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Morphological and molecular characterization of a new member of the phylum Rhodelphidia

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Abstract

Rhodelphidia is a recently discovered phylum within the supergroup Archaeplastida, comprising only two known representatives (*Rhodelphis marinus* and *Rhodelphis limneticus*). Despite its close phylogenetic relatedness to red algae, Rhodelphidia differ markedly by being nonphotosynthetic eukaryotrophic flagellates with gene- and intron- rich genomes. Here, we describe a new freshwater Rhodelphidia species, *Rhodelphis mylnikovi* sp. n., strain Rhod- M. It shows clear morphological differences with the two other *Rhodelphis* species, including larger cell body size, presence of two contractile vacuoles, short and blunt pseudopodia, absence of cysts, and tendency to cannibalism. 18S rRNA- based phylogenetic analysis placed it sister to the freshwater species *R. limneticus*.

KEY WORDS

18S rRNA gene phylogeny, flagellates, morphology, protists, *Rhodelphis mylnikovi*

RHODELPHIDIA is a recently described eukaryotic phylum closely related to Rhodophyta (red algae) and Picozoa (Gawryluk et al., 2019; Schön et al., 2021). Together with Glaucophyta and Chloroplastida (green algae and land plants) they comprise the supergroup Archaeplastida, which ancestrally acquired their (primary) plastid through the direct endosymbiosis of a cyanobacterium (Archibald, 2009; Ponce- Toledo et al., 2017). So far, the phylum Rhodelphidia contains only two species, *Rhodelphis limneticus* and *R. marinus*, isolated from freshwater and marine environments, respectively (Gawryluk et al., 2019).

Despite their close phylogenetic relatedness to red algae, the known Rhodelphidia species differ markedly from their photosynthetic relatives in terms of both phenotype and genome complexity. Rhodelphidia are nonphotosynthetic eukaryotrophic flagellates with gene- and intron-rich genomes. Red algae, in contrast, lost their flagellar apparatus and generally have gene- and intron-poor genomes and reduced metabolism (Qiu et al., 2015). The flagellum of Rhodelphidia resembles that of glaucophytes and Picozoa, having virtually identical ciliary transition zones (Cavalier-Smith, 2022). Rhodelphidia and Picozoa are also similar in having tubular mitochondrial cristae in contrast with the flattened cristae found in photosynthetic Archaeplastida (Adl et al., 2019; Gawryluk et al., 2019; Seenivasan et al., 2013).

Rhodelphis species possess a combination of general ultrastructural features found across different eukaryotic supergroups as well as unique characteristics. The latter include a smooth endoplasmic reticulum forming a double-layered sac around the nucleus and mitochondria, and the presence of umbrella-like glycostyles covering the plasma membrane and the posterior flagellum (Gawryluk et al., 2019). Although no plastids have ever been detected in *Rhodelphis* by electron microscopy, its genome encodes homologues of several plastid import proteins and many proteins with a putative plastid function, supporting that a relict primary plastid still exists (Gawryluk et al., 2019). This suggests that *Rhodelphis* diverged from an already photosynthetic common ancestor shared with Archaeplastida and has implications for our understanding of the early evolution of red algae and archaeplastids in general. Microscopy and genomic data suggest that the common ancestor of Rhodelphidia and red algae was a mixotrophic flagellate, akin to the archaeplastid common ancestor. The discovery of *Rhodelphis* also implies that phagotrophy was independently lost multiple times within Archaeplastida. The transitions from a mixotrophic ancestor to exclusively photosynthetic organisms involved dramatic changes in cell morphology and structure that are difficult to trace. Because of their phylogenetic position and peculiar features, gaining a deeper knowledge about Rhodelphidia can help deciphering the underlying evolutionary path that led to such transitions.

In this study, we characterize a new member of this clade isolated from freshwater, strain Rhod- M. 18S rRNA-based phylogenetic analysis placed it as a separate lineage closely related to the freshwater species *R. limneticus*. Based on the phylogeny, cell morphology and behavioral traits of the new strain, we describe the new species *Rhodelphis mylnikovi* sp. n.

MATERIALS AND METHODS Sample collection and culture establishment

Samples were collected from the freshwater pond Étang du Manet (48°45'39.0" N 2°00'43.7" E) near Montigny-le-Bretonneux, Ile-de-France, France. Water collected at 40 cm depth

together with the upper layer of bottom sediments was placed into a sterile 50- mL plastic Falcon tube and transported to the laboratory. An inoculum of this sample was placed in a Petri dish with 5 mL of sterile Volvic mineral water enriched with 0.1% YT medium (100 mg yeast extract and 200 mg tryptone in 100 mL distilled water, as in the protocol from the National Institute for Environmental Studies [NIES, Japan]) and maintained at 15°C in the dark to promote bacterial growth. We observed the presence of flagellated eukaryotrophic cells and let them multiply; cells were then isolated manually using a glass micropipette by picking out one cell at a time and placing them in wells of a 48- well plate containing the bodonid prey *Bodo saltans* Ehrenberg, 1832, in freshwater Volvic medium with 0.1% YT at 15°C. We obtained in this way a clonal culture, strain Rhod- M, currently stored in a collection of live protist cultures at the Université Paris- Saclay, France. Prey culture strain K1 was obtained from lake Konstanz, Germany (Blom et al., 1998) and kindly provided to us by Dr. Julius Lukeš.

Light and electron microscopy

Light microscopy observations of strain Rhod- M were made using an inverted Zeiss Axiovert 40 CFL microscope equipped with phase contrast objectives (20× and 40×) and an upright Zeiss Axioplan 2 microscope equipped with an oil- immersion DIC objective (100×) and a water- immersion phase contrast/DIC N- Achromplan objective (63×). Videos were recorded with a Sony α9 digital camera.

For scanning electron microscopy, a concentrated cell culture was mixed with 4% glutaraldehyde (final concentration 2%) diluted in 0.2 M sodium cacodylate buffer (pH 7.2), deposited on a 9- mm circular coverslip coated with poly- L- lysine and fixed for 1 h. After fixation, cells were washed with 0.2 M sodium cacodylate buffer (pH 7.2) and dehydrated in a graded series of ethanol baths (30%, 50%, 70%, 80%, 90%, 100% ×3 times, 10 min each). Subsequently, coverslips with cells were washed in 100% hexamethyldisiloxane three times for 15 min and dried at room temperature. Dry coverslips were mounted on aluminum stubs, coated with platinum, and observed using a GeminiSEM 500 (Carl Zeiss) scanning electron microscope.

18S rRNA gene PCR amplification, sequence alignment, and phylogenetic analyses

The 18S rRNA gene was amplified with GoTaq polymerase reaction mix (Promega) in 25 μL reaction volumes and four combinations of different universal eukaryotic primers: (A) EK- 1F (5'- CTGGT TGA TCC TGC CAG- 3') and EK- 1520R (5'- CYGCA GGT TCA CCTAC- 3'); (B) 18S- 42F (5'- CTCAA RGA YTA AGC CATGCA- 3') and EK- 1520R; (C) EK- 82F (5'- GAAAC TGC GAA TGG CTC- 3') and 18S- 1498R (5'- CACCT ACG GAA ACC TTGTTA- 3'); and (D) 18S- 42F and 18S- 1498R.

PCR amplifications were carried out following a nested approach. For the first PCR reaction, we used the primer combinations A and B, adding manually isolated single *Rhodolphis* cells to PCR reaction tubes. Then, we used 2 μL of the resulting PCR products as template for subsequent PCR reactions using primer combinations C and D. The amplification steps were

as follows: initial denaturing period (95°C for 3 min) followed by 35 cycles of denaturation (95°C for 45 s), annealing (55°C for 45 s), extension (72°C for 2 min), and a final extension period (72°C for 5 min). Sanger sequencing of eight amplification products (2 from PCR combination A + C, two from PCR combination A + D, and four from PCR combination B + C) was performed by GENEWIZ. We used MAFFT (Kato et al., 2002) with default settings to align the eight sequences and obtain a consensus sequence using the cloud-based platform Benchling (<https://www.benchling.com/>). The resulting 18S rRNA gene sequence was submitted to GenBank under accession number OQ924990.

The Rhod- M strain sequence was added to an alignment containing 90 sequences representing all major Archaeplastida clades and related groups and aligned with MAFFT with default settings (Kato et al., 2019). Ambiguously aligned positions were trimmed off with trimAL v.1.2 (Capella-Gutiérrez et al., 2009) using a gap threshold of 60% and minimum percentage of the positions in the original alignment to conserve 20%. The number of analyzed sites after trimming were 1751 bp. The trimmed alignment was analyzed by maximum likelihood (ML) with IQ-TREE v.1.6.11 (Nguyen et al., 2015) using the GTR + G sequence evolution model. ML statistical support was calculated with 1000 nonparametric bootstrap replicates. The tree was visualized using FigTree v.1.4.4 (github.com/rambaut/figtree/) and the online platform iTOL v.6.7.6 (<https://itol.embl.de/>).

RESULTS AND DISCUSSION

Protist isolation and molecular phylogeny

Using single cell picking, we isolated a new flagellate (Rhod- M strain) from freshwater sediment samples (see Materials and Methods). We then amplified and Sanger-sequenced its 18S rRNA gene. The amplicon sequence was 1656 bp long (GenBank OQ924990). Preliminary Blast (Altschul et al., 1990) searches against the nonredundant nr GenBank database indicated that the new sequence was similar to sequences of Archaeplastida species. Therefore, we included it in a multiple sequence alignment containing sequences representative of the main Archaeplastida lineages and their phylogenetically related groups and used it to reconstruct a maximum likelihood (ML) phylogenetic tree. This analysis placed the strain Rhod- M within Rhodelphidia with full support (Figure 1A and Figure S1) and as sister of the freshwater species *Rhodelphis limneticus*, with which it shared 95.16% nucleotide identity in the 18S rRNA gene.

Cell morphology

The overall cell morphology of the strain Rhod- M is similar to the previously described Rhodelphidia species, *R. limneticus* and *R. marinus* (Gawryluk et al., 2019). Cells are oval in shape, slightly laterally compressed, with an oblique anterior end having a small notch, and with a rounded or slightly elongated posterior end (Figure 1B– M). However, cell body sizes are larger than in the other *Rhodelphis* species, 12– 18.5 × 8.5– 14 μm ($N = 22$) versus 10– 13 × 7.5– 8.5 μm in *R. limneticus* and 10– 12 × 7.5– 8.0 μm in *R. marinus* (Gawryluk et al., 2019).

Rhod- M has two heterodynamic perpendicularly oriented flagella emerging subapically from the slight depression on the cell surface (Figure 1E,G). The anterior flagellum is about 1– 1.5 times longer than the cell, directed forward, and makes fast undulating and lasso- like movements. The posterior flagellum is 1.5– 2.5 times longer than the cell, directed backward, and trails behind the cell, sometimes making waving movements or bending in the distal part. The large round nucleus is located medially (Figure 1F,H). One or more, most often two, large contractile vacuoles are located in the anterior apical part of the cell (Figure 1D,I). This is a characteristic difference from the closely related freshwater species *R. limneticus*, which contains only one contractile vacuole (Gawryluk et al., 2019). Usually, one large and several small food vacuoles are located in the posterior part of the cell (Figure 1D,F– I). The surface of the cell body appears to be striated under the light microscope (Figure 1H). This feature was not mentioned in the description of *R. limneticus* and *R. marinus* and the nature of such striation is unknown. This striation cannot be visualized under SEM, since the surface of cells is always covered with an organic layer, which is also present in *R. limneticus* and *R. marinus* (Gawryluk et al., 2019). Many equal- sized granules are visible directly under the cell membrane. Given that extrusome- like structures were observed in TEM sections of *R. limneticus* (Gawryluk et al., 2019), these granules are also probably extrusomes (Figure 1D,H). Rarely, one or several short and blunt pseudopodia not associated with the feeding process are visible, extending from the anterior or the posterior end of the cell (Figure 1F,I).

We observed cell movement features similar to those described for *R. limneticus* and *R. marinus*. Cells swim both at the surface of the substrate and in the water column, in circles or in sinusoidal lines. However, Rhod- M cells almost never rotate around their longitudinal axis, unlike *R. limneticus* and *R. marinus*. The cell division is simple binary and cells cleave in an anterior– posterior direction (Figure 1C,J). Like the other species of *Rhodelphis*, Rhod- M is capable of consuming both bacterial and eukaryotic prey (bodonid heterotrophic flagellates) but does not survive on prokaryotic prey alone. The capture of the prey occurs with emerging pseudopodia (Figure 1K– M; Movie S1). Feeding pseudopodia always appear on the posterior pole of the cell body in the form of a funnel, enveloping the prey from all sides. Capture of the prey cell is fast, within 15– 20 s. Compared to *R. limneticus* and *R. marinus*, Rhod- M does not consume all eukaryotic prey cells in the culture and does not multiply actively, therefore never reach high density of cells in the culture. We observed numerous attempts of cannibalism in starving cultures (Figure 1K; Movies S2 and S3), although they were unsuccessful. As in the capture of bodonid prey, the capture of *Rhodelphis* cells by other *Rhodelphis* occurs through protruding posterior pseudopodia, which sometimes almost completely engulf the attacked cell, although sooner or later the predator releases it. Cysts were not observed, in contrast with the presence of roundish cysts found in the closely related freshwater species *R. limneticus* (Gawryluk et al., 2019).

Based on the characteristic morphological features and the phylogenetic position of Rhod- M, as well as the 18S rRNA sequence nucleotide divergence with other described for the genus *Rhodelphis*, including the general *Rhodelphis* strains, we can confidently assign our strain cell body shape, length and orientation of the flagella, to a new species of the genus *Rhodelphis*. The general mor- the location of the nucleus and contractile vacuoles, the phological characteristics of Rhod- M agree with those cell movement, and the eukaryotrophic lifestyle

with the ability to also consume bacteria. However, it bears clear morphological differences with the known species of the genus, *R. marinus* and *R. limneticus*, notably its larger cell body size, the presence of two contractile vacuoles and short and blunt nonfeeding pseudopodia, the absence of cysts, and a tendency to cannibalism.

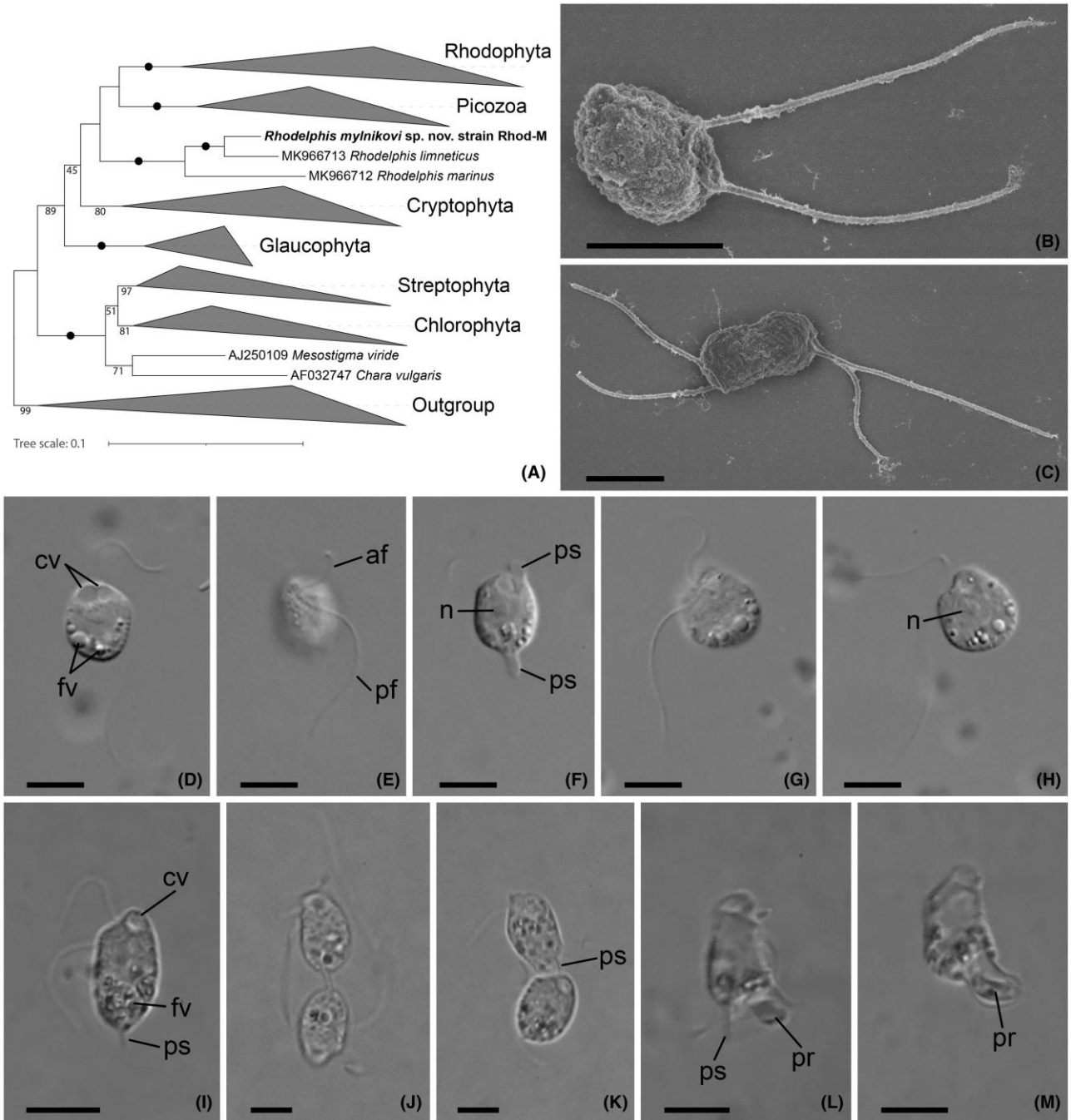


FIGURE 1 (A) Maximum likelihood (ML) 18S rRNA phylogenetic tree showing the position of *Rhodelphis mylnikovi* sp. n. strain Rhod-M within Archaeplastida. The tree contains 91 sequences of representatives of this supergroup and related clades. The tree was constructed using the GTR + G sequence evolution model. Branches with 100% ML bootstrap values are indicated by black dots, values

lower than 50% are not shown. (B– M) External morphology of *Rhodelfhis mylnikovi* sp. n. strain Rhod- M. (B and C) Scanning electron microscopy of fixed cells showing an individual cell (B) and a dividing cell (C). (D– M) Light micrographs of living cells visualized by differential interference contrast (D– H) and phase contrast (I– L), showing individual cells (D– I), a dividing cell (J), a cell attempting cannibalism (K), and a cell capturing prey (*Bodo saltans*) with pseudopodia (L and M). af, anterior flagellum; cv, contractile vacuole; fv, food vacuole; n, nucleus; pf, posterior flagellum; pr, prey; ps, pseudopodium. Scale bars = 10 µm. For the complete tree, see Figure S1.

Taxonomic summary

Taxonomy: Eukaryota; Archaeplastida;
Rhodelphidia

Rhodelfhis Tikhonenkov et al., 2019 in Gawryluk et al. (2019) Tikhonenkov, Gawryluk, Mylnikov et Keeling in Gawryluk et al. (2019) *Rhodelfhis mylnikovi* sp. n.

Description. Cells are 12– 18.5 µm long, 8.5– 14 µm wide. Anterior flagellum is about 1– 1.5 times longer than the cell, directed forward and with fast undulating and lasso- like movements. Posterior flagellum is 1.5– 2.5 times longer than the cell, directed backward and trailing behind the cell, making waving movements or bending in the distal part. Feeding pseudopodia always appear at the posterior pole of the cell in the form of a funnel. One or several short and blunt pseudopodia, not associated with feeding, may emerge from anterior and posterior parts of the cell. One or two contractile vacuoles in the anterior apical part of the cell. Cells usually actively swim in circles or following a sinusoidal line without rotating around their longitudinal axis. No cysts.

Type material. A SEM stub of chemically fixed and platinum- coated cells of the type strain, Rhod- M, is deposited in the DEEM collection of type materials, CNRS and Université Paris- Saclay (France). This constitutes the name bearing type of the new species (a hapantotype).

Type strain. Strain Rhod- M is deposited in the DEEM culture collection, CNRS and Université Paris- Saclay (France).

Type Figure. Figure 1D.

Type locality. Freshwater pond Étang du Manet, near town Montigny- le- Bretonneux, Ile- de- France, France.

Etymology. Named after Dr. Alexander Mylnikov, one of the researchers who discovered and described the phylum Rhodelphidia.

Gene sequence. The 18S rRNA gene sequence has the GenBank Accession Number OQ924990.

Zoobank Registration. LSID for this publication: urn:lsid:zoobank.org:pub:5AF881FF- E3F8- 47F3- 99FE- F3B90E6B3F87. LSID for the new species: urn:lsid:zoobank.org:act:9330112F- 4656-4 4FD- 92E0- 23 EB608749EC.

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