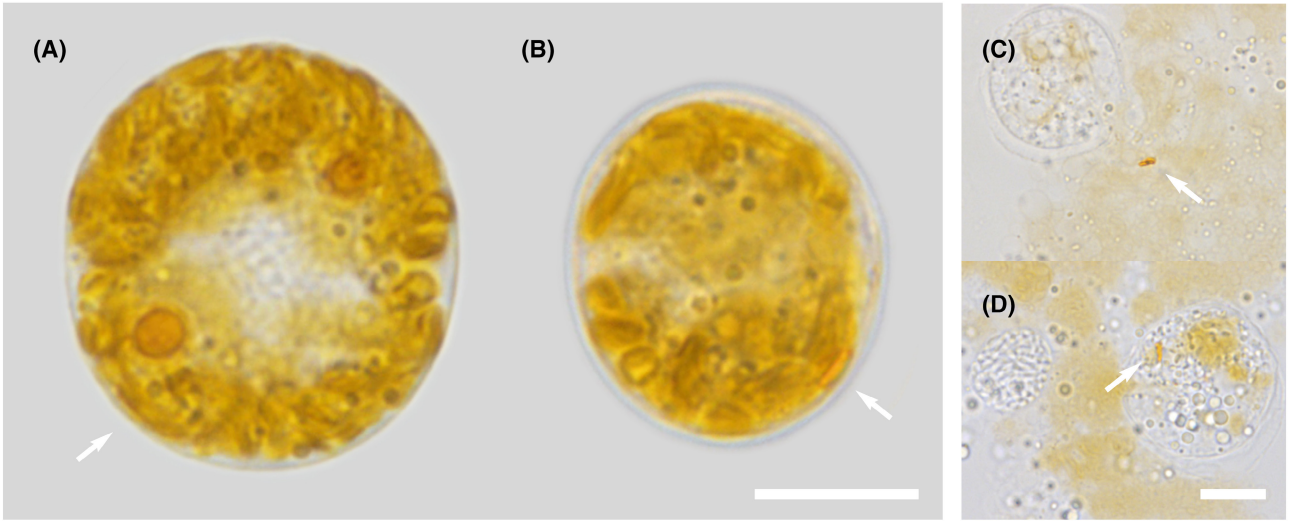

FIGURE 3 Eyespot of *Borghiella ovum* (GeoK*077) in LM (white arrows). (A, B) Lateral views with the eyespot presumably in the hyposome. (C, D) Burst cells with disc-shaped eyespots still intact. Scale bar: 10 μm.

varying width. On the episome, a furrow with small knobs as part of the LAC was observed (Figure 1L,M,O,P). It was ca 3 μm long, accompanied by two pentagonal amphiesmal vesicles on each side, and extended diagonally through the apex from dorsal-left to ventral-right. Chloroplasts were numerous (Figure 1C,H) and were predominantly found in the periphery of the cell. As inferred from the red color, mostly one, occasionally more lipid globule(s) were found (Figure 1A,E–G). The orange eyespot (Figure 3) was intraplastidic, disc-shaped with varying outline, and ca 3 μm in diameter. It was difficult to observe in LM and within the cell, it was always present in a similar position (presumably in the hyposome). The eyespot remained intact even after cell rupture.

Coccoid cells were mostly of ovoid shape (Figures 2A–E,H–K and 5D) but occasionally also almost globular (Figure 4A) or ellipsoid. The mostly spherical, sometimes ellipsoid or ovoid protoplast was surrounded by a coat of unknown material, which was frequently varying in thickness within each cell: In LM, such cells resembled a longisection of a rotationally symmetric egg, with the yolk embedded in the egg white. The cell size ranged from 16 to 36 μm in length (mean: 25 μm; SD: 3.5 μm; n = 109) and 15–31 μm in width (mean: 20 μm; SD: 2.7 μm; n = 109). In SEM, the surface of the coccoid cells appeared smooth through microgranular. At its thickest region, the coat occasionally showed a small inclusion in LM. Coloration was varying from yellow-brown (similar to motile cells) to more translucent, and the latter type showed big, red accumulation bodies in the center. As inferred from autofluorescence, all coccoid cells showed chloroplasts, with reduced numbers in the more translucent cells. Despite multiple attempts, no nucleus could be stained using DAPI in coccoid cells, but was successfully demonstrated with astra blue staining, as it was used for the preparation of the type material (Figure 5). The origin and fate of such coccoid cells could not be determined.

A second type of coccoid cells was represented by division stages (ratio ca 1:10): Two (or rarely four) presumably flagellated cells were found within a shared pellicle without flagella (Figures 4B–J and 5B,C). Such cells exhibited a single red globule each, with an apparently consistent position, though it was unclear whether in epi- or hyposome. However, the two presumptive sister cells took either point symmetric (Figure 4D,E) or axial symmetric positions (Figure 4G,H) to each other as inferred from the red globules. Origin and fate of such cells could not be determined but rarely, presumable exuviae lay on the ground of the vessels. Two ovoid coccoid cells, connected by a stalk-like structure of unknown material (Figure 4A), were a unique observation. Motile, and fusing cells were never observed.

DNA-metabarcoding and molecular phylogenectis

In the metabarcoding project, we found an ASV being identical to the DNA sequence gained from strain GeoK*077. This ASV represented the second most abundant dinophyte of the entire data set. It was detected between Nov 2021 and Mar 2022, but was entirely absent since April 2022 (Figure 6). This is the time around the vernal equinox, when the day length is increasing fastest, but a month before water temperature increased perceptibly. We included the corresponding DNA sequence also in the phylogenetic analysis.

The SSU + ITS + LSU alignment was 1818 + 789 + 3492 bp long and comprised 369 + 545 + 933 parsimony informative sites (30.3%, mean of 26.4 per terminal taxon) and 3144 distinct RAxML alignment patterns. The internal topology of the best-scoring ML tree (Figure 7) showed high if not maximal statistical support for many crucial nodes. †Suessiales (100 LBS, 1.00 BPP) were monophyletic

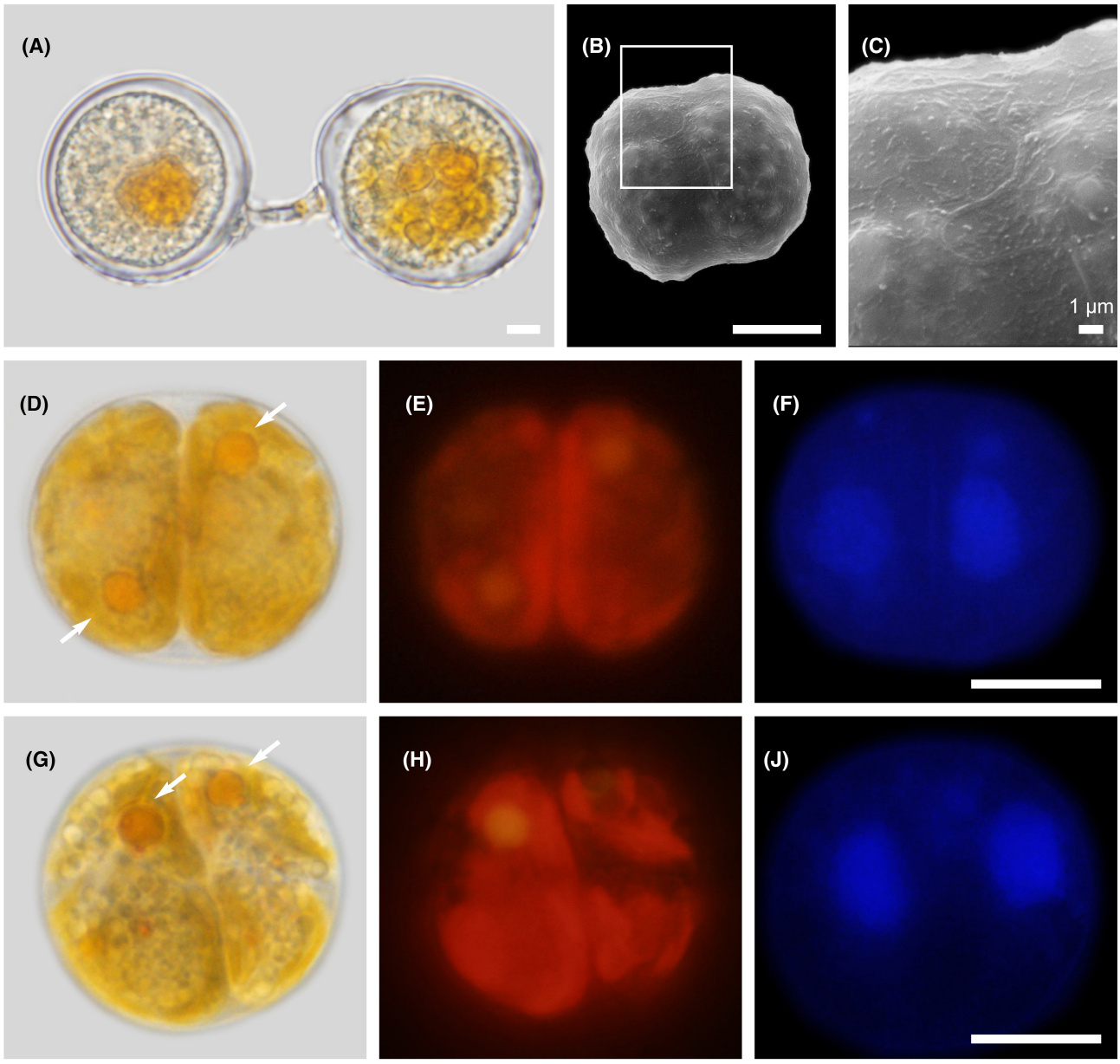

FIGURE 4 Cell pairs of *Borghiella ovum* (GeoK*077) in LM (A, D, G), fluorescence LM (E, F, H–J), and SEM (B, C). (A) Two coccoid cells connected by an unknown structure (unique observation). (B, C) Presumably, two cells are enclosed in one pellicle, in between showing a seam-like structure on the cell surface (magnification in C). (D–F) The same two cells share one pellicle in point inversion (as inferred from red globules in distinct positions), each with a single nucleus (F DAPI-stained). (G–J) The same two cells share one pellicle in axial symmetry (as inferred from red globules in distinct positions), each with a single nucleus (f DAPI-stained). Scale bar: 10 µm if not otherwise stated.

with respect to the outgroup (Gymnodiniales: 83 LBS, 0.98 BPP and Dinophysales: 100 LBS, 1.00 BPP) and segregated into three lineages, namely Glenodiniaceae (79 LBS, 0.98 BPP), Borghiellaceae (100 LBS, 1.00 BPP) and Symbiodiniaceae sensu lato (*s.l.*; 100 LBS, 1.00 BPP). *Baldinia* (97 LBS, 1.00 BPP) was part of Glenodiniaceae, but not of Borghiellaceae, and showed a close relationship to a lineage including accessions of *Cystodinium* G.A.Klebs and *Phytodinium* G.A.Klebs (100 LBS, 1.00 BPP). However, *Cystodinium* was polyphyletic, and another accession was nested in *Glenodinium* Ehrenb. (= *Sphaerodinium* Wołosz.: 100 LBS, 1.00 BPP).

All species of *Borghiella* with DNA sequence information available [i.e. *B. andersenii* Daugbjerg, Andreasen, Happel, Pandeir., Gert Hansen, Craveiro, Calado & Moestrup, *B. dodgei* Moestrup, Gert Hansen & Daugbjerg, *B. pascheri* (Suchl.) Moestrup, *B. ovum*, sp. nov., *B. tenuissima* (Lauterborn) Moestrup, Gert Hansen & Daugberg, *B. verrucosa* (Baumeister) Knechtel & Gottschling] were distinct from each other, but clear relationships within Borghiellaceae could not be inferred. Additionally to DNA sequences gained from strain GeoK*077 (equivalent to the type), the new species was detected by an identical environmental DNA amplicon

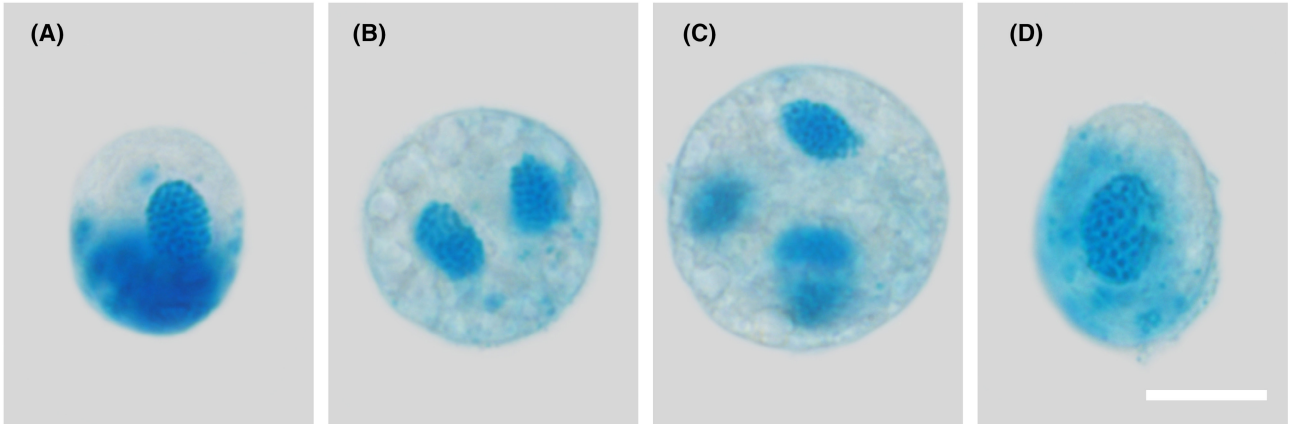

FIGURE 5 Type material of *Borghiella ovum* (GeoK*077) in LM, note the distinct staining, particularly of the nucleus exhibiting the condensed chromosomes. (A) Presumable flagellated cells, note that many such cells of the slides show unequal distribution of stained material either in the epi- or hyposome. (B, C) Two and four cells share one pellicle, as inferred from the stained nuclei. (D) Presumable coccoid cell of ovoid shape, note that the stained cytoplasmic material is also unequal in distribution. Scale bar: 10 μm.

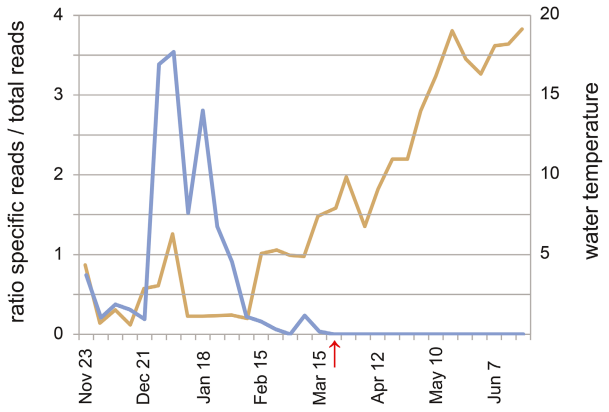

FIGURE 6 Temporal dynamics of *Borghiella ovum* (ASV0054) in the Botanical Garden Munich-Nymphenburg as inferred from metabarcoding data, note that the species is absent during May through October in the plankton. The blue line shows reads of *Borghiella ovum*; the brown line represents the temperature; the red arrow indicates the equinox.

(ASV0054) and a slightly deviating GenBank sequence from Lake Baikal. The distinctiveness in the V4 region of the SSU between *B. ovum* and all other species of *Borghiella*, from which corresponding sequences were available, was also displayed in a distance tree inferred from the results of a NCBI Blast Search (Figure S1).

DISCUSSION

The second most abundant dinophyte in the metabarcoding study has no name but is the same as GeoK*077

There are sanguine opinions that the inventory of all species will be largely completed within the next few decades (Costello et al., 2013). This may certainly be the case for morphologically well-recognizable taxa although even here, the discovery of many new species from remote habitats such as the deep sea or the last dense patches of tropical rainforests could take longer than expected. Moreover, DNA sequencing techniques have led to the recognition of genetically but not morphologically differentiated (so-called cryptic) species across all taxonomic groups (Bickford et al., 2007; Caron et al., 2012; Struck et al., 2018), most of which have not yet been formally assessed. With respect to the morphology of the flagellated cells, *Borghiella* appears as well as a species complex rather than easily discernible taxonomic units.

The situation of biodiversity assessment still seems particularly precarious in the microscopy realm, where species are often only recognizable to a limited extent due to the lack of diagnostic traits. Especially, the metabarcoding studies of recent years with their extensive share of dark diversity (Jang, 2022; Mahé et al., 2017) have contributed to this insight. Some of these studies have been the basis for research into hitherto completely unknown evolutionary lineages such as *Picomonas* Seenivasan, Sausen, Medlin & Melkonian (Seenivasan et al., 2013) or *Ancoracysta* Janoušk., Tikhonenkov, Burki, A.T.Howe, F.L.Rohwer, A.P.Mylnikov & P.J.Keeling (Janouškovec et al., 2017) with isolated positions in the Tree of Life. Under the impression of these approaches, it is hardly conceivable to completely inventory the entirety of microscopic species from soil and sediments as well as from the sea and freshwater or even the phyllosphere in the near future (at least, this would require larger human capacities than are currently available).

These conclusions are also supported by the current study: If there are still frequent species in the microscopy realm already among phototrophic organisms (which are easy to culture) from supposedly well-studied regions that have not yet been recognized, such as *B. ovum*, then the magnitude of the task is incalculable for heterotrophic organisms (which are difficult or impossible to

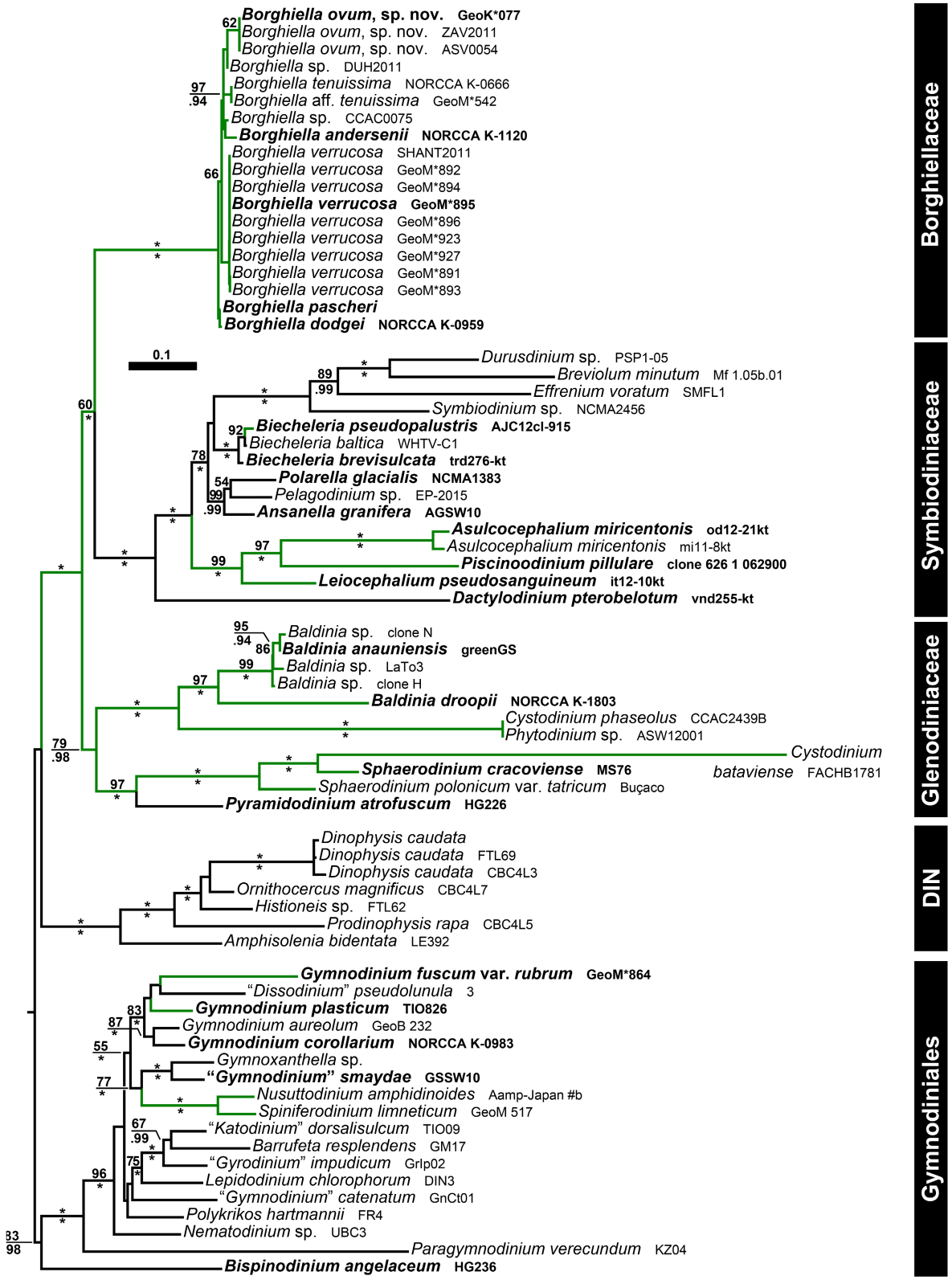

FIGURE 7 Molecular phylogeny of †Suessiales (= Phytodiniales). Maximum likelihood (ML) reference tree of systematically representative suessialean accessions and all sequences available from *Borghiella* (with strain number information) as inferred from a SSU+ITS+LSU nucleotide alignment (1847 parsimony-informative positions). Major clades are indicated, and sequences gained from type material (or equivalents) are highlighted in bold. Freshwater taxa are indicated by green color. Numbers on branches are ML bootstrap (above) and Bayesian support values (below) for the clusters (asterisks indicate maximal support values, values under 50 and 0.90, respectively, are not shown). DIN, Dinophysales. Note that there are environmental DNA sequences identical to those obtained from holotype equivalents of *Borghiella ovum*.

cultivate) from more remote habitats. In addition, many species show complex interactions with other organisms (for example parasites or endosymbionts), which are even more complex to inventory. As long as we do not know the true biodiversity and its species, it is impossible to even approximate the importance of these organisms for the ecosystem and their services.

Taxonomic delimitation

The species of Borghiellaceae are difficult to delimitate in LM, but are clearly distinct by DNA-sequence comparison. This also applies to the presented new species, *B. ovum*. There are currently eight species assigned to *Borghiella* (Moestrup & Calado, 2018), five of which with linked DNA-sequence information (see also the comparative table 1 in Knechtel et al., 2020). Morphologically, *Baldinia* is different from *Borghiella* due to the absence of a LAC (observable only in SEM) and the presence of a central pyrenoid, from which the chloroplast radiates (Moestrup & Calado, 2018). Species of *Borghiella* including *B. ovum* have multiple chloroplasts, distributed in the periphery of the cell as demonstrated also in the present study, and may prefer colder habitats than *Baldinia*, which appears as an important ecological trait for taxonomic delimitation.

Flagellated cells of *Borghiella sylvatica* (Er.Lindem.) Moestrup (Lindemann, 1923) and *B. tenuissima* (Lauterborn, 1894) are larger and dorsoventrally more flattened than those of the other *Borghiella* species including *B. ovum*. Moreover, *B. pascheri* (Suchlandt, 1916) is distinct because of the carmine-red cell coloration and the snow habitat, forming sometimes extensive patches in the field (Nicholls, 2017) similar to the green algae *Chlamydomonas nivalis* (F.A.Bauer) Wille (Hoham & Remias, 2020). The flagellated cells of the remaining *Borghiella* species including *B. ovum* are overall very similar. There is some variation in the general shape of the cells and size and constitution of the LAC but with the exception of the three mentioned species, flagellated cells of *Borghiella* cannot be determined reliably without DNA-sequence information.

It is the morphology of the coccoid cells that allows distinction, at least of some *Borghiella* species. Species other than *B. ovum* have coccoid cells of spherical through ellipsoid (*B. andersenii*: Daugbjerg et al., 2014, *B. dodgei*: Flaim et al., 2010), sometimes obtusely polygonal shape (*B. sylvatica*: Lindemann, 1923), occasionally with a notably thick shell (*B. pascheri*: Suchlandt, 1916) and mostly with smooth or exceptionally wrinkled surface (*B. verrucosa*, why it was initially described under *Dinastridium* Pascher: Knechtel et al., 2020). *Borghiella ovum* is unique by the characteristically ovoid shape of the coccoid cell, primarily caused by different thickness of the coat on opposite poles of the cell as demonstrated in LM. Even in the admirable compilation of all dinophyte coccoid cells documented from freshwater habitats (Mertens et al., 2012), no cell comparable to *B. ovum* is illustrated.

For *B. ovum*, the only issue with the morphological approach for species recognition is the coccoid cell of *B. pascheri* likewise having varying thickness of the coat occasionally (Moestrup et al., 2018). However, *B. pascheri* shows intensely carmine-red coloration (Moestrup et al., 2018; Nicholls, 2017) rather than the golden-brown coloration widely present in dinophytes such as *B. ovum*. Moreover, roughly similar coccoid cells are reported from the marine environment, but they are assigned to taxonomically different lineages (e.g. scrippsielloid: Satta et al., 2013, gonyaulacoid: Matsuoka & Fukuyo, 2000). The developmental origin of the ovoid coccoid cells remains elusive in *B. ovum*—as fusing cells (i.e. gametes) have never been observed in the cultured material of the present study, vegetative production by mitosis is likely. Therefore, they can be expected to have the same ploidy level as flagellated cells. As autofluorescence of chlorophyll has been detected, such coccoid cells are potentially photosynthetically active and rather do not represent developmental stages of dormancy.

Overall, little is known about mitotic cell division of flagellated cells across suessialean dinophytes. However, the second type of coccoid cells of *B. ovum* can be interpreted as the division stage (functionally a sporocyst) and the expression of what is known in dinophytes as eleuteroschisis (Bold & Wynne, 1978). Two (or four or eight) cells included in a shared pellicle have been variously documented from peridinialean (Kretschmann et al., 2018; Schilling, 1891) and tovellialean dinophytes (Lindemann, 1929; Pandeirada et al., 2017), but also from suessialean *Borghiella* (Daugbjerg et al., 2014; Lindemann, 1929; Nicholls, 2017). As no processes such as cell fusion have been observed in *B. ovum*, those cells likely represent vegetative replication rather than sexual reproduction, and this might also be true for sporocysts including four cells and therefore only putatively indicating meiosis. More research is needed to relate division modes with other evolutionary traits and phylogenetic relationships in dinophytes.

In ultrastructure studies, an intraplastidial type B eyespot (Moestrup & Daugbjerg, 2007) has been shown for *Borghiella*, so that the presence of such an organelle (though difficult to observe in LM) does not come as a surprise in *B. ovum*. Whether the absence of an eyespot in *Borghiella marylandica* (R.H.Thompson) Moestrup, *B. sylvatica*, *B. tenuissima*, and *Borghiella woloszynskae* (Pascher) Moestrup is true (the structure might be very inconspicuous as in *B. pascheri*: Moestrup et al., 2018), remains a topic for future research.

Biological implications

Previous DNA-metabarcoding studies in the microscopy realm have considered large taxonomic groups rather than particular species and have an emphasis on the spatial (Boenigk et al., 2018; Gollnisch, 2022; Rimet

et al., 2018; Šupraha et al., 2022) rather than the temporal occurrence of protists (Bruhn et al., 2021; Mordret et al., 2023; Siano et al., 2021; Sildever et al., In press). Water temperature is considered one of the most important environmental variables filtering the presence of protists in a given habitat (Rose & Caron, 2007; Weisse et al., 2016). This is certainly true for *B. ovum*, which is detected at values below 6°C in the field (and is also maintained in cultivation at rather low temperatures, see Materials and Methods). However, the decline in late winter during the course of a year starts much earlier than the rise of the water temperature and hence, the latter cannot be considered the trigger for the development. Recently, photoperiod in temperate habitats is identified as another dominant factor related to protist turnover and community replacement (Longobardi et al., 2022) and thus day-length change (particularly pronounced at the equinox of March 21) may better explain the trajectories of winter-dominant dinophytes such as *B. ovum* than water temperature alone.

As in many other protist groups, a stable dinophyte taxonomy is still only developing. The synonymization of *Glenodinium* and *Sphaerodinium*, for example, is not universally accepted (Moestrup & Calado, 2018), but we sympathize with the taxonomic conclusions of Wołoszyńska (1918), Loeblich I-II (1980), and Fensome et al. (1993) that in fact, *Glenodinium*, with its type species *Glenodinium cinctum* Ehrenb., is the accepted name. Furthermore, the possible relationship between *Baldinia* and *Borghiella* was only moderately through weakly supported before the present study, in which comprehensive alignments of concatenated sequences from different rRNA regions are evaluated. As a result, *Baldinia* appears feasibly supported as an element of Glenodiniaceae rather than Borghiellaceae and thus, the present approach might outperform phylogenetic analyses based on separate SSU, ITS, or LSU alignments.

It is particularly notable that accessions of the morphologically distinct, crescent-shaped *Cystodinium* (including a new, long rRNA sequence of strain CCAC 2439B) do not constitute a monophyletic group: *Cystodinium phaseolus* Pascher together with an organism determined as *Phytodinium* sp. constitute the sister lineage of *Baldinia*, while a Chinese, morphologically documented accession of *Cystodinium bataviense* G.A.Klebs (张琪 et al., 2015), the type species of *Cystodinium*, appears nested within *Glenodinium*. The latter is characterized by a unique horseshoe-shaped eyespot that is found in dinophytes from freshwater habitats only (Craveiro et al., 2010; Ehrenberg, 1837; Wołoszyńska, 1916). A similar structure is reported from species of more distantly related *Tovellia* Moestrup, K. Lindb. & Daugbjerg (Wołoszyńska, 1918), but also from some species of apparently more closely related *Cystodinium* (Pascher, 1928). The molecular tree presented here raises the question of whether this unusual (type F) eyespot is homologous at least between *Cystodinium* and *Glenodinium*. Much more research is necessary to disentangle phylogenetics and character evolution of glenodiniacean dinophytes.

The identification of three highly supported lineages in molecular phylogenetics, namely Glenodiniaceae, Borghiellaceae, and Symbiodiniaceae *s.l.*, is a clear advantage for an improved classification of suessialean dinophytes. †*Suessia* Morbey comprises fossils from the Triassic Period (Helby et al., 1987; Morbey, 1975) and is the type of the †Suessiales. A group of similar fossils is documented from the first half of the Mesozoic, which has been extinct since the Jurassic (Fensome et al., 1993). Despite a fossil gap of ca 180 Ma, extant forms such as *Polarella* Montresor, Procaccini & Stoecker, and the coral endosymbionts of *Symbiodinium* Freud. ex Gert Hansen & Daugbjerg have been associated with this fossil group (Loeblich III, 1984; Montresor et al., 1999). However, contemporary molecular phylogenetics shows that such dinophytes are deeply nested in the DNA trees, and even the stem group has been dated not older than 207 mya (Chacón & Gottschling, 2020). As long as the relationship between the fossils and the extant forms is not reliably established, usage of available non-fossil names for supraspecific taxa such as Symbiodiniaceae (instead of †Suessiaceae: Moestrup & Calado, 2018) and Lophodiniales or Phytodiniales (instead of †Suessiales) appear more appropriate, as also already proposed previously (Janouškovec et al., 2017). Irrespective of the superordinate names, the taxonomic assessment of this group comprises much dark diversity and is far from being completed, as it is also illustrated by the inventory of *B. ovum* presented here.

FORMAL TAXONOMY

Borghiella ovum **A.Müll.bis & Gottschling**, sp. nov.—TYPE [slide with non-fossil specimens]: Germany: Bavaria, Lower Bavaria, Rottal-Inn, Reut (48°18.715′ N, 12°56.484′ E, 448 m), 11 Nov 2020: M. Gottschling & S. Schottenhammel [S. Schottenhammel GeoK*077] D221 (holotype, designated here: CEDiT-2023H171!, isotypes, designated here: B 400046321! M-0331206!) [http://phycobank.org/104050].

Description: Dinophytes small, phototrophic, and athecate. Flagellated cells 22 µm long, 17 µm wide, widely through very widely ovoid; surface made of pentagonal or hexagonal vesicles; LAC 3 µm long. Compartments distinct; nucleus in a central position; chloroplasts numerous, in the periphery of the cells; eyespots disc-shaped. Coccoid cells 25 µm long, 20 µm wide, ovoid, resembling the longisection of a rotationally symmetric egg; surface smooth. Division stages present; two cells included in the pellicle.

Note: A detailed description of the strain, from which the type material was prepared, is provided in the Results section, and a diagnosis in the Discussion section. More original material is available as B 400046320! CEDiT-2023RM172! M-0331203! M-0331204!

Etymology: The epithet refers to the shape of the coccoid cells.

Note: The taxonomy presented here follows the rules of the International Code of Nomenclature for algae, fungi, and plants (ICN; Turland et al., 2018).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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