Morpho-molecular Characterization of the Litostomatean Predatory Ciliate *Phialina pupula* (Müller, 1773) Foissner, 1983 (Haptoria, Lacrymariidae)

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Abstract. The morphology and phylogenetic position of a haptorian ciliate, *Phialina pupula* (Müller, 1773) Foissner, 1983, isolated from microaerobic sandy sediments of the floodplain area of the Boise River, Idaho, U.S.A., were studied using live observation, protargol impregnation, scanning electron microscopy, and the 18S rRNA gene as well as the ITS region. The Boise population of *P. pupula* is characterized by a size of about 60–130 × 20–50 µm, an elliptical macronucleus with a single micronucleus, highly refractive dumbbell-shaped inclusions scattered throughout the cytoplasm and concentrated in the anterior body half, a single subterminal/terminal contractile vacuole, about 10 µm long rod-shaped extrusomes, and an average of 15 ciliary rows. In phylogenetic analyses, the newly obtained sequences from *P. pupula* and *Lacrymaria olor* clustered within the family Lacrymariidae with full to moderate statistical support. Neither the genus *Phialina* nor the genus *Lacrymaria* was depicted monophyletic both in the single gene and multigene phylogenetic inferences. Specifically, the genus *Phialina* was shown as a paraphyletic assemblage containing members of the polyphyletic genus *Lacrymaria*. This indicates that the phialinid bauplan, i.e., an anterior body end differentiated into a head-like structure directly attached to the trunk, might represent the ground pattern in the family Lacrymariidae. On the other hand, the long highly contractile neck carrying the head-like structure probably evolved later and convergently in multiple *Lacrymaria* species from *Phialina*-like ancestors.

Keywords: 18S rRNA gene; Boise; Floodplain sand; ITS region; Lacrymariidae; Phylogeny

INTRODUCTION

*Phialina pupula* (Müller, 1773) Foissner, 1983 represents a free-living, predatory ciliate belonging to the subclass Haptoria Corliss, 1974 of the highly diverse class Litostomatea Small and Lynn, 1981. Müller (1773) described this species for the first time as a cone-shaped microorganism with an apical head-like structure. Later on, Bory de Saint-Vincent (1824) classified all ciliates with an apical head into the genera *Phialina* Bory de Saint-Vincent, 1824 and *Lacrymaria* Bory de Saint-Vincent, 1824. He distinguished the two genera by the localization of the cell mouth: *Phialina* has a lateral while *Lacrymaria* possesses a terminal cytostome. These generic characters were, however, revealed to be problematic and consequently most species were assigned to *Lacrymaria* (Ehrenberg 1838, Dujar-
According to molecular data, *Phialina* and *Lacrimarya* form a monophyletic group, the family Lacrymaryidae from 1876, but the phylogenetic position of the family within the class Litostomatea remains unresolved (Gao et al. 2008, Vďačný et al. 2011, Zhang et al. 2012, Kwon et al. 2014, Vďačný and Rataj 2017, Wu et al. 2017, Huang et al. 2018, Wang et al. 2019). Neither the genus *Phialina* nor the genus *Lacrimarya* is monophyletic and their species are intermingled in single gene and also in multigene phylogenies (Wu et al. 2017, Huang et al. 2018, Wang et al. 2019). This fact indicates that a new taxonomic concept is needed to reconcile the conflicts between morphologic and molecular classifications.

Although notable progress has been achieved in the morphological and molecular research on the family Lacrymaryidae in the past 35 years, there are still many "old" species that need to be investigated using modern alpha-taxonomic methods. Moreover, the molecular sampling of lacrymaryiids is also limited and sequences from more taxa and genes are needed to reconstruct the evolutionary history of this peculiar group of predatory ciliates more robustly. Therefore, we provide in this study a comprehensive morphological re-description of an insufficiently known species, *P. pupula*, and multigene phylogenetic analyses of the family Lacrymaryidae.

**MATERIAL AND METHODS**

**Sampling, morphologic methods and terminology**

Populations of *P. pupula* and *Lacrimarya olor* (Müller, 1786) Bory de Saint-Vincent, 1824 were collected in Boise, Idaho, northwestern U.S.A. (Supplementary Table S1). The former species was isolated from sand percolates of the floodplain area of the Boise River near the Glenwood Bridge (43°39′47.57″ N, 116°16′56.99″ W). The latter species was gathered from sediments of a pond in the Julia Davis Park (43°36′23.84″ N, 116°11′46.24″ W). Both species were isolated directly from the environmental samples after transport to the laboratory at Boise State University.

Living specimens were studied using a Zeiss Axioskop 2 Plus microscope at 100–1000× magnification. Protargol impregnation was carried according to the Wilbert’s method (Ji and Wang 2018). Specimens for scanning electron microscopy (SEM) were prepared following the method described in Foissner (2014). Briefly, ciliates were fixed with 1:1 solution of 2.5% glutaraldehyde and 2% osmium tetroxide, dehydrated in ethanol (50, 70, 90 and three changes of 100%), dried in a critical point dryer (EMS 850, Electron Microscopy Sciences, Hatfield, PA, USA), sputtered with gold in an Agar sputter coater (Electron Microscopy Sciences, Hatfield, PA, USA), and examined at 15 kV in a Hitachi S-3400N scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan). Living specimens were measured from images captured with a Flex Digital camera (Diagnostic Instruments, Sterling Heights, MI) using the calibrated software ImageJ (Schneider et al. 2012). Protargol-impregnated cells were measured directly under the optical microscope using an ocular micrometer. Illustrations of live specimens and impregnated cells were based on microphotographs. Unfortunately, the quality of *L. olor* preparations was insufficient for a thorough morphologic description. Therefore, we morphologically characterize only *P. pupula* in detail here but provide sequence data for both species.

General terminology follows Lynn (2008) and specific terminology is according to Foissner and Xu (2007) as well as Vďačný and Foissner (2012).

**Molecular methods**

After identification, several specimens from both species were picked, washed and transferred into the cell lysis buffer. The DNEasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used to extract the genomic DNA. Amplification of the 18S rRNA gene followed Vďačný et al. (2011) while that of the ITS1-5.8S-ITS2 region was according to Vďačný et al. (2012). PCR products were enzymatically purified and ligated into a plasmid with the pGEM®-T and pGEM®-T Easy Vector Systems (Promega, Madison, Wisconsin, USA). After a 12-hour incubation of the ligation mixture at 4 °C, recombinant plasmids were introduced into the
competent Escherichia coli cells (strain JM109). The efficiency of transformation was checked by the blue-white selection method. Molecularly cloned recombinant plasmids were again subjected to PCR but using the M13F and M13R primers under the same conditions as described in Vďačný et al. (2011). The resulting PCR products were enzymatically purified and then sequenced on an ABI 3730 automatic sequencer (Macrogen Inc., Amsterdam, The Netherlands) using the M13F and M13R primers.

**Sequences and phylogenetic analyses**

The obtained sequence fragments were checked, trimmed and assembled into contigs using BioEdit ver. 7.2.5 (Hall 1999). All 18S rRNA gene and ITS1-5.8S-ITS2 region sequences were deposited in the GenBank database. Their length, GC content and GenBank accession numbers are provided in Supplementary Table S1. Multi-sequence alignments were conducted using the MAFFT algorithm and were masked with the cutoff value of 0.93 (Supplementary Table S2) on the Guidance2 server (http://guidance.tau.ac.il/ver2/) (Sela et al. 2015).

Maximum likelihood and Bayesian inference were used to analyze ten alignments, as specified in Supplementary Table S2. The best evolutionary substitution models under the Akaike information criterion were selected using jModelTest ver. 0.1.1 (Guindon and Gascuel 2003, Posada 2008). Parameters of the best fitting substitution models for both Bayesian and maximum likelihood analyses were summarized in Supplementary Table S2. Maximum likelihood analyses were conducted with PhyML ver. 3.0 on the South of France bioinformatics platform (http://www.atgc-montpellier.fr/phylml/) (Guindon et al. 2010) using the SPR tree-rearrangement and one thousand non-parametric bootstrap replicates. Following Hillis and Bull (1993), bootstrap values <70% were considered as low, 70–94% as moderate, and ≥95% as high. Bayesian inference was performed with MrBayes ver. 3.2.6 (Ronquist et al. 2012) on the CIPRES Portal ver. 3.1 (http://www.phylo.org), using four independent chains, five million generations and a sampling frequency of one thousand. The burn-in fraction was specified as 25% of the first sampled trees. Posterior probabilities <0.94 were considered as low while ≥0.95 as high (Alfaro et al. 2003). Bayesian and maximum likelihood trees were visualized in FigTree ver. 1.2.3 (Rambaut 2015).

**RESULTS**

**Phialina pupula** (Müller, 1773) Foissner, 1983

**Zoobankregistrationnumber:** urn:lsid:zoobank.org:pub:BFF7D5C4-A4F7-42F9-9FCC-DBE7814F0000.

**Improved diagnosis (based on Boise population):** In vivo size about 60–130 × 20–50 μm. Body shape highly variable depending on state of contraction, ranging from clavate in extended condition through fusiform, obpyriform, elliptical to almost globular in semi-contracted and contracted state. Macronucleus elliptical with a single micronucleus. Highly refractive dumbbell-shaped inclusions scattered throughout cytoplasmin and usually concentrated in anterior body part. Contractile vacuole subterminal in extended condition, terminal in contracted state. Extrusomes about 10 μm long, rod-shaped, attached to oral bulge and forming bundles in cytoplasm. On average 15 ciliary rows, each row anteriorly differentiated into a dorsal brush composed of one to four dikinetids.

**Type locality:** Müller (1773) did not specify the type locality. He mentioned only that he found the species in water and ice from dunghills during November and December.

**Type material and voucher slides:** No type material is available from Müller’s (1773) specimens. Three voucher slides containing protargol-impregnated specimens from the Boise population have been deposited at Department of Zoology, Comenius University in Bratislava.

**Material studied:** Specimens from lower micro-aerobic layers of the interstitial sandy sediments from the floodplain area of the Boise River near the Glenwood Bridge, Boise, Idaho, U.S.A.

**Etymology:** Not given in the original description. The feminine Latin noun pupula is a diminutive form of pupa (doll, puppet or pupa of an insect), obviously referring to the doll- or pupa-like body shape of the ciliate. The name is treated as a noun in the nominative singular standing in apposition to the generic name [Art. 11.9.1.2 of the International Commission on Zoological Nomenclature (1999)].

**Description of Boise population:** Size in vivo 60–130 × 20–50 μm, usually about 85 × 30 μm, as calculated from some in vivo measurements and morphometric data adding 15% preparation shrinkage; length/width ratio on average 2.2:1 in vivo and 2.9:1 (n = 32) in protargol preparations (Table 1). Body shape highly variable depending on state of contraction, ranging from clavate in extended condition through fusiform, obpyriform, elliptical to almost globular in semi- or fully contracted and contracted state. Head barrel-shaped, about 8.5 × 6.0 μm in size after protargol impregnation, distinct from trunk but without neck-like region, sometimes retracted into trunk creating an impression of shoulders. Posterior body end tapered and tail-like in extended condition, narrowly to broadly rounded in semi- or fully contracted and contracted state (Figs 2A, E, F, 3A, C, E, F, H–M). Contraction occurs slowly.

Nuclear apparatus located in or slightly posterior to mid-body, usually slightly lateral of cell center. Macronucleus elliptical, on average 15 × 10 μm (n = 32) in size after protargol impregnation. Macronucleus adja-
Table 1. Morphometric data on Phialina pupula (Boise population).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean</th>
<th>M</th>
<th>SD</th>
<th>SE</th>
<th>CV</th>
<th>Min</th>
<th>Max</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body, length</td>
<td>75.3</td>
<td>74.5</td>
<td>14.2</td>
<td>2.5</td>
<td>18.8</td>
<td>53.0</td>
<td>115.0</td>
<td>32</td>
</tr>
<tr>
<td>Body, width</td>
<td>26.4</td>
<td>26.5</td>
<td>6.1</td>
<td>1.1</td>
<td>23.1</td>
<td>17.0</td>
<td>42.0</td>
<td>32</td>
</tr>
<tr>
<td>Body length:width, ratio</td>
<td>2.9</td>
<td>2.8</td>
<td>0.6</td>
<td>0.1</td>
<td>20.6</td>
<td>1.7</td>
<td>4.4</td>
<td>32</td>
</tr>
<tr>
<td>Head, height</td>
<td>8.4</td>
<td>9.0</td>
<td>1.2</td>
<td>0.2</td>
<td>13.9</td>
<td>5.0</td>
<td>11.0</td>
<td>32</td>
</tr>
<tr>
<td>Head, width</td>
<td>6.1</td>
<td>6.0</td>
<td>0.9</td>
<td>0.2</td>
<td>15.4</td>
<td>5.0</td>
<td>8.0</td>
<td>31</td>
</tr>
<tr>
<td>Anterior body end to macronucleus, distance</td>
<td>34.9</td>
<td>35.0</td>
<td>11.0</td>
<td>2.0</td>
<td>31.5</td>
<td>10.0</td>
<td>59.0</td>
<td>32</td>
</tr>
<tr>
<td>Macronucleus, length</td>
<td>15.2</td>
<td>15.0</td>
<td>2.6</td>
<td>0.5</td>
<td>17.1</td>
<td>10.0</td>
<td>20.0</td>
<td>32</td>
</tr>
<tr>
<td>Macronucleus, width</td>
<td>9.5</td>
<td>9.0</td>
<td>2.2</td>
<td>0.4</td>
<td>23.5</td>
<td>5.0</td>
<td>16.0</td>
<td>32</td>
</tr>
<tr>
<td>Extrusome, length</td>
<td>9.7</td>
<td>10.0</td>
<td>0.8</td>
<td>0.2</td>
<td>8.1</td>
<td>8.0</td>
<td>11.0</td>
<td>25</td>
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<tr>
<td>Somatic ciliary rows, number</td>
<td>15.0</td>
<td>16.0</td>
<td>1.3</td>
<td>0.3</td>
<td>8.9</td>
<td>12.0</td>
<td>16.0</td>
<td>20</td>
</tr>
<tr>
<td>Somatic ciliary rows, distance in between</td>
<td>3.9</td>
<td>4.0</td>
<td>0.5</td>
<td>0.1</td>
<td>13.0</td>
<td>3.0</td>
<td>4.5</td>
<td>16</td>
</tr>
<tr>
<td>Kinetids in a ciliary row, total number</td>
<td>21.8</td>
<td>21.0</td>
<td>5.7</td>
<td>1.7</td>
<td>26.3</td>
<td>13.5</td>
<td>33.0</td>
<td>12</td>
</tr>
<tr>
<td>Kinetids in a ciliary row, distance in between</td>
<td>3.2</td>
<td>3.0</td>
<td>0.5</td>
<td>0.1</td>
<td>16.6</td>
<td>2.0</td>
<td>4.0</td>
<td>18</td>
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<tr>
<td>Brush dikinetids in a kinety, number</td>
<td>3.7</td>
<td>3.8</td>
<td>1.1</td>
<td>0.5</td>
<td>29.1</td>
<td>2.0</td>
<td>5.0</td>
<td>6</td>
</tr>
</tbody>
</table>

* Data based on protargol-impregnated and semi-contracted to extended specimens. Measurements in µm. CV, coefficient of variation (%); M – median; Max – maximum; Mean – arithmetic mean; Min – minimum; n – number of individuals investigated; SD – standard deviation; SE – standard error of arithmetic mean.

Somatic cilia about 8 µm long in vivo, arranged in an average of 15 rows, each row composed of about 22 monokinetids with some dikinetids (dividing basal bodies) irregularly interspersed. Somatic kineties ordinally spaced, extend meridionally to slightly helically depending on state of contraction (Table 1; Figs 2A, 4A, C). Dorsal brush at anterior end of all somatic kineties, very inconspicuous not only in vivo but also in protargol preparations and in SEM because composed of only two to five dikinetids (SEM measurements): first brush dikinetid bears a short, 1.5–2.0 µm-long, rod-like cilium followed by an ordinary cilium about 6.5 µm long; second dikinetid associated with a minute, 0.3 µm-long, stump-like cilium followed by an ordinary cilium; all following brush dikinetids with anterior basal body unciliated and posterior basal body bearing an ordinary cilium (Table 1; Figs 2E, 4A, B).

Oral apparatus occupies apical end of head. Oral bulge contains tip of extrusomes, posteriorly delimited by circumoral kinety as usual in congeners. Circumoral kinety and its structure very difficult to recognize in protargol preparations, very likely composed of dikinetids. Head kineties helical and narrowly spaced, extend between circumoral kinety and dorsal brush, composed of densely arranged monokinetids bearing about 10 µm long cilia in vivo and almost completely covering head in SEM (Figs 2A, E, 3A, 4A).
**Phylogenetic analyses**

In total, ten alignments containing 18S rRNA gene sequences, ITS region sequences and their concatenations, were analyzed using Bayesian inference and maximum likelihood (Supplementary Table S2). Six alignments contained representatives of all main litostomatean lineages and members of the class Armophorea served to root the trees. The remaining four alignments included only sequences from representatives of the family Lacrymariidae. To test the robustness of results, each unmasked alignment has its counterpart masked with a cutoff value of 0.93. All analyses resulted in similar topologies with respect to statistically supported nodes. Therefore, we present here only trees inferred from the unmasked concatenated 18S rRNA gene-ITS region dataset containing 80 litostomatean taxa (Fig. 5) and from the masked 18S rRNA gene alignment containing 22 lacrymariid taxa (Fig. 6).

The class Litostomatea was recognized as a monophyletic group with full statistical support. The order Helicoprorodontida and the family Chaeneidae were placed as deep-branching lineages but the statistical support for their positions was weak in the maximum likelihood analyses, very likely because of long branch attraction. Relationships among the remaining litostomateans were poorly resolved although the main lineages were usually strongly statistically supported. Rynchostomatians formed a fully statistically supported cluster both in the Bayesian and the maximum likelihood tree. Haptorians with one- or two-rowed dorsal brush and meridionally extending somatic kineties (Pleurostomatida, Homalozoonidae, and Haptorida) were clustered together in the Bayesian inference tree with high statistical support but this group was not corroborated in the maximum likelihood analyses. The order Spathidiida was depicted as paraphyletic, containing endocommensals from the subclass Trichostomatia, and this whole assemblage received full statistical support in the Bayesian tree but very low statistical support in the maximum likelihood tree. The family Lacrymariidae was fully to moderately statistically supported and was placed in a polytomy of the subclass Haptoria (Fig. 5).

Evolutionary relationships among members of the family Lacrymariidae were investigated in detail on the basis of the 18S rRNA gene (Fig. 6). Neither the genus *Phialina* nor the genus *Lacrymaria* were monophyletic.
Fig. 2. Phialina pupula from life (A–D, F) and after protargol impregnation (E). (A) Overview of a representative semi-contractioned specimen. (B) Details of dumbbell-shaped inclusions from various views. (C) Extrusomes are rod-shaped and about 10 μm long. (D) Surface view showing cortical granulation. (E) Ciliary pattern. (F) Variability of body shape in extended, semi-contracted and contracted cells. CK – circumoral kinety; CV – contractile vacuole; DB – dorsal brush; DI – dumbbell-shaped inclusions; EB – extrusome bundle; EX – extrusomes; G – cortical granules; OB – oral bulge; MA – macronucleus; MI – micronucleus; SK – somatic kineties. Scale bars: 20 μm.
Fig. 3. Phialina pupula from life under differential interference contrast (A–G) and bright field (H–M) illumination. (A) Overview of a semi-contracted specimen, showing the general body organization. The head is attached directly to the broadly fusiform trunk. Note that the contractile vacuole is located terminally due to the body contraction. The macronucleus is elliptical and situated slightly below the mid-body. (B) Detail of the highly refractive dumbbell-shaped inclusions scattered throughout the cytoplasm. (C) A semi-contraction specimen, showing an accumulation of the dumbbell-shaped inclusions in the anterior body half. (D) Detail of the nuclear apparatus. The macronucleus is elliptical, and the micronucleus is attached to the anterior pole of the macronucleus. (E) A contracted specimen, showing many refractive, dumbbell-shaped inclusions scattered throughout the cytoplasm and an elliptical macronucleus accompanied by a single micronucleus. (F) A strongly squeezed specimen, showing the nuclear apparatus, multiple extrusome bundles and some lipid droplets scattered throughout the cytoplasm. Left inset shows optical section through the cortex (opposed arrowhead), containing inconspicuous elliptical granules. (G) Detail of a cytoplasmic rod-shaped extrusome. (H, J) Fusiform, slightly curved cells with narrowly rounded posterior body end. (I) A cylindrical cell. (K) An extended, fusiform exemplar with tail-like posterior end. (L) A sigmoid cell with narrowly rounded ends. (M) A semi-contraction, obpyriform specimen with broadly rounded posterior body end. CV – contractile vacuole; DI – dumbbell-shaped inclusions; EB – extrusome bundles; EX – extrusomes; G – cortical granules; H – head; LD – lipid droplets; MA – macronucleus; MI – micronucleus; OB – oral bulge; T – trunk. Scale bars: 20 μm.
Fig. 4. Phialina pupula in the scanning electron microscope (SEM). (A) Detail of the anterior body half. The head is localized at the anterior body end and is attached directly to the trunk, as typical of the genus Phialina. The head is covered by very narrowly spaced cilia arranged in helically extending rows. Note that the cortex of the trunk is distinctly furrowed by slightly helically extending ciliary rows. According to protargol preparations, each somatic ciliary row has two to five brush dikinetids at its anterior end (see Fig. 2E). SEM observations show that the anterior basal body of a brush dikinetid bears a minute to short cilium or is unciliated, while the posterior basal body bears an ordinary somatic cilium. Therefore, the brush is very difficult to recognize in the SEM and in vivo. (B) Detail of the anterior end of somatic ciliary rows, showing that the anterior basal body of a brush dikinetid bears a short cilium (arrowheads) or is unciliated. The posterior basal body of a brush dikinetid bears an ordinary somatic cilium. Such an inconspicuous brush is a typical feature of lacrymariids and also of the possibly related chaeneids. (C) Detail of a somatic ciliary row, showing a dikinetid (dividing basal bodies) followed by monokinetids that bear ordinary cilia. As typical for haptorians, the anterior cilium of dividing basal bodies is short and stump-like while the posterior cilium is ordinarily long. AC – anterior stump-like cilium of dividing basal bodies; G – tips of cortical granules; H – head; HC – head cilia; SC – somatic cilia; T – trunk.
The genus *Phialina* was depicted as paraphyletic containing the polyphyletic genus *Lacrymaria*. The newly obtained *P. pupula* sequences formed a fully supported clade that was placed at the very base of the *Lacrymaria* clade. Two variably supported *Phialina* clusters were further recognized: (i) the *P. caudata + P. clampi + Phialina* sp. MF474346 group and (ii) the *P. salinarum + P. vertens + Phialina* sp. FJ870088 and FJ876972 group. The latter group was depicted as sister to the *L. marina + L. maurea + L. olor + Lacrymaria* sp. assemblage. Only *Lacrymaria* sp. 1 did not cluster with congeners, causing the polyphyly of the genus *Lacrymaria* (Fig. 6). However, *Lacrymaria* sp. 1 clusters with the other *Lacrymaria* species when more molecular characters are included (e.g., Huang *et al.* 2018).

**DISCUSSION**

**Comparison of *Phialina pupula* populations**

Müller (1773) described *P. pupula* very briefly and without illustration under the name *Enchelis pupula*. Later, Müller (1786) provided a description with figures that, however, did not enable unambiguous identification of the species. Multiple descriptions of populations identified as *P. pupula* occur sporadically in the literature, for instance, in Kahl (1930), Gajewskaja (1933), Dragesco (1960), Vuxanovici (1963) as well as in Song and Wilbert (1989). All basically match in the body shape, the nuclear and contractile vacuole apparatus as well as in the extrusome pattern. However, most authors very likely depicted only semi-contracted, mostly elliptical specimens with a rounded posterior body end. The single exception is the study of Vuxanovici (1963) who described and illustrated almost the whole range of shape variability, including obconical, obpyriform, elliptical and even sigmoidal cells.

Kahl (1930) described very peculiar dumbbell-shaped inclusions scattered throughout the cytoplasm and especially concentrated in the anterior body half of *P. pupula*. Gajewskaja (1933) illustrated these remarkable inclusions in her specimens and we also observed them in the Boise exemplars. However, they were not present in every cell, which possibly explains why these dumbbell-shaped inclusions were not mentioned by Vuxanovici (1963).

Some *P. pupula* populations differ in two taxonomically important features, the body size and the number of the ciliary rows, indicating that they might be not conspecific. Specifically, Kahl’s (1930) specimens were 120–180 µm long, Dragesco’s (1960) individuals were 160 µm long, Gajewskaja’s (1933) as well as Song and Wilbert’s (1989) exemplars were only 60–90 µm long, and Vuxanovici (1963) did not mention the length at all. By contrast, Boise specimens were within the range provided by Kahl (1930) and Gajewskaja (1933) as well as Song and Wilbert (1989), i.e., they measured 60–130 × 20–50 µm, usually about 85 × 30 µm. Nevertheless, we cannot exclude that *P. pupula* is highly variable in body size, possibly reflecting contractility and nutritional factors. Indeed, the body length in phialinids usually spans a comparatively wide range (e.g., Foissner 1983, Foissner *et al.* 2002, Wang *et al.* 2019). *Phialina pupula* populations also differ conspicuously in the number of ciliary rows. There are about eight rows on one side according to Kahl (1930) and ten rows on one side according to Gajewskaja (1933) but 30 rows in total according Dragesco (1960) and 43–52 rows in total according to Song and Wilbert (1989). Boise specimens display about eight ciliary rows on one side, matching Kahl’s (1930) and Gajewskaja’s (1933) observations quite well.

To summarize, the Boise population might be conspecific with *P. pupula* sensu Kahl (1930), Gajewskaja (1933) and Vuxanovici (1963). However, *P. pupula* sensu Dragesco (1960) and Song and Wilbert (1989) very likely represent a different species due to the markedly higher number of ciliary rows.

**Comparison of *Phialina pupula* with similar species**

*Phialina pupula* can be easily distinguished from all congeners by having highly refractive dumbbell-shaped inclusions scattered throughout the cytoplasm and usually concentrated in the anterior body half (Kahl 1930, Gajewskaja 1933, present study). Interestingly, Kahl (1930) observed dark granules also in *P. coronata* (Claparède and Lachmann, 1859) Foissner, 1897 but, as he explicitly mentioned, they were never dumbbell-shaped. Moreover, *P. coronata* occurs in salt water in contrast to the freshwater *P. pupula* (Kahl 1930, Foissner *et al.* 1995).

There are three freshwater species, viz., *P. vermicularis* (Müller, 1786) Bory de Saint-Vincent, 1824, *P. vertens* (Stokes, 1885) Foissner and Adam, 1979 and *Lacrymaria phialina* Švec, 1897, which resemble *P. pupula* in body shape, characteristics of the nuclear and contractile vacuole apparatus, and the extrusome pattern. *Phialina vermicularis*, as redescribed by Foissner (1983), also differs from *P. pupula* in body length (40–60 µm vs. 60–180 µm) and the shape of the cortical
Fig. 5. Phylogeny based on the 18S rRNA gene and the ITS1-5.8S-ITS2 region of 80 litostomatean taxa and two armophoreans serving as outgroup (CON-lit alignment). Posterior probabilities for the Bayesian inference and bootstrap values for maximum likelihood were mapped onto the 50%-majority rule Bayesian consensus tree. Note that monophyly of the family Lacrymariidae is moderately to strongly statistically supported. Sequences in bold face were obtained during this study. The scale bar indicates five substitutions per one hundred nucleotide positions. For GenBank accession numbers, see Supplementary Table S3.
Fig. 6. Phylogeny based on the 18S rRNA gene of 22 taxa from the family Lacrymariidae (18S-lac1 alignment). Note that the genus *Phialina* is paraphyletic and contains the polyphyletic genus *Lacrymaria*. Posterior probabilities for the Bayesian inference and bootstrap values for maximum likelihood were mapped onto the 50%-majority rule ML tree. Sequences in bold were obtained during this study. The scale bar indicates nine substitutions per one thousand nucleotide positions.

granules (conspicuous and rod-shaped vs. inconspicuous and broadly elliptical to elliptical). *Phialina vertens*, as redescribed by Foissner (1983), is distinguished from *P. pupula* by the contractile vacuole surrounded by slightly yellowish granules. And, finally, *L. phialina*, as re-described by Penard (1922), has almost twice the number of ciliary rows as *P. pupula* (about 30 vs. 15).

**Molecular and morphological evolution of the family Lacrymariidae**

According to multiple phylogenetic analyses, the family Lacrymariidae represents a monophyletic and distinct lineage within the subclass Haptoria (e.g., Gao et al. 2008, Vďačný et al. 2011, Zhang et al. 2012, Kwon et al. 2014, Wu et al. 2017, Huang et al. 2018, Wang et al. 2019), which is also in accordance with the present results (Figs 5, 6). In the pioneer studies, the genera *Phialina* and *Lacrymaria* were each depicted as being monophyletic (Zhang et al. 2012, Kwon et al. 2014). However, with an increasing sequence pool, both genera have become non-monophyletic (Wu et al. 2017, Huang et al. 2018, Wang et al. 2019, present study). Although the generic home of most lacrymariid taxa is questionable and unstable (e.g., Penard 1922, Kahl
The final edited and typeset version of record will appear in future

tested by further molecular markers.

- marsiovia, plesiomorphies or homoplasies, needs to

- maridae and Chaeneidae (Kwon

- ing the sister-group relationship of the families Lacry-

- and separation of the dorsal brush from the anterior

(e.g., body contractility, head-like anterior body end,

2014. There are also further morphological features

- relationship with the family Chaeneidae Kwon

- posterior basal body of brush dikinetids associated

- (e.g., monokinetid) corroborating

- the sister-group relationship of the families Lacry-

- and Chaeneidae (Kwon et al., 2014, Vďačný and Rataj 2017). Whether these features are synap-

morphic features (e.g., body contractility, head-like anterior body end, and separation of the dorsal brush from the anterior body end by files of somatic monokinetids) corroborating the sister-group relationship of the families Lacrymariidae and Chaeneidae (Kwon et al., 2014, Vďačný and Rataj 2017). Whether these features are synapo-

morphies, plesiomorphies or homoplasies, needs to be tested by further molecular markers.

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15-0147 and by the Grant Agency of the Ministry of Education,

Science, Research and Sport of the Slovak Republic and Slovak

Agency of Sciences under the Grant VEGA 1/0041/17.

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Holophryidae, Prorodontidae, Plagiocampidae, Colepidae, En-


einiger neuer und wenig bekannter terrestrieller, limnis-

cher und mariner Ciliaten (Protozoa: Ciliophora) aus den Klassen

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Ciliophora). Holophryidae, Prorodontidae, Plagiocampidae, Cole-

pidae, Enchelyidae and Lacrymariidae. In: Klassen und Ordnun-

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Foissner W., Berger H., Schaumburg J. (1999) Identification and
ecology of limnetic plankton ciliates. Informationsberichte des

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a, Ciliophora) from Namibia (southwest Africa) with emphasis on
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fusoirs proprement dits comprenant de nouvelles recherches

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pèces nouvelles ou peu connus. G. Masson, Paris
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Received on 29th April, 2019; revised on 7th June, 2019; accepted on 4th July, 2019
Supplementary Table S1. Characterization of new 18S rRNA gene and ITS region sequences obtained during this study.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Collection site</th>
<th>Clone No.</th>
<th>18S rRNA gene Length (nt)</th>
<th>GC (%)</th>
<th>GenBank entry</th>
<th>ITS-5.8S-ITS2 region Length (nt)</th>
<th>GC (%)</th>
<th>GenBank entry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phialina pupula</td>
<td>Sediments from the floodplain area of the Boise River near the Glenwood Bridge, Boise, Idaho, U.S.A.</td>
<td>1</td>
<td>1636</td>
<td>42.85</td>
<td>MN030551</td>
<td>1190</td>
<td>43.36</td>
<td>MN030617</td>
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<td></td>
<td>2</td>
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<td>42.72</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacrymaria olor</td>
<td>Water and sediments from the shore area of a pond at the Julia Davis Park, 700 S. Capitol Blvd. Boise, Idaho, U.S.A.</td>
<td>1</td>
<td>1642</td>
<td>42.75</td>
<td>MN030553</td>
<td>1293</td>
<td>42.30</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1642</td>
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<td>42.30</td>
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<td></td>
<td></td>
<td>3</td>
<td>1642</td>
<td>42.75</td>
<td>MN030555</td>
<td>1293</td>
<td>42.23</td>
<td>MN030620</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>1642</td>
<td>42.69</td>
<td>MN030556</td>
<td>1293</td>
<td>42.23</td>
<td>MN030621</td>
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<td></td>
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<td>42.69</td>
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<td>1293</td>
<td>42.23</td>
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<td>–</td>
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<td>42.23</td>
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<td></td>
<td></td>
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<td>–</td>
<td>1293</td>
<td>42.23</td>
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</tr>
</tbody>
</table>

*These sequences also contain a variably long 5’-end of the 28S rRNA gene.

Supplementary Table S2. Characterization and parameterization of the GTR evolutionary models of the alignments analyzed.

<table>
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<tr>
<th>Characteristics†</th>
<th>Alignment</th>
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<td>No. of taxa</td>
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<td>No of characters</td>
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</tr>
<tr>
<td>Cutoff value‡</td>
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<td>A</td>
<td>0.2817</td>
</tr>
<tr>
<td>C</td>
<td>0.1843</td>
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<tr>
<td>G</td>
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<tr>
<td>T</td>
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</tr>
<tr>
<td>[AC]</td>
<td>1.6547</td>
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<tr>
<td>[CG]</td>
<td>0.6655</td>
</tr>
<tr>
<td>[GT]</td>
<td>1.0000</td>
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<tr>
<td>I</td>
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</tr>
<tr>
<td>Γ</td>
<td>0.3420</td>
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</tbody>
</table>

†The best fitting evolutionary models were selected for each dataset under the Akaike information criterion in jModelTest. A, C, G, T, base frequencies; [AC], [AG], [AT], [CG], [CT], [GT], rate substitution matrices; I, proportion of invariable sites; Γ, gamma distribution shape parameter.

‡Unreliably aligned columns were removed from the alignment at the cutoff value of 0.93. Dash indicates no masking strategy.

§The CON-lit dataset was created by combining the 18S-lit2 and the ITS-lit2 alignment.

αThe CON-lac dataset was created by combining the 18S-lac2 and the ITS-lac2 alignment.
**Supplementary Table S3.** List of ciliate taxa with GenBank accession numbers of corresponding 18S rRNA gene sequences and ITS1-5.8S-ITS2 region sequences included in phylogenetic analyses. Newly obtained sequences are in bold face.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>GenBank entry 18S rRNA gene</th>
<th>GenBank entry ITS region</th>
<th>Taxon</th>
<th>GenBank entry 18S rRNA gene</th>
<th>GenBank entry ITS region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Armophorea (outgroup)</td>
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<td></td>
<td>Rimaleptus binucleatus</td>
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<td>Clevelandella panthestiae</td>
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<td>KC460347</td>
<td>Rimaleptus mucronatus</td>
<td>H581675</td>
<td>JX07865</td>
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<td>Nycotherus ovalis</td>
<td>AJ222678</td>
<td>AJ006714</td>
<td><em>Rurikoplites armatus</em> (Korea)</td>
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<td>MF288138</td>
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<td>Chaeneida</td>
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<td><em>Rurikoplites armatus</em> pop. 1 (Slovakia)</td>
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<td>KP868778</td>
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<td>Chaenea sp. 2</td>
<td>MF474336</td>
<td>MF474336</td>
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<tr>
<td>Chaenea sp. 3</td>
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<td>Rurikoplites longirichus</td>
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<td>Chaenea vorax</td>
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<td>Helicoprorodon maximus</td>
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<td><em>Apobryophyllum schmidigeri</em> (Germany)</td>
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<td>KY556653</td>
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<td>Homalozoon vermiculare</td>
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<td>MF474368</td>
<td>Arcuospathidium cultriforme scarliforme</td>
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<td>Lacymaryidae</td>
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