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SHORT COMMUNICATION

Validation of *Polytomella piriformis* nomen nudum (Chlamydomonadaceae): a Distinct Lineage Within a Genus of Nonphotosynthetic Green Algae

Shelley M. MacDonald & Robert W. Lee

Department of Biology, Dalhousie University, 1355 Oxford St., Halifax, Nova Scotia, B3H 4R2, Canada

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Correspondence
R.W. Lee, Department of Biology, Dalhousie University, 1355 Oxford St., Halifax, Nova Scotia, B3H 4R2, Canada
Telephone number: +1 902-494-2554; FAX number: +1 902-494-3736; e-mail: robert.lee@dal.ca

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ABSTRACT

Polytomella strain SAG 63-10 was first described by Pringsheim (1963) as *Polytomella piriformis* nomen nudum. The current study validates the name *Polytomella piriformis* following the International Code of Nomenclature for algae, fungi, and plants (ICN). We present 18S rRNA sequences of SAG 63-10 and several other *Polytomella* strains, which, along with existing mitochondrial DNA sequences, clearly distinguishes *P. piriformis* n. sp. from other available *Polytomella* species. The first type material of the species is presented, as well as an illustration and micrographs. Our own observations of *P. piriformis* SAG 63-10 are compared to Pringsheim's description and to descriptions of other valid *Polytomella* spp.

POLYTOMELLA is a genus of unicellular, colorless, chlorophycean algae, whose members inhabit freshwater environments rich in organic matter (Pringsheim 1955). Vegetative cells store starch and are quadriflagellated, wall-less, and often variations of an egg shape. Most strains form spherical cysts, which are smaller than the vegetative cells and have rigid cell walls (Pringsheim 1955). There are conflicting reports on whether *Polytomella* spp. can undergo sexual reproduction (reviewed by de la Cruz and Gittleson 1981). Readers are directed to Pringsheim (1955), de la Cruz and Gittleson (1981), and Ettl (1983) for reviews of the genus.

Pringsheim (1963) introduced *Polytomella piriformis* nomen nudum, which is currently maintained at the Culture Collection of Algae at the University of Göttingen (SAG) as strain 63-10. He did not designate a type specimen as required by Art. 40.1 of the International Code of Nomenclature for algae, fungi, and plants (ICN) (McNeill et al. 2012), include an image (ICN Art. 44.2), or provide a Latin description as required at the time (ICN Art 44.1). His description also did not adequately distinguish the material from other *Polytomella* species (ICN Art. 38).

We aim to validate *P. piriformis* nomen nudum by (i) discussing molecular data distinguishing *P. piriformis* SAG 63-10 from three other identified *Polytomella* lineages, (ii) presenting our morphological observations of SAG 63-10 in comparison to Pringsheim (1963), and (iii) comparing *P. piriformis* SAG 63-10 to other validly described *Polytomella* species. Validation of the name *P. piriformis* is timely, as the name has been used by the University of Texas Culture Collection (UTEX) (Starr and Zeikus 1987) and in research reports (for example, Smith and Lee 2014).

MATERIALS AND METHODS

Culture and strains

According to the SAG database (http://sagdb.uni-goettingen.de/, accessed 1 May 2015), *P. piriformis* SAG 63-10 was isolated prior to 1963 from garden soil of a heated green house in Göttingen, Germany by E. G. Pringsheim. Our laboratory received this strain from SAG in 2002 or 2003, made it axenic by antibiotic treatment, and maintained it in liquid culture (Mallet and Lee 2006) until 2010, after which it was maintained as cysts on filter paper (Reed et al. 1976) at −20 °C until 2014 and −80 °C thereafter.

Cells were grown at 22 °C in *Polytomella* medium (Sheeler et al. 1968) out of direct light, without shaking, in 20 × 150-mm closure-topped test tubes with 10 ml of medium (for microscopy) or in cotton-plugged 250-ml side-arm Erlenmeyer flasks with 50 ml of medium (for RNA extraction). $OD_{750\ nm}$ was measured with a Bausch & Lomb Spectronic 20 (Rochester, NY). Measurements of $OD_{750\ nm}/10^6$ cells ml^{-1} were taken in the logarithmic phase of growth when the $OD_{750\ nm}$ was ~0.05–0.30. Cultures were harvested for RNA extraction at the following $OD_{750\ nm}$ values: 0.19 (*P. piriformis* SAG 63-10), 0.25 (*Polytomella parva* SAG 63-3), 0.18 (*Polytomella capuana* SAG 63-5), and 0.23 (*Polytomella magna* SAG 63-9).

Sequencing and molecular evolutionary analyses

Polytomella RNA was extracted using the Qiagen RNeasy Kit and Qiagen RNase-free DNase (Qiagen Sciences, Germantown, MD). The transcriptome of *P. parva* SAG 63-3 has been described previously (Smith and Lee 2014). Illumina sequencing and transcriptome assembly were performed for the remaining three strains by the McGill University and Génome Québec Innovation Centre (Montreal, Canada). See Table S1 for a summary of transcriptome assembly metrics.

To recover homologous transcripts, the contigs of each of the four *Polytomella* transcriptomes were aligned individually to the *Chlamydomonas reinhardtii* 18S rRNA sequence (GenBank accession M32703) using the "Map to Reference" function in Geneious v7.1.7 (Kearse et al. 2012). This step used the "highest sensitivity" option with index and word lengths of 10 nucleotides and a maximum total gap length of 20% per contig. *Polytomella* 18S rRNA contigs were trimmed to the length of the *C. reinhardtii* reference sequence. The 18S rRNA sequences of *P. piriformis* SAG 63-10 (1,833 nt), *P. parva* SAG 63-3 (1,839 nt), *P. capuana* SAG 63-5 (1,821 nt), and *P. magna* SAG 63-9 (1,789 nt) have been deposited into GenBank under accessions KP299172, KP299173, KP299174, and KP299175, respectively. Our current sequences are considerably longer than those used by Smith and Lee (2011) and Smith et al. (2013), which were ~1,032 nt in length.

The four *Polytomella* 18S rRNA sequences were added to an alignment from Nakada et al. (2008) of 34 *Reinhardtinia*-clade (defined by Nakada et al. 2008) taxa with an outgroup of three *Desmotetra* sequences (Table S2) using MUSCLE (Edgar 2004) implemented through Geneious, with default parameters and the maintenance of any existing gaps. Regions of ambiguous alignment were manually removed and the *Polytomella* sequences were trimmed to the length of the reference alignment. Phylogenetic analyses were performed on the resulting 1,679 nt alignment with RAxML 7.2.6 (Stamatakis 2014) under the GTR + GAMMA model, with 1,000 bootstrap replicates.

Microscopy

We observed iodine-treated and live *P. piriformis* SAG 63-10 cells using a Zeiss Axiovert 200M microscope (Zeiss, Oberkochen, Germany), equipped with a Zeiss Axiocam HR digital camera, using differential interference contrast and a 100X oil-immersion lens with and without a 1.6X Optovar. Micrographs of live cells (slides prepared with ~10 μl of log-phase culture) were used as references to produce a drawing of *P. piriformis* SAG 63-10. Micrographs were also taken of *P. parva* SAG 63-3 for comparison. Cell size was measured using log-phase cultures, before cells became visibly distorted after the minimum addition of 2.5% iodine tincture required to inhibit the mobility of most cells (starting with 1 μl in 1 ml of culture).

RESULTS AND DISCUSSION

Phylogeny

Our phylogenetic analysis using 1,679 nt of the 18S rRNA gene (Fig. 1A) agrees with the topology of previous phylogenies including these *Polytomella* strains (Smith et al. 2013) and is consistent with previous analyses in showing accelerated rates of evolution in *Polytomella* spp. compared to other *Reinhardtinia*-clade (defined by Nakada et al. 2008) taxa (e.g. Fan and Lee 2002; Nakayama et al. 1996; Smith et al. 2010). Past phylogenies including all four available *Polytomella* lineages have been relatively small, whereas phylogenies with a large set of taxa have included only *P. parva* (UTEX 193) (Gerloff-Elias et al. 2005; Nakada et al. 2008). The tree in Fig. 1A and a second, more inclusive tree produced by the same method using 18S rRNA sequences from these four *Polytomella* strains and 316 other chlamydomonadalean taxa used by Nakada et al. (2008), resulting in an alignment of 1,741 nt, agree in topology and support *Polytomella* as a monophyletic group within the *Reinhardtinia* clade (data not shown).

Microscopy

Our illustration and micrographs of *P. piriformis* n. sp. (SAG 63-10) are shown in Fig. 1B and S1, respectively. Micrographs of *P. parva* (SAG 63-3), the closest known relative of *P. piriformis*, are provided for comparison (Fig. S2). In agreement with Pringsheim's 1963 description, *P. piriformis* cells are typically pear- to egg-shaped and with a distinct papilla. Flagellae, which are more easily observed in iodine-treated cells compared to untreated ones, are usually slightly longer than the body, also agreeing with Pringsheim (1963). Pear-shaped cells with a narrowed or pointed posterior (Fig. S1A) are more common in *P. piriformis* than in *P. parva*, with cells of *P. parva* typically being rounder in the posterior half. However, there is variation in posterior shape within each species and this variation overlaps between species, making it difficult to distinguish between them by morphology alone.

Vegetative cell and cyst size have historically been used to help distinguish *Polytomella* species. Pringsheim (1963) reported iodine-treated *P. piriformis* cells to have a size of 17–21 μm long × 12–14 μm wide. We observed consider-

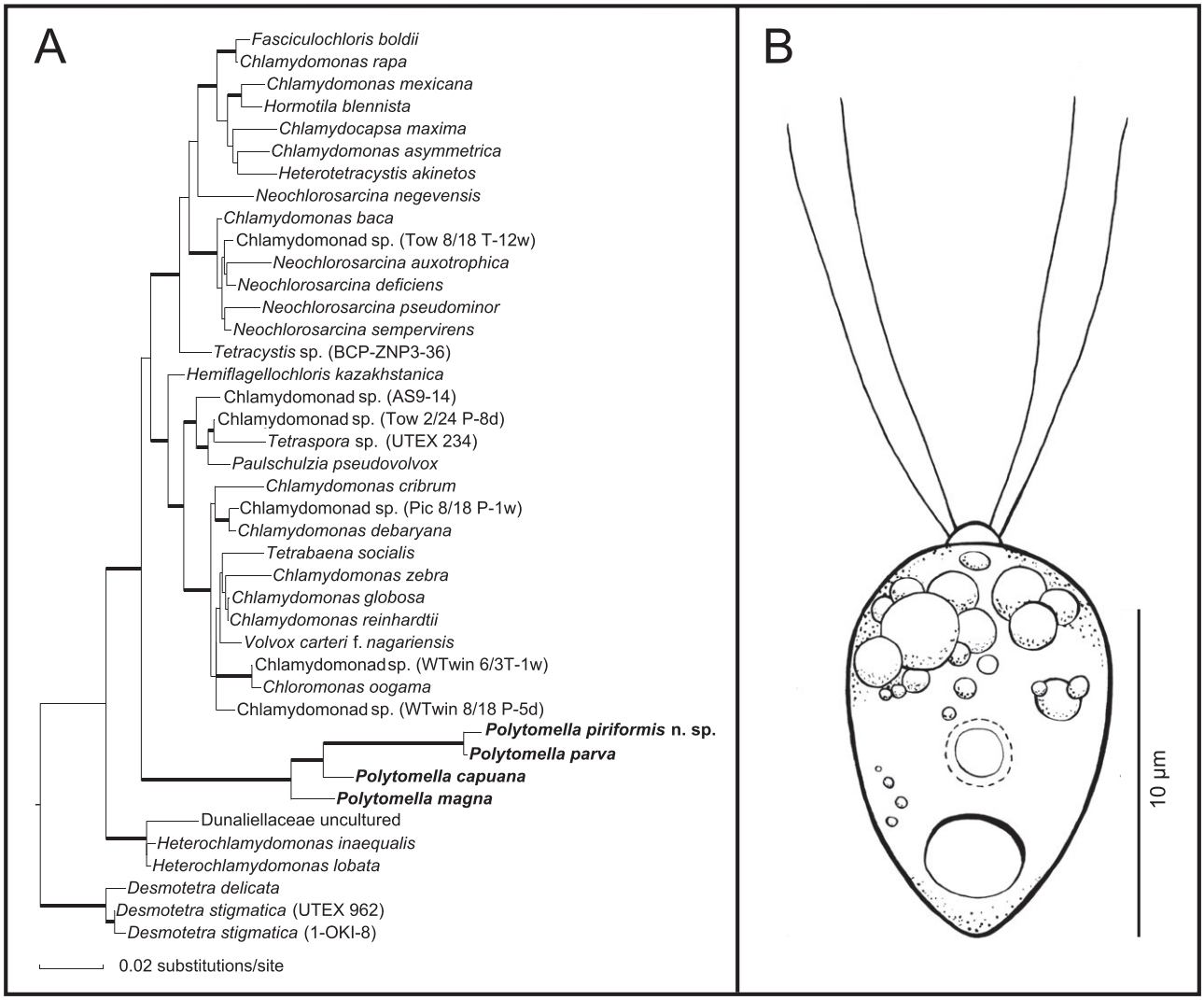

Figure 1 Morphology and molecular phylogeny of *Polytomella piriformis* n. sp. (SAG 63-10). **A.** Maximum likelihood tree inferred from a 1,679 nt alignment of 18S rRNA sequences from four *Polytomella* species (bold font) and 34 other chlamydomonadalean reference taxa. The tree was rooted using three *Desmotetra* homologs. Bold branches indicate bootstrap support ≥ 0.75 from 1,000 bootstrap replicates. Strains are shown in parentheses for uncharacterized taxa. GenBank accessions and strain designations for all taxa are listed in Table S2. **B.** Drawing of *P. piriformis* n. sp. as viewed by light microscopy. Starch granules are visible in the anterior portion of the cell and a vacuole is evident in the cell posterior, below the central nucleus.

ably smaller cells. Our iodine-treated *P. piriformis* SAG 63-10 cells measured 7.5–20 µm × 5–15 µm, with an average length of 14.1 (±1.2) µm and an average width of 10.1 (±0.8) µm (n = 101). Moreover, Pringsheim described *P. piriformis* as larger than *P. parva*; however, our observations of both cell size (Table S3) and mass, based on $OD_{750\ nm}/10^6$ cells ml^{-1} (Table S4), of *P. piriformis* SAG 63-10 and *P. parva* SAG 63-3 showed no significant difference between these two strains. Assuming that SAG 63-10 and SAG 63-3 are derived from Pringsheim's original materials, differences between our observations and Pringsheim's may reflect differences in culture conditions or the difficulty of accurately observing *Polytomella* spp., but are also a possible result of evolution of the strains, which were maintained as metabolically active cultures for more than 50 yr. Our measurement of average cyst diameter, the first reported for *P. piriformis*, is 10 (±1) µm (n = 45) with a range of 7.5–12.5 µm. This range is inclusive of the cyst diameter of *P. parva* reported by Pringsheim (1955) and observed in our laboratory (Table S3).

Polytomella piriformis distinct species status

There are 10 validly named species of *Polytomella*, half of which are not currently available in a public culture collection, to which we compare *P. piriformis* (SAG 63-10) in Table S3. *Polytomella aphanochloris* is distinct from *P. piriformis* in having an eyespot, while *P. magna* and *Polytom-*

ella zerovii are distinguished from *P. piriformis* by both presence of an eyespot and larger size. In addition, molecular data places *P. magna* (SAG 63-9) in a distinct lineage, separate from *P. piriformis* (SAG 63-10), as shown in Fig 1A and Smith et al. (2013). Other than the presence/absence of an eyespot, morphological features of *Polytomella* spp. are few, frequently overlap between species, and are notoriously difficult to determine accurately because of rapid movement and susceptibility to changes in shape during observation, as noted by others (Kater 1925; Massjuk and Kostikov 1986; and Pringsheim 1955). Pringsheim (1955) considered early descriptions of *Polytomella* spp. to be unreliable. Of particular note are the conflicting morphological descriptions of *Polytomella agilis* (Table S3). Ettl (1983) and others (Gittleson and Jahn 1968; Kater 1925) attach the description by Doflein (1916), which includes an eyespot, to the name *P. agilis*. *Polytomella globosa* and *Polytomella citri* lack an eyespot and are similar in size to *P. piriformis*. Pascher (1927) described *P. globosa* as commonly having a spherical shape, unlike most cells of *P. piriformis*. Kater (1925) described *P. citri* cells as pear-shaped, like *P. piriformis*, but observed common variants with 2–3 posterior "tail" shapes, a characteristic unique to the description of *P. citri*. Finally, in our opinion, *P. parva* (SAG 63-3 and UTEX 193), *Polytomella papillata* (SAG 63-2), *Polytomella caeca* (SAG 63-1b and SAG 63-2b), and *P. capuana* (SAG 63-5) are difficult to reliably distinguish from *P. piriformis* by morphology, but available sequence data from this and other studies (Mallet and Lee 2006; Smith and Lee 2011; Smith et al. 2013) places them among two other *Polytomella* lineages separate from *P. piriformis* SAG 63-10. We thus argue that SAG 63-10 represents a distinct species, and provide a taxonomic summary below to validate *P. piriformis* nomen nudum.

Phylum Chlorophyta emend. Lewis and McCourt, 2004

Class Chlorophyceae Wille in Warming, 1884 emend. Mattox and Stewart, 1984

Order Chlamydomonadales Fritsch, 1927 (= Volvocales sensu Nakada et al. 2008)

Family Chlamydomonadaceae Stein, 1878

Genus *Polytomella* Aragão, 1910

Polytomella piriformis Pringsheim ex. MacDonald and Lee, 2015

Diagnosis. Vegetative cells pear to broad egg-shaped, often slightly rounded at the posterior end, with small and distinct papilla on the anterior end. Iodine-treated cells, 7.5- to 20-μm long, and 5- to 15-μm wide. Flagellae often slightly longer than the cell. No stigma. Cysts, 7.5–12.5 μm in diam., usually 10 μm. Differs from morphologically similar species *P. parva*, *P. capuana*, and *P. magna* in 18S rRNA sequence (current study) and mitochondrial DNA sequence (Smith et al. 2010, 2013).

Strain. *P. piriformis* SAG 63-10, collected prior to 1963 from soil of a heated greenhouse in the Old Botanical Garden, University of Göttingen, Germany (latitude 51.537 807; longitude 9.935947).

Etymology. Specific epithet from L. pirum-, "pear" + L. -formis, "formed" or "shaped".

Type material. The holotype material of the species is maintained as permanently cryopreserved cysts at SAG as strain 63-10. Cultures of strain 63-10 in liquid medium are also available through SAG.

Sequence data. The 18S rRNA sequence of *P. piriformis* SAG 63-10, with a length of 1,833 nt and a GC content of 45.0%, is deposited in GenBank under accession number KP299172. All *P. piriformis* sequence data currently available in GenBank was obtained from our laboratory, in which *P. piriformis* SAG 63-10 was maintained in *Polytomella* medium for 7 to 8 yr prior to DNA extraction.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. *Polytomella piriformis* n. sp. (SAG 63-10), differential interference contrast (DIC) micrographs of live vegetative cells showing common variations in shape (**A**–**C**) and cysts (**D**). All scale bars represent 10 μm.
Figure S2. *Polytomella parva* (SAG 63-3), differential interference contrast (DIC) micrographs of live vegetative cells showing common variations in cell shape (**A**–**D**). All scale bars represent 10 μm.
Table S1. RNA-seq de novo transcriptome assembly metrics for *Polytomella piriformis* n. sp. (SAG 63-10), *Polytomella capuana* (SAG 63-5), and *Polytomella magna* (SAG 63-9).
Table S2. Taxa used in the phylogenetic analysis of Fig. 1A, with sequences from the present study in bold.
Table S3. Comparison of *Polytomella piriformis* n. sp. (SAG 63-10) to all valid *Polytomella* spp. descriptions.
Table S4. Optical density of *Polytomella piriformis* n. sp. (SAG 63-10) and *Polytomella parva* (SAG 63-3) at a wavelength of 750 nm/10^6 cells ml^{-1}.