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ORIGINAL ARTICLE

Pseudovampyrella gen. nov.: A genus of *Vampyrella*-like protoplast extractors finds its place in the Leptophryidae

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Abstract

Vampyrellid amoebae are predatory protists, which consume a variety of eukaryotic prey and inhabit freshwater, marine and terrestrial ecosystems. Although they have been known for almost 150 years, much of their diversity lacks an in-depth characterization. To date, environmental sequencing data hint at several uncharacterized lineages, to which no phenotype is associated. Furthermore, there are numerous historically described species without any molecular information. This study reports on two new vampyrellid strains from moorlands, which extract the protoplasts of *Closterium* species (Zygnematophyceae). Our data on morphology, prey range specificity and feeding strategy reveal that the studied vampyrellids are very similar to the historically described *Vampyrella closterii*. However, phylogenetic analyses demonstrate that the two strains do not belong to the genus *Vampyrella* and, instead, form a distinct clade in the family Leptophryidae. Hence, we introduce a new genus of algivorous protoplast extractors, *Pseudovampyrella* gen. nov., with the species *P. closterii* (= *V. closterii*) and *P. minor*. Our findings indicate that the genetic diversity of morphologically described vampyrellid species might be hugely underrated.

KEYWORDS
algae, amoebae, Cercozoa, Endomyxa, Rhizaria, rhizopoda, Zygnematophyceae

INTRODUCTION

THE vampyrellid amoebae (Vampyrellida, Rhizaria) represent a morphologically and ecologically diverse order of predatory protists, which prey on a variety of (mainly) eukaryotic prey including streptophyte and chlorophyte green algae, cryptophytes, diatoms, fungi and even microscopic animals (Cienkowski, 1865; Grell, 1985; Hess, 2017a, 2017b; Hess et al., 2012; More et al., 2019, 2021; Röpstorf et al., 1994; Zopf, 1885; Zwillenberg, 1953). They inhabit freshwater, marine and terrestrial ecosystems and are globally distributed—from mountain glaciers to the deep sea (Schoenle et al., 2021; Vimercati et al., 2019). Vampyrellids have been known for almost 150 years, and yet much of their diversity lacks an in-depth characterization. Currently, there are five phenotypically known subclades, four of which are formally described as families, plus several uncharacterized lineages that have only been detected by environmental sequencing (Berney et al., 2013; Hess & Suthaus, 2022). In addition, there are numerous historically described taxa which still lack a molecular identity and phylogenetic placement. For example, there are more than 20 described species for the genus *Vampyrella* Cienkowski, 1865 (Hess & Suthaus, 2022), but so far only three of them (*V. lateritia*, *V. pendula* and *V. vorax*) were rediscovered and subjected to phylogenetic studies (Hess et al., 2012). The fact that *V. vorax* (currently treated as *Leptophrys vorax*) is phylogenetically distinct from the other two species and belongs to a different

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family (Leptophryidae) indicates that the prediction of phylogenetic relationships and generic affiliations based on morphology alone is extremely difficult for vampyrellids. Hence, some of the other *Vampyrella* species described in the historic literature might turn out to belong to other genera or even families as well.

Here, we report on two new vampyrellid strains (VC.01 and VC.02) from the acidic ponds of moorlands. These amoebae extract the protoplasts of *Closterium* species (Zygnematophyceae, Streptophyta) after annular perforation of the algal cell walls. They possess an orange cytoplasm, multiple nuclei and filopodia studded with numerous motile granules ("membranosomes"); known differences between strains VC.01 and VC.02 concern the cell size and prey range. Overall, the new strains resemble *Vampyrella closterii* Poisson & Mangenot, 1933, a specific predator of *Closterium* species that was originally discovered in the moors of Bretony and still lacks phylogenetic placement (Poisson & Mangenot, 1933). Based on molecular data (SSU rRNA gene phylogenies), we demonstrate that the two new strains have no close affinity to the other sequenced *Vampyrella* species (including the type species *V. pendula*). Instead, they form a formerly unrecognized branch of the family Leptophryidae. Because of the surprising phylogenetic position and the distinctive feeding habit among the known leptophryid amoebae, we introduce a new genus, *Pseudovampyrella* gen. nov., with the species *P. closterii* comb. nov. (for strain VC.01) and *P. minor* sp. nov. (for strain VC.02).

MATERIALS AND METHODS

Establishment and maintenance of cultures

Cells of strain VC.01 were discovered feeding on *Closterium gracile* in samples from the Thielenbrucher Moor, Cologne, Germany. Those of strain VC.02 were discovered feeding on an unknown strain of *Closterium* in samples from the Simmelried Moor, Constance, Germany. Vampyrellid cells were isolated from natural samples with glass micropipettes as described in Hess et al. (2012) and maintained in half-strength Waris-H medium (W/2) at 15°C under dim light from a white LED (14:10 h light: dark cycle, photon fluence rate 10–30 μmol m^{-2} s^{-1}). The cultures were fed every 2 weeks with *Closterium cornu* (strain CCAC 1125; for VC.01) and *Closterium limneticum* (strain CCAC 2687; for VC.02). The vampyrellid cultures and prey algae are available from the corresponding author (S.H.) upon request.

Light microscopy

Vampyrellid cultures and feeding experiments were observed and documented with the Motic AE2000 (Motic Hong Kong Limited) inverted microscope equipped with brightfield and phase contrast optics and with a MikroLive 6.4MP CMOS camera (MikroLive, Oppenau). High-resolution micrographs and fluorescence images were taken with the ZEISS Axio Observer 7 inverted microscope (ZEISS), equipped with differential interference contrast optics, a digital CCD camera (ZEISS Axiocam 512 color) as well as the objective lenses Plan-Neofluar 20×/0.5, Plan-Neofluar 40×/1.3 and Plan-Neofluar 100×/1.3. Dimensions of cells and cellular structures were measured with Fiji (Schindelin et al., 2012). Nuclei of live cells were stained with Hoechst 33528 (Invitrogen) at 1 μg/mL for 5 min at room temperature and then imaged with the above-mentioned Zeiss Axio Observer 7 equipped with the ZEISS Colibri 5 LED illumination system (RGB-UV) and the filter set 96 HE BFP (excitation 390/40 nm, emission 450/40 nm). Adobe Photoshop CS4 (Adobe Systems) was used to adjust color balance and contrast of light micrographs.

Feeding experiments

Microalgae for feeding experiments (see results for a list of strains) were obtained from Karl Heinz Linne von Berg (private collection; University of Cologne), from natural samples as well as from the Central Collection of Algae Cultures (CCAC) of the University of Duisburg Essen (https://www.uni-due.de/biology/ccac/). New microalgae used in this study were identified by Karl Heinz Linne von Berg or with an identification key (Lenzenweger, 1996) based on morphology, sometimes tentatively. Taxa which could not be identified to the species level based on morphological characteristics were kept unidentified. Potential prey cultures were suspended in sterile W/2 medium, distributed to petri dishes and inoculated with starving trophozoites of strains VC.01 and VC.02, respectively. The cultures were monitored daily for 2 weeks to determine whether the vampyrellids were fed and multiplied. Feeding was identified based on the presence of emptied prey cells and the digestive cysts. The results were summarized in two categories, feeding and growth (+) and no feeding and no growth (−). The algal cultures used in the feeding experiments are available from the corresponding author or from the CCAC (except strains N044 *Closterium praelongum* and N30 *Closterium kuetzingii*; deceased).

DNA amplification, sequencing and sequence assembly

Single starving trophozoites were isolated from cultures with the aid of a glass micropipette, washed several times in nuclease-free water and subjected to whole genome amplification using the Repli-G Advanced Single Cell Kit (Qiagen GmbH). After amplification, the DNA was diluted 1:100 in nuclease-free water and used as a

template for PCR. The nuclear SSU rRNA gene was amplified using the DreamTaq™ DNA Polymerase (Fermentas) according to the manufacturer's instructions with the universal eukaryotic primers EukA and EukB (Medlin et al., 1988). The following PCR protocol was used: an initial denaturation step (95°C for 180″) was followed by 30 cycles including denaturation (95°C for 45″), annealing (55°C for 60″) and elongation (72°C for 180″) with a final elongation (72°C for 300″). PCR products that showed single clear bands by gel electrophoresis were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and subjected to commercial Sanger sequencing (Eurofins Scientific) with the primers mentioned above. The partial sequences resulting from forward and reverse sequencing reactions were quality-checked and assembled with AlignIR (LI-COR Biosciences). Doubtful nucleotide reads at the beginning and the end of the sequences were cropped. The resulting SSU rRNA gene sequences of strains VC.01 (1714 bp) and VC.02 (1647 bp) were used for assessing the pairwise distance and deposited at GenBank under the accession numbers OQ591878 and OQ591879, respectively.

Alignments and phylogenetic analyses

BLAST searches with the SSU rRNA gene sequences of strains VC.01 and VC.02 (nr/nt database; https://blast.ncbi.nlm.nih.gov/Blast.cgi) returned hits associated with vampyrellid amoebae with high genetic identity (e.g. *Theratromyxa weberi* GQ377666, 94.14%). The sequences were added to an alignment of vampyrellid SSU rRNA sequences, which was used in a previous study (Hess & Suthaus, 2022), and aligned with MUSCLE in Seaview 5.0.4, followed by manual curation. We performed maximum likelihood (ML) analyses with raxmlGUI 2.0.5 (Edler et al., 2021) using the model GTR+ Γ+I and 100 runs and retrieved bootstrap support values with 1000 replicates. Additionally, we performed Bayesian Inference (BI) with Beast 2.7.3. (Bouckaert et al., 2014) using the model GTR+ Γ+I, 5,000,000 generations (trees sampled every 1000 generations) and a 25% burn-in (1,250,000 generations discarded). Stationarity of the BI was confirmed using Tracer 1.7.2. (Rambaut et al., 2018). As previous studies robustly recovered the Sericomyxidae (formerly known as "Clade C") as the deepest branching clade of the vampyrellids (Berney et al., 2013; More et al., 2021), we here used this clade to root the phylogenetic trees.

Scanning electron microscopy

The remnants of *Closterium* cells that remained in the vampyrellid cultures were placed on poly-L-lysine (Sigma-Aldrich) coated coverslips and allowed to settle for 30 min. Subsequently, the coverslips were passed through a series of water:ethanol mixtures of increasing ethanol concentrations (50–70–100%; 5 min each), then incubated in hexamethyldisilazane (Carl Roth) for 10 min and air-dried after removal of excess liquid. The dry sampled were sputter-coated with gold and examined with a ZEISS Neon 40 scanning electron microscope at 2.5 kV acceleration voltage (ZEISS).

Generation of type material

Vampyrellid cultures were grown in small petri dishes (diameter 55 mm, VWR Collection). Once suitably dense, the cells were fixed in the petri dish using 2.5% glutaraldehyde in MT buffer (30 mM HEPES, 15 mM KCl, 5 mM $MgSO_4$, 5 mM EGTA, 100 µM DTT, pH 7.0) for 20 min at room temperature. The glutaraldehyde fixative was removed, the cells rinsed with distilled water, postfixed with an aqueous solution of osmium tetroxide (0.04%) for 20 min, then rinsed twice with water and detached from the petri dish with a cell scraper. The suspended cells were applied to a poly-L-lysine coated coverslip and let sediment for 30 min. After a centrifugation step (10 min at 1000 *g*), the cell-bearing coverslips were passed through an ascending series of ethanol solutions (20–40–60–80–90–100%, 5 min each), then incubated in 100% isopropanol for 20 min and finally mounted with 1:1 Euparal resin (Carl Roth)/Isopropanol.

Nomenclatural acts

This published work and the nomenclatural acts it contains have been registered in ZooBank (http://zoobank.org/), the proposed online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information can be viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/." The LSID for this publication is as follows: urn:lsid:zoobank.org:pub:57ADAF8F-DD52-4615-A362-9EE7C28DD7C9.

RESULTS

Phylogenetic position of *Pseudovampyrella* species

BLAST searches of the SSU rRNA gene sequences of both strains, VC.01 and VC.02, clearly indicated a vampyrellid affinity, with >93% similarity to sequences of *Theratromyxa weberi* (GQ377666), *Kinopus chlorellivorus* (MW694332), *Platyreta germanica* (AY941201) and other vampyrellid representatives. Hence, we analyzed the sequences in the framework of a published Vampyrellida dataset (Hess & Suthaus, 2022) that was

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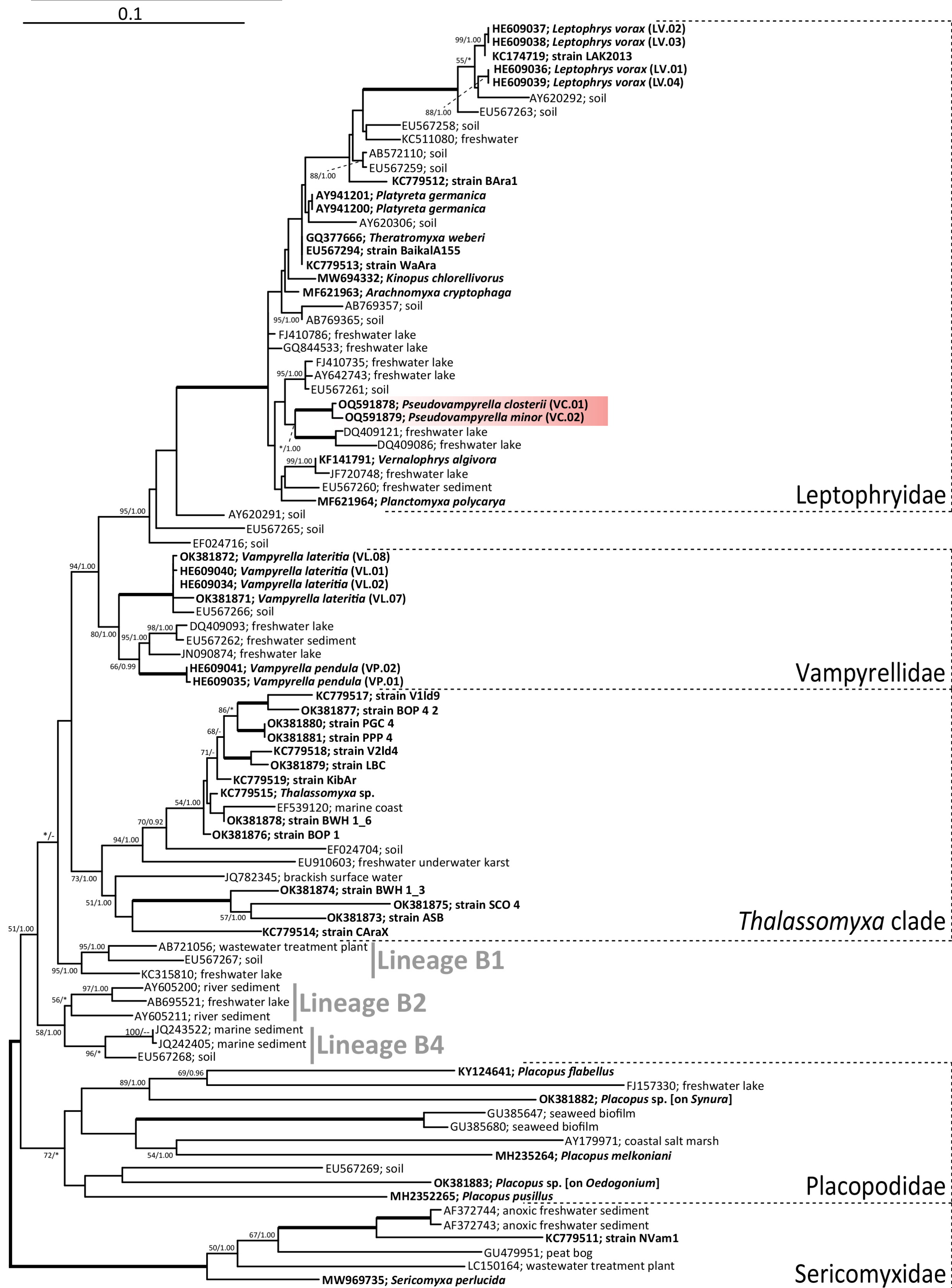

FIGURE 1 Maximum likelihood phylogeny of the Vampyrellida inferred from SSU rRNA gene sequences (91 sequences, 1497 sites, GTR+Γ+I model). The best tree of 100 inferences was rooted with the deepest branching clade (Sericomyxidae) and shows bootstrap support values (ML) and posterior probabilities (BI) at the branches (ML/BI), except for bold branches that have full support (100/1.00) or support <50/0.9 (* or omitted). Hyphens (−) denote branches that were not present in the resulting tree from BI. Sequences with associated phenotypic information (cultures or imaged cells) are in bold. The scale bar corresponds to 0.1 expected substitutions per site.

updated with the sequence of the recently described vampyrellid *Kinopus chlorellivorus* (Zhang et al., 2022). The best ML tree obtained resolved all known vampyrellid lineages, but with varying support (Figure 1). BI showed similar results as indicated by the bootstrap values. The Leptophryidae were well supported (100/1.00); however, as previously reported (Hess, 2017b) the internal branching of this family remained largely unresolved. The strains VC.01 and VC.02 formed a clade within the family Leptophryidae with maximum support, most closely related to environmental sequences with no associated phenotype. Strains VC.01 and VC.02 were clearly distant from the known leptophryid genera *Leptophrys*, *Platyreta*, *Theratromyxa*, *Arachnomyxa*, *Planctomyxa*, *Vernalophrys** and *Kinopus*. Comparisons of the near full-length SSU rRNA gene sequences of the two studied strains revealed a genetic identity of 99.39% (seven mismatches in 1642 aligned nucleotides).

Description of Pseudovampyrella closterii comb. nov., strain VC.01

Trophozoite morphology: The trophozoites in actively growing cultures were isodiametric or fan-shaped and mostly exhibited a crawling locomotion along the substrate (Figure 2A,B). However, cells could also float in the water column, then being spherical with radiating filopodia. The trophozoites generally appeared to be flexible as indicated by temporary deformations and typically ranged from 30 to 100 μm in size. During locomotion over the substrate, the trophozoites formed tapering, unbranched filopodia at the leading edge (anterior), which traveled toward the cell's posterior as the trophozoite progressed and finally merged with the main body. These filopodia measured up to 50 μm in length and exhibited a large number of refractive, motile granules (about 1 μm in diameter) at their basal portion (Figure 2A–C), which resemble the "membranosomes" described for *Vampyrella lateritia* (Hülsmann, 1985). The main cell body was characterized by an opaque, granular cytoplasm of orange color. As revealed by fluorescence microscopy of Hoechst-stained cells, the trophozoites contained multiple nuclei of about 3 μm in diameter (Figure 2D). These nuclei, each with a single nucleolus, were very indistinct in transmitted light microscopy, but could be observed in squashed cells (Figure 2E). Strain VC.01 readily formed plasmodia by cell fusion when cultures grew older and food was almost depleted (Figure 2F). The plasmodia exhibited various shapes, including fan-like or ribbon-like morphologies, sometimes exceeding 1 mm in size. On occasion, the plasmodia split into smaller units. In some instances, we observed trophozoites whose filopodia lacked the characteristic motile granules. The origin, function and fate of these cells are currently unknown.

Feeding and cyst stages: Six out of 22 tested *Closterium* strains proved suitable as food for strain VC.01 (Table 1). Other desmids (e.g. *Euastrum*, *Micrasterias*, *Netrium*, *Penium*, *Pleurotaenium*) as well as other common prey algae of vampyrellids of diverse phylogenetic affiliation and structure (e.g. *Oedogonium*, *Zygnema*, *Chroomonas*) were not consumed (Table 1). The trophozoites ingested the cell contents of suitable *Closterium* strains by typical protoplast extraction as known from *Vampyrella* and *Placopus* species (Hess, 2017a; Hess et al., 2012). Before the feeding act, *P. closterii* approached the *Closterium* cell most often in the fan-like configuration, whereupon it crawled across the algal cell surface and then attached firmly to its prey, frequently at the cell poles (Figure 3A). The lysis of the algal cell wall occurred in 15–60 min, as indicated by the rupturing *Closterium* cell and rapid movement of cell contents into a large feeding cup (the future digestive vacuole) of the trophozoite (Figure 3A). Once these turgor-driven processes ceased, the trophozoite withdrew the remaining algal protoplast with a hyaline-feeding pseudopodium. Time-lapse photography revealed that the large algal chloroplast was partitioned into smaller pieces during the process (Video S1). In most cases, the entire algal protoplast was consumed. Upon completion, the trophozoite withdrew the feeding pseudopodium and either left the alga or developed into a digestive cyst. The algal remains exhibited elliptic perforations of about 10 μm, frequently with a "lid" or "flap" due to annular degradation of the algal wall (see below for details). Strain VC.01 was observed to extract one to three *Closterium* cells before forming a digestive cyst. Well-fed trophozoites contained numerous food vacuoles with algal contents in different stages of digestion as indicated by the green to brown color (Figure 3B), as did young digestive cysts (Figure 3C). Over a period of 1–2 days, the cytoplasm of the digestive cyst turned orange (Figure 3D) and the food remnants accumulated in a central vacuole, typically in the form of brown granule (Figure 3E). Digestive cysts had an oval or round outline, measured 30–60 μm in their largest dimension and were always attached to a substrate, frequently at the cell wall of *Closterium* cells in the live or emptied state (Figure 3E). Digestive cysts possessed a thin, smooth velum, which stuck closely to the secondary cyst wall (Figure 3D). When digestion was completed, the trophozoite(s) hatched from the cyst through one or several holes (Figure 3F). Strain VC.01 divided by external plasmotomy, that is during or after hatching. Different domains of the cell moved in opposite directions, so that the cell lengthened and finally split as the connecting strand(s) between the domains ruptured (Figure 3F). In older cultures when food algae were scarce or conditions adverse, VC.01 formed orange resting cysts (spores) of up to 28 μm in size. These developed within digestive cysts as indicated by the presence of food remnants and the digestive cyst wall (Figure 3H).

* We here did a mandatory change in spelling of *Vernalophrys algivora* in accordance with articles 31.2. (Agreement in gender) and 34.2. (Species-group names: mandatory change in spelling) of The International Code of Zoological Nomenclature (ICZN, 1999).

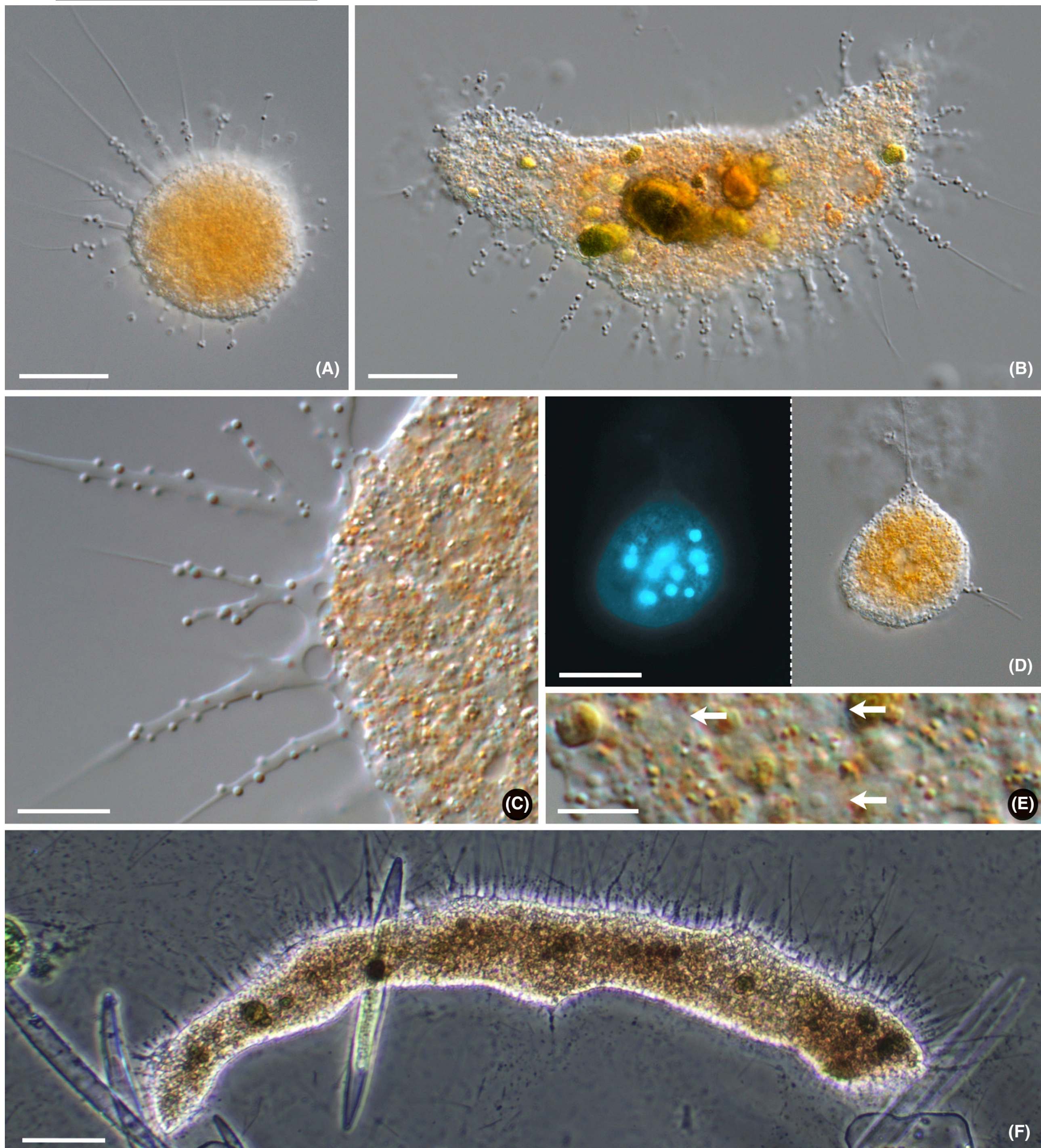

FIGURE 2 Morphological characteristics of *Pseudovampyrella closterii* strain VC.01; differential interference contrast (A–E), fluorescence (D) and phase contrast (F). (A) Trophozoite with spherical cell body and anterior filopodia. (B) Fan-shaped trophozoite with anterior filopodia. (C) Detail of filopodia with refractive granules. (D) Hoechst-stained trophozoite with multiple nuclei (left = fluorescence signal, right = DIC). (E) Cytoplasm of squashed cell revealing a spherical nucleus with discernible nucleolus. (F) Expanded plasmodium moving along the substrate in late-stage culture. Scale bars: 20 μm in A-D, 5 μm in E, 100 μm in F.

Ultrastructure of algal remains: We studied the cell walls of three *Closterium* strains, which had been consumed by strain VC.01, by scanning electron microscopy. These algae (*C. cornu*, *C. intermedium* and an undetermined *Closterium* species) differed in cell wall thickness and structure and were all readily taken as food by the vampyrellid. The micrographs confirm that strain VC.01 degraded the algal walls in an annular pattern as known from other protoplast feeders (Hess & Melkonian, 2013; Old, 1969). Frequently, we observed cell wall flaps still attached to the holes (Figure 4A,B). The incomplete perforations in the cells of *Closterium* cf. *intermedium* showed a destructed layer of amorphous polysaccharides (probably pectic substances; (Baylson et al., 2001) and exposed

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TABLE 1 Results of the feeding experiment with *Pseudovampyrella* species categorized in "feeding and growth" (+) and "no feeding and no growth" (−).

Algal lineage	Algal species[a]	Strain	*P. closterii* (VC.01)	*P. minor* (VC.02)
Chlorophyceae	*Oedogonium stellatum*	CCAC 2231 B	−	−
Cryptophyceae	*Chroomonas* sp.	CCAC 4848	−	−
Zygnematophyceae	*Closterium abruptum*	C007	−	−
	Closterium baillyanum var. *crassum*	D249	−	−
	Closterium cf. *intermedium*	C250_SH[b]	+	−
	Closterium cf. *pseudolunula*	D459	−	−
	Closterium cf. *striolatum*	C009	−	−
	Closterium closterioides var. *closterioides*	D084	−	−
	Closterium closterioides var. *closterioides*	Sch24	−	−
	Closterium cornu	CCAC 1125	+	−
	Closterium costatum	C084	−	−
	Closterium cynthia var. *cynthia*	D022	−	−
	Closterium didymotocum	C013	−	−
	Closterium gracile	Cgrac01_SH[b]	+	+
	Closterium intermedium	C035a	−	−
	Closterium intermedium	C450	−	−
	Closterium kuetzingii	N30	+	N/A
	Closterium limneticum	CCAC 2687	+	+
	Closterium littorale	C290	−	−
	Closterium moniliferum	F168	−	−
	Closterium praelongum	N044	−	−
	Closterium pritchardianum	D050	−	−
	Closterium sp.	C120_SH[b]	+	−
	Closterium striolatum	C459	−	−
	Euastrum humerosum	N022	−	−
	Euastrum oblongum	N058	−	−
	Micrasterias americana	C514	−	−
	Micrasterias rotata	D465	−	−
	Netrium digitus	Ndig_01_AS[b]	−	−
	Penium margaritaceum	CCAC 0215	−	−
	Pleurotaenium trabecula	N059	−	−
	Zygnema pseudogedeanum	CCAC 0199	−	−

Abbreviation: CCAC, Central Collection of Algal Cultures.
[a]Determination on the basis of morphological traits, sometimes tentative.
[b]Strain isolated in this study.

cellulose microfibrils in a confined degradation zone (Figure 4C–E). Additionally, we observed changes in the surface properties in a wider, elliptic area around the incomplete perforations in *Closterium* sp. (Figure 4F). These were even more evident in micrographs taken with the backscattered electron detector (Figure 4G).

Description of *Pseudovampyrella minor* sp. nov., strain VC.02

Trophozoite morphology: The trophozoites of strain VC.02 exhibited a similar overall morphology and locomotion to *Pseudovampyrella closterii*, strain VC.01. They were somewhat smaller, ranging from 13 to 30 μm often with a spherical or elliptic cell body (Figure 5A,B). The latter showed a clear differentiation between a colorless, vacuolated ectoplasm and an orange, granular endoplasm, responsible for a pale orange coloration of the cells. In locomoting cells, the unbranched filopodia were mostly anterior and studded with colorless granules (about 0.7 μm in diameter) at their bases (Figure 5A,B). The filopodia reached up to 60 μm in length and thus could exceed the size of the cell body (Figure 5A). The trophozoites contained multiple spherical nuclei of about 2 μm in size as revealed by fluorescence microscopy of Hoechst-stained individuals (Figure 5C). In older cultures, we observed large (>500 μm), ribbon- or

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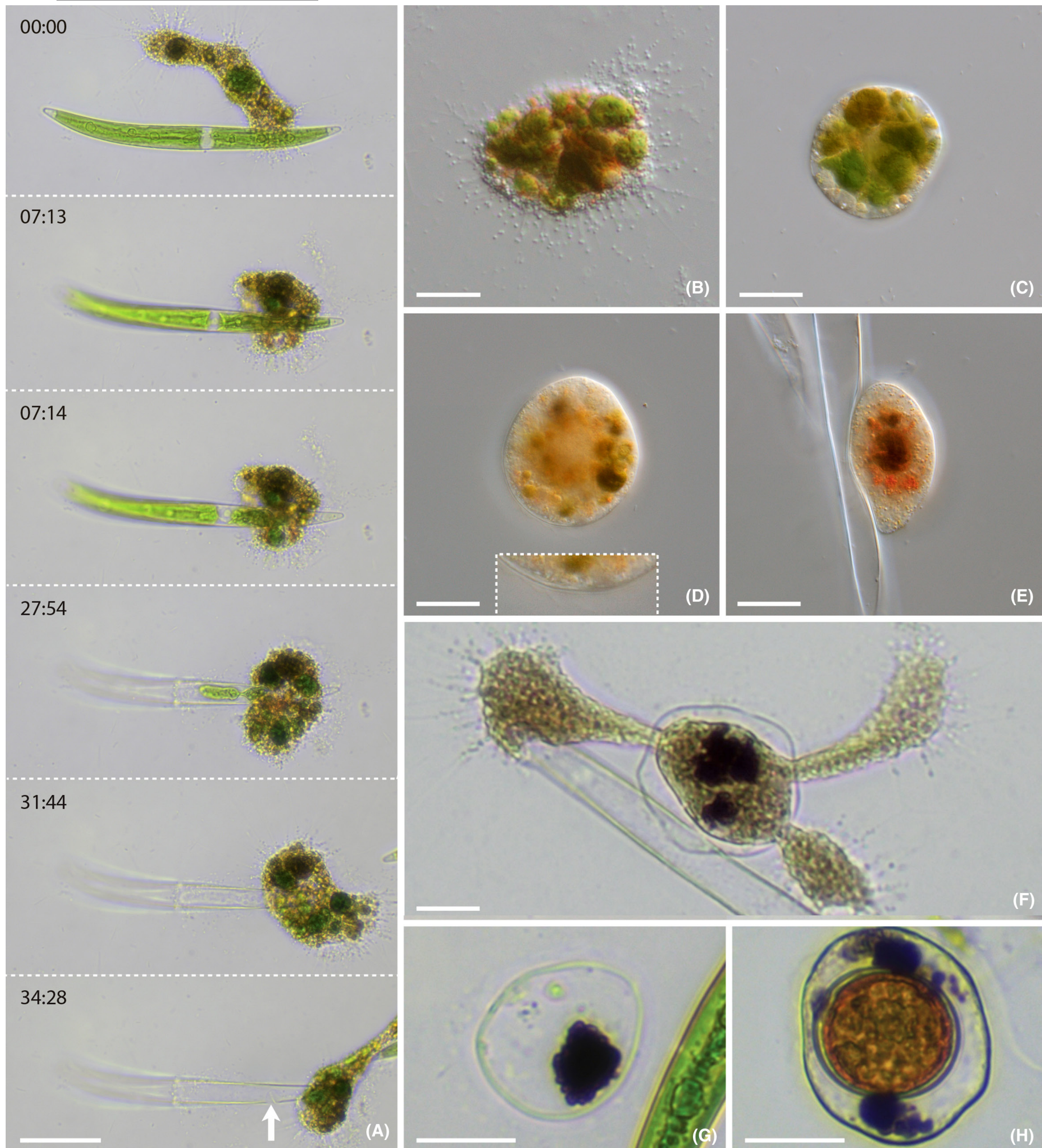

FIGURE 3 Feeding process and life history of *Pseudovampyrella closterii*, strain VC.01; brightfield (A, F–H) and differential interference contrast (B–E). (A) Time series of the feeding act on *C. cornu*. Time stamps are shown in mm:ss, and the white arrow indicates the cell wall perforation. (B) Trophozoite with numerous green food inclusions. (C) Early-stage digestive cyst with green and brownish food inclusions. (D) Intermediate-stage digestive cyst with brown food inclusions and orange cytoplasm. Dotted rectangle magnifies faintly visible velum. (E) Late-stage digestive cyst attached to an emptied *Closterium* cell. The undigested algal remains concentrated in a central vacuole. (F) Hatching cyst with three cellular domains emerging from separate holes. (G) Empty digestive cyst with dark brown food remnant. (H) Resting cyst with dark brown food remnants and orange spore. Scale bars: 50 μm in A, 20 μm in B–H.

fan-shaped plasmodia with colorless ectoplasm and a tendency to branch (Figure 5D).

Feeding and cyst stages: Strain VC.02 fed exclusively on *Closterium* species (Table 1, Figure 5E,F). Only two out of 22 *Closterium* strains in culture proved suitable for cultivation (*C. limneticum* and *C.gracile*). Additionally, strain VC.02 was found feeding on an unidentified species of *Closterium* in the natural sample. When prey

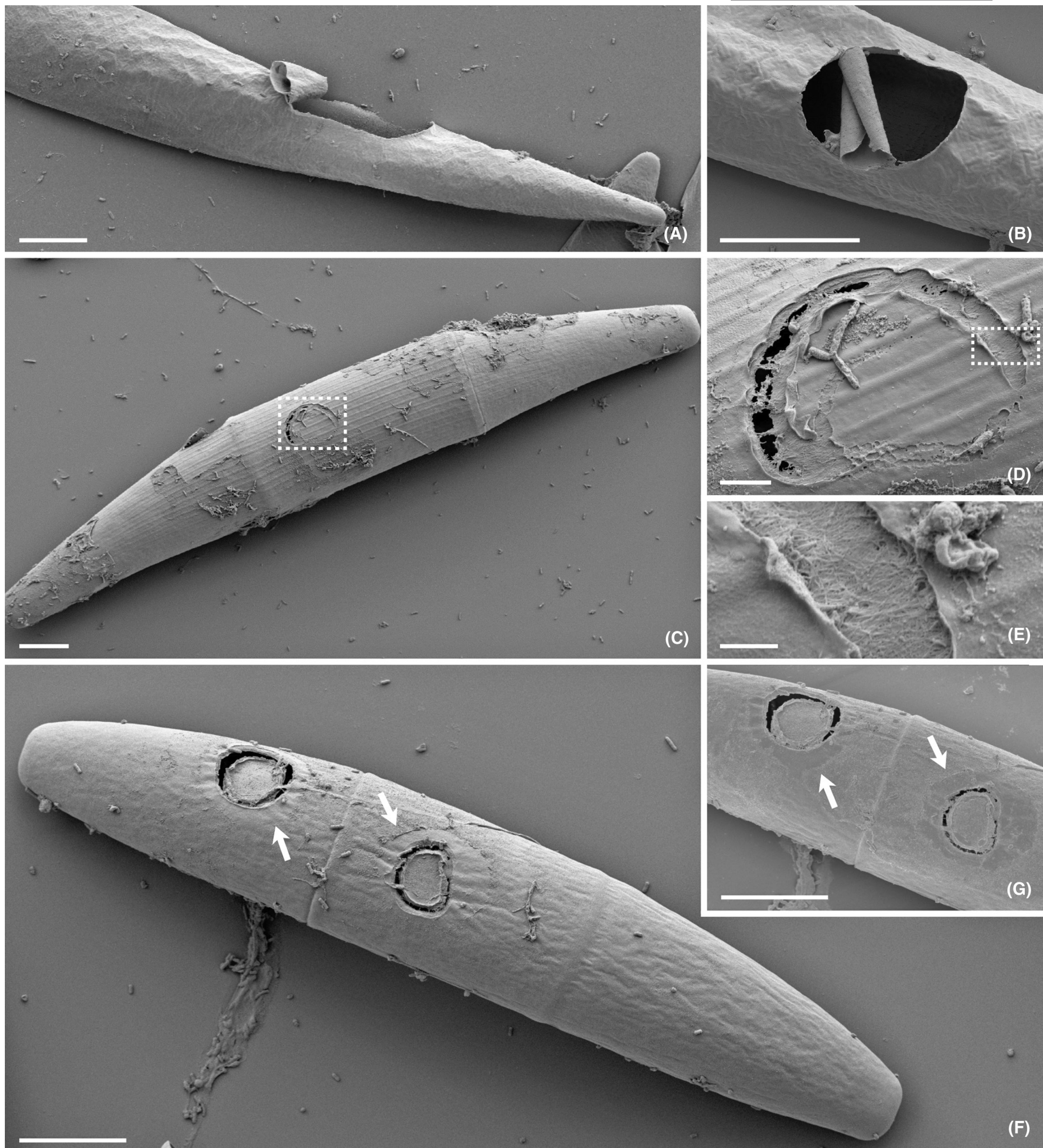

FIGURE 4 Scanning electron micrographs of *Closterium* cells perforated by *Pseudovampyrella closterii*, strain VC.01. (A, B) Perforated cell walls of *Closterium cornu*, with attached cell wall flap. (C–E) Incomplete perforation of *Closterium* cf. *intermedium* (strain C250_SH) at different magnifications. Dotted rectangles highlight magnified areas. Note the degraded outer layer (pectic substances) and the exposed cellulose microfibrils. (F, G) Perforations in the cell wall of *Closterium* sp. (strain C120_SH) with lids (excised cell wall discs) still in place imaged with the secondary electron detector (F) and the backscattered electron detector (G). Arrows indicate changes in cell surface properties well around the perforations. Scale bars: 10 μm in A-C, F, G, 2 μm in D, 600 nm in E.

was suitable, *Pseudovampyrella minor* fed to near depletion over a prolonged period. However, cultures did not grow to high density. The feeding process is comparable to *Pseudovampyrella closterii* strain VC.01, though it took place on a smaller scale. *Pseudovampyrella minor* attached to the cell wall of a *Closterium* cell and pierced the cell wall and extracted the protoplast. The cell wall perforations were circular or elliptic (Figure 5G) and produced by annular cell wall degradation. *Pseudovampyrella minor* fed on 2–4 *Closterium limneticum* cells

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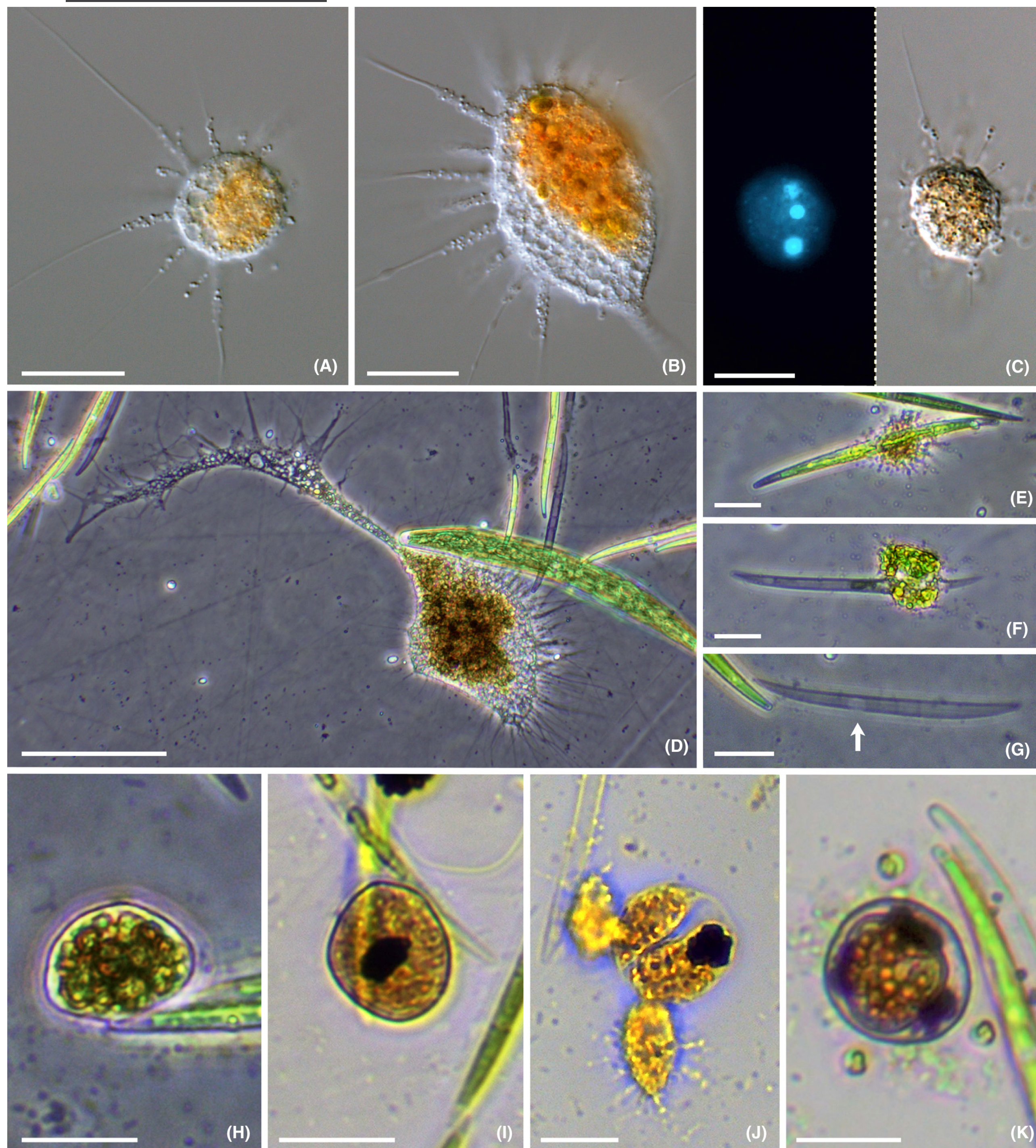

FIGURE 5 Morphology and life history of *Pseudovampyrella minor*, strain VC.02. Differential interference contrast (A–C), fluorescence (C), phase contrast (D–H) and brightfield (I–K). (A) Motile trophozoite with spherical cell body and long filopodia. (B) Motile, elliptic trophozoite with anterior filopodia. (C) Hoechst-stained trophozoite with several nuclei (left = DIC, right = fluorescence signal). (D) Expanded plasmodium. (E) Trophozoite during attack on a *Closterium limneticum* cell (F) Trophozoite in a late stage of the feeding act with most of the algal protoplast extracted. (G) Emptied cell of *Closterium limneticum* with elliptical perforation (arrow). (H) Early-stage digestive cyst with green food inclusions. (I) Late-stage digestive cyst with orange cytoplasm and dark brown food remnant in a central vacuole. (J) Two trophozoites hatching from digestive cyst after internal plasmotomy. (K) Resting cyst with orange spore and food remnants. Scale bars: 20 µm in A–C, E–K, 100 µm in D.

before encystation. The digestive cysts had a round or elliptic outline, with up to 38 µm in their largest dimension. They were first green due to the food inclusions (Figure 5H) and turned orange during the maturation (Figure 5I). Division occurred through internal plasmotomy and resulted in 1–4 trophozoites (Figure 5J). Strain

VC.02 also formed resting cysts that had a similar structure to those of *P. closterii*, but the orange spores with up to 20 μm were somewhat smaller (Figure 5K).

DISCUSSION

In this study, we characterized two isolates of vampyrellid protoplast extractors from acidic ponds of moorlands. Both strains feed specifically on species of the desmid genus *Closterium*, exhibit a similar morphology and autecology and have a genetic identity of >99% in the SSU rRNA gene. And yet, the two strains differ in prey range, cell size and details of their cell division (external vs. internal plasmotomy), suggesting that they might represent two biological entities. Certain morphological characteristics (orange cytoplasm, isodiametric cells, motile granules on the filopodia) and, in particular, the feeding behavior of the strains are reminiscent of algivorous *Vampyrella* species, two of which (*V. lateritia*, *V. pendula*) have been studied by molecular phylogenetics and are part of the Family Vampyrellidae (Hess et al., 2012).

Surprisingly, our phylogenetic analyses reveal that the two new strains are not closely related to these *Vampyrella* species and instead belong to the family Leptophryidae. They form a distinct branch separate from known leptophryid genera and are nested in environmental sequences, indicating that we phenotypically sampled a new fraction of the leptophryid diversity. Within this family, protoplast extraction was only reported for *Platyreta germanica* (Bass et al., 2009; Pakzad, 2003) and a number of mycophagous amoebae that are putative leptophryids but lack genetic identity (Anderson & Patrick, 1980; Old & Darbyshire, 1978; Old & Patrick, 1979). *Platyreta germanica* was isolated from soil and consumes the contents of diverse fungi and a few tested algal species (Pakzad, 2003), but there is no detailed documentation of its interaction with algal cells. Interestingly, this species opens fungal cells by a ring-like (annular) perforation of the wall, which resembles the situation in the strains VC.01 and VC.02. The exact process of prey cell wall degradation in vampyrellids is not well known, but it might involve lytic enzymes applied in an annular pattern as demonstrated for another rhizarian protoplast feeder, *Orciraptor agilis* (Moye et al., 2022). The changes in material density (SEM) observed around the cell wall perforations produced by strain VC.01 indicate an alteration of the pectic cell wall layer in an area much larger than the perforation. This might be related to adhesive processes and based on protein-carbohydrate interactions, which deserve further study. Members of the other known leptophryid genera consume algal cells by free capture (e.g. *Leptophrys*, *Kinopus*, *Vernalophrys*, *Planctomyxa*), colony invasion (e.g. *Arachnomyxa*), or prey on micrometazoa (*Theratromyxa*; Gong et al., 2015; Hess, 2017b; Hess et al., 2012; Weber et al., 1952; Zhang et al., 2022; Zwillenberg, 1953). Considering our findings on *Pseudovampyrella*, the Leptophryidae exhibit a stunning diversity of feeding strategies and prey types, highlighting the ecological versatility of this family.

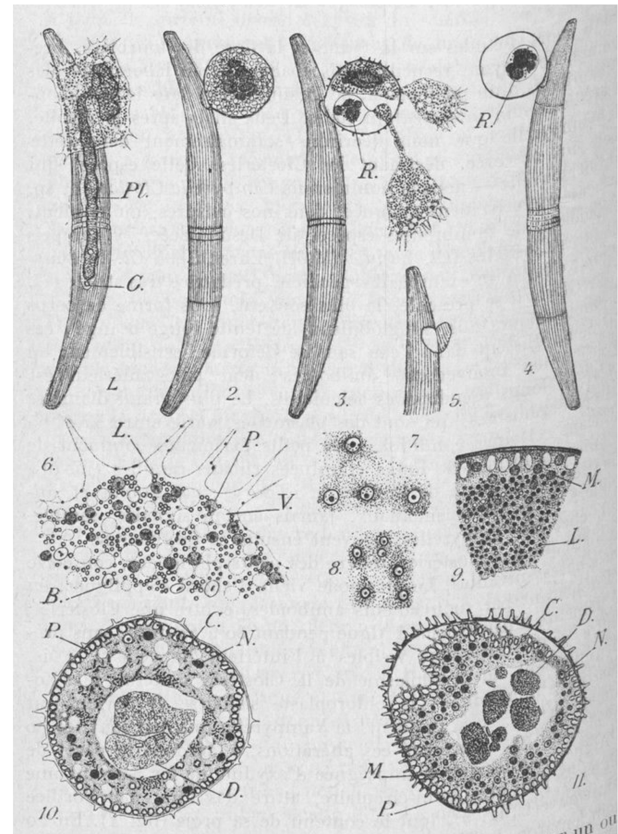

FIGURE 6 Illustrations of *Vampyrella closterii* from its original description (Poisson & Mangenot, 1933). The drawings depict various stages of the feeding process and life history (1., 2., 3., 4.) as well as morphological details such as filopodia with granules (6.), nuclei (7., 8.) and the flap remaining at the perforation site (5.).

Strain VC.01 bears a remarkable similarity to the historically described species *Vampyrella closterii* Poisson & Mangenot, 1933 (Poisson & Mangenot, 1933). This species was found in moorlands of Bretony and specifically fed on some bog-dwelling *Closterium* species with marked cell wall striations and girdle bands (potentially *Closterium intermedium*, Figure 6). Furthermore, the original description reports trophozoites with orange cytoplasm and globular to ribbon-like morphology. Poisson and Mangenot revealed that these amoebae contained multiple nuclei of the vesicular type (with a single nucleolus each) by staining their DNA and depicted small granules on the filopodia (Figure 6). *Vampyrella closterii* was also reported to form large plasmodia exceeding 1500 μm in size and to leave behind a characteristic “flap” at the cell wall perforation after extracting the algal prey. Overall, strain VC.01 is very similar to *V. closterii* in morphology, ecology and feeding behavior. As we demonstrated in the feeding experiment, strain VC.01

fed only a on a small selection of *Closterium* species including those which were isolated from moorlands and resemble the *Closterium intermedium* depicted in the original description of *Vampyrella closterii*. Hence, we consider strain VC.01 and *Vampyrella closterii* as the same organism. Strain VC.02, instead, is considerably smaller than *Vampyrella closterii* and did not feed on *Closterium intermedium*-type species in our experiments. It may represent a new species, even though the genetic difference between the strains VC.01 and VC.02 in the SSU rRNA gene is surprisingly low (<1%). More variable markers (e.g. ITS sequences) might be suitable to better resolve closely related strains, but are not yet established for vampyrellids. Currently, there is a lack of knowledge concerning biological species and sexual processes of vampyrellids. Future experimental and genomic studies have yet to show whether such closely related vampyrellid isolates with distinct phenotypes are genetically isolated species. However, the marked phenotypic differences observed in strains VC.01 and VC.02 apply to various aspects of their life history (including trophozoites, feeding processes and cell division), and we are inclined to propose a new species for strain VC.02 to account for the observed biological diversity (see below for taxonomic details).

Due to the phylogenetic position of the new strains in the Leptophryidae, we cannot assign them to the genus *Vampyrella* (Vampyrellidae). In fact, *Vampyrella closterii* was already transferred to another genus, *Gobiella* Cienkowski, 1881, for unknown reasons (Cienkowski, 1881; Röpstorf et al., 1994). *Gobiella* has repeatedly been considered a vampyrellid genus (Cienkowski, 1881; Röpstorf et al., 1994; Valkanov, 1940), but an examination of the original description of its type species, *G. borealis* (Cienkowski, 1881), revealed that the latter closely resembles *Chlamydomyxa labyrinthuloides*, a heterokont, amoeboid alga (Archer, 1875; Wenderoth et al., 1999). Hence, we consider the genus name *Gobiella* inadequate for the studied strains (and vampyrellids in general) and establish the new genus *Pseudovampyrella* with two species.

TAXONOMY

Vampyrellida West, 1901

Leptophryidae Hess et al., 2012

Pseudovampyrella gen. nov.

LSID: urn:lsid:zoobank.org:act:8951622E-EC61-4D65-B938-EFA9CA731E94.

Etymology: *ψευδής (pseudes)* [Ancient Greek], false; *Vampyrella*, existent genus name. Referring to the resemblance to known *Vampyrella* species.

Description: Trophozoites fan-shaped, elliptic or spherical with orange cytoplasm and hyaline filopodia and numerous nuclei. Filipodia studded with refractive granules at their bases. Cells feed on green algae by protoplast extraction after annular perforation of the prey cell wall.

Type species: *P. closterii* (Poisson & Mangenot, 1933) Suthaus & Hess, 2023.

Pseudovampyrella closterii comb. nov.

Basionym: *Vampyrella closterii* Poisson & Mangenot, 1933.

LSID: urn:lsid:zoobank.org:act:64579FED-C292-4F28-9668-0BCCB1AB0088.

Description: Trophozoites have dynamic, granular, orange cell bodies. Cell outline variable in motion on substrate, frequently fan-shaped, cell outline spherical when floating in water column. Typical cell bodies range from 30 to 100 μm. Filopodia are hyaline, nonbranching and tapering, up to 50 μm in length, with basal, motile, refractive granules about 1 μm in diameter. Movement is crawling, with posterior absorption of filopodia. Trophozoites are multinucleate with spherical nuclei (3 μm in diameter) and off-center spherical nucleolus. Feeds on *Closterium* spp. through protoplast extraction. An operculum is frequently visible after feeding. Typical digestive cysts 30–60 μm in their largest dimension with circular to oval outline. Fed digestive cysts green due to prey inclusions, which changes during digestion to orange. Food inclusions bundled into a central digestive vacuole.

Differential diagnosis: Differs from *Vampyrella* spp. in prey spectrum (*Closterium* spp. rather than filamentous green algae), variable morphology (expanded morphotype), more dynamic cell bodies, presence of large numbers of basal refractive granules at the filopodia and cell division via external plasmotomy. Differs from *Pseudovampyrella minor* in prey spectrum (broader spectrum of *Closterium* spp. with larger *Closterium* strains consumed). Cell bodies and cyst size are nearly twice as large as *P. minor* in range. Nuclei and refractive granules larger in size than *P. minor*. Filopodia shorter than *P. minor* in relation to cell body size. Orange granulation evenly distributed in cytoplasm with no clear differentiation between ecto- and endoplasm, resulting in strong orange coloration, unlike *P. minor*. Cell division via external plasmotomy during hatching, in contrast to *P. minor* (i.e. internal plasmotomy).

Type material: A permanent slide (aldehyde/osmium tetroxide fixed cells for DIC microscopy), constituting the name-bearing hapantotype (article 73.3, ICZN), has been deposited in the "Protists Collection" at the Department of Life Sciences of the Natural History Museum in London (Cromwell Road, London, UK) with registration number NHM 2023.8.3.1. Cells of the hapantotype are shown in Figure 7A,B.

Type generating strain: VC.01.

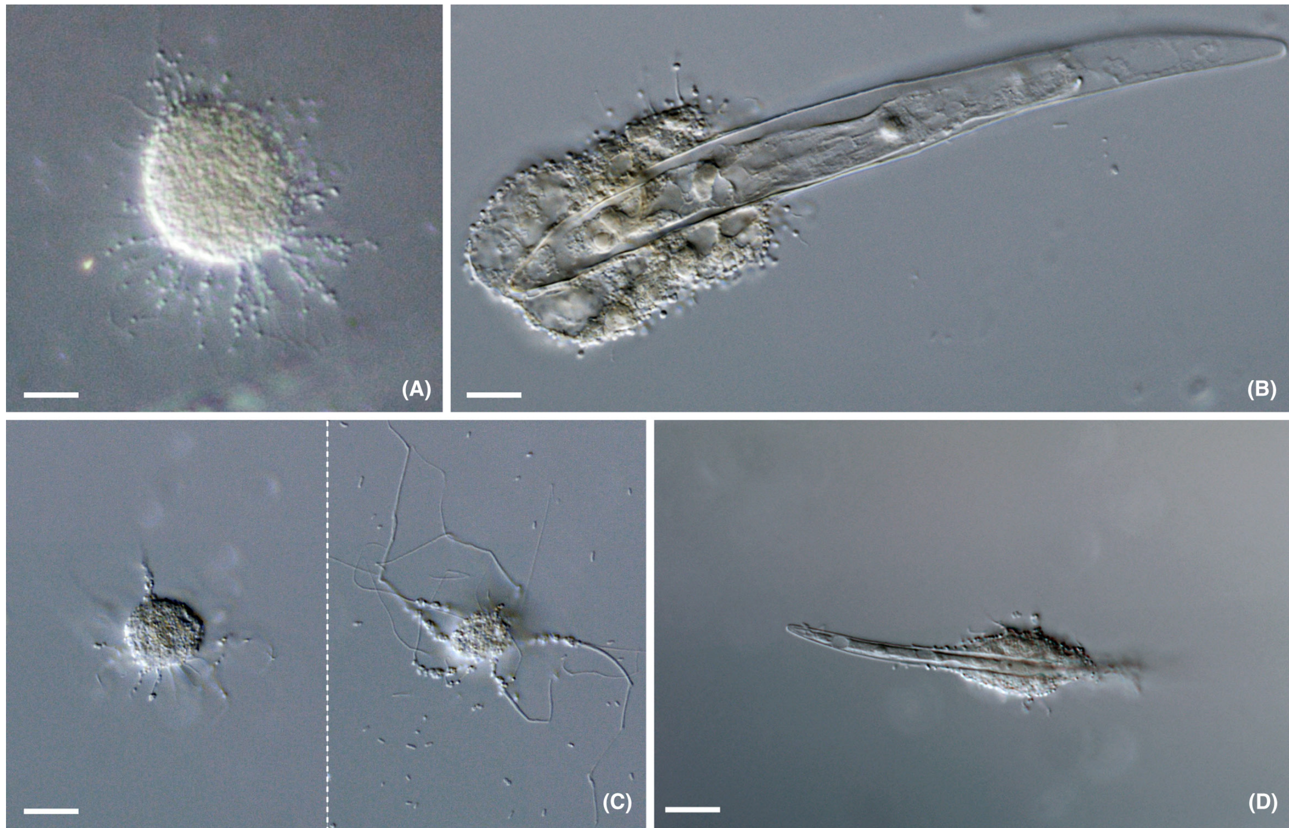

FIGURE 7 Preserved cells from type material (permanent slides) of *Pseudovampyrella* strains illustrating the marked size difference between the species; differential interference contrast. (A) Trophozoite of *P. closterii*. (B) Trophozoite of *P. closterii* during extraction of the protoplast from *Closterium cornu*. (C) Trophozoite of *P. minor* in two focal planes. (D) *P. minor* attached to *Closterium limneticum* during feeding. Scale bars: 10 μm.

Sequence of type generating strain (SSU rRNA gene): OQ591878.

Type habitat and locality: Organic sediment of acidic pond; Thielenbruch moorland, Cologne, Germany; 50.987959, 7.078928.

Pseudovampyrella minor sp. nov.

LSID: urn:lsid:zoobank.org:act:D6430D34-6328-4A43-9381-1AE3F752ECC3.

Etymology: *minor, minōris* [Latin], smaller. Referring to the relatively small cell size compared to the other known species of the genus.

Description: Trophozoites have dynamic spherical or elliptical cell bodies with colorless, vacuolated ectoplasm and orange, granular endoplasm. Typical cell bodies range from 13 to 30 μm. Filopodia are hyaline, nonbranching, up to 60 μm in length, with basal, motile, refractive granules about 0.7 μm in diameter. Movement is crawling, with posterior absorption of filopodia. Cells are multinucleate with spherical nuclei (2 μm in diameter). Feeds on *Closterium* spp. via protoplast extraction. Typical digestive cysts range from 15 to 38 μm in diameter and are oval, circular or occasionally irregular in outline. Fed digestive cysts green due to prey inclusions, which changes during digestion to orange. Food inclusions bundled into a central digestive vacuole.

Differential diagnosis: Differs from *Vampyrella* spp. in prey spectrum (*Closterium* spp. rather than filamentous green algae), variable morphology (expanded morphotype), more dynamic cell bodies and presence of large numbers of basal refractive granules at the filopodia. Differs from *Pseudovampyrella closterii* in prey spectrum (narrow spectrum of *Closterium* spp. with smaller *Closterium* strains consumed). Cell bodies and cyst size smaller than *P. closterii* in range. Nuclei and refractive granules smaller than *P. closterii*. Filopodia longer than *P. closterii* in relation to cell body, frequently exceeding cell body in size. Orange granulation localized in granular endoplasm with vacuolated hyaline ectoplasm, resulting in pale orange coloration, unlike *P. closterii*. Cell division via internal plasmotomy during hatching, in contrast to *P. closterii* (i.e. external plasmotomy).

Type material: A permanent slide (aldehyde/osmium tetroxide fixed cells for DIC microscopy), constituting the name-bearing hapantotype (article 73.3, ICZN), has been deposited in the "Protists Collection" at the Department of Life Sciences of the Natural History Museum in London (Cromwell Road, London, UK)

with registration number NHM 2023.8.3.2. Cells of the hapantotype are shown in Figure 7C–E.

Type generating strain: VC.02.

Sequence of type generating strain (SSU rRNA gene): OQ591879.

Type habitat and locality: Organic sediment of acidic pond; Simmelried moorland, Constance, Germany; 47.717767, 9.09375.

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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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