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A taxonomic reassessment of *Chlamydomonas meslinii* (Volvocales, Chlorophyceae) with a description of *Paludistella* gen.nov.

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Abstract

Chlamydomonas (Volvocales, Chlorophyceae) is a large polyphyletic genus that includes numerous species that should be classified into independent genera. The present study aimed to examine the authentic strain of *Chlamydomonas meslinii* and related strains based on morphological and molecular data. All the strains possessed an asteroid chloroplast with a central pyrenoid and hemispherical papilla; however, they were different based on cell and stigmata shapes. Molecular phylogenetic analyses based on 18S *r*DNA, *atp*B, and *psa*B indicated that the strains represented a distinct subclade in the clade *Chloromonadinia*. The secondary structure of ITS-2 supported the separation of the strains into four species. Based on the results, we propose a novel genus, *Paludistella*, including *P. meslinii comb. nov.*, *P. chlorostelatta comb. nov.*, *P. asymmetrica sp. nov.*, and *P. triangulocculus sp. nov.*

Introduction

Chlamydomonas (*Cd.*) C. Ehrenb (Volvocales, Chlorophyceae) is one of the largest genera of green microalgae and includes 400–600 species (Ettl, 1983). Traditionally, members of the genus *Chlamydomonas* were described as unicellular green algae with two equal flagella, a cell wall, chloroplast(s) possessing pyrenoid(s), and no "distinct" features (Ettl 1983). However, molecular phylogenetic studies have revealed that *Chlamydomonas,* in that sense, was apparently polyphyletic (e.g., Buchheim *et al*. 1990, 1996, Nozaki *et al*. 2000, Pröschold *et al*. 2001, Nakada *et al*. 2008, 2019). Pröschold *et al*. (2001) proposed the delimitation of *Chlamydomonas* to include only the clade comprising the conserved type species, *Cd. reinhardtii* Dangeard (Pröschold *et al*. 2001, Pröschold & Silva 2007, Wiersema *et al*. 2015). Based on this definition, *Chlamydomonas* is considered to include only limited species (Nakada *et al*. 2010, 2019, Pröschold *et al*. 2018). In addition, based on the revision, some species of *Chlamydomonas* in the traditional sense have been transferred to other genera, such as *Oogamochlamys* Pröschold, Marin, Schlösser & Melkonian, *Lobochlamys* Pröschold, Marin, Schlösser & Melkonian (Pröschold *et al*. 2001), *Microglena* C. Ehrenb. (Demchenko *et al*. 2012), *Dangeardinia* Tempère, *Ixipapillifera* Nakada, *Rhysamphichloris* Nakada (Nakada *et al*. 2016), and *Edaphochlamys* Pröschold & Darienko (Pröschold *et al*. 2018). Nevertheless, a large number of species remain in *Chlamydomonas* in the traditional sense.

The genus *Chloromonas* (*Cr.*) Gobi traditionally included *Chlamydomonas*-like algae without pyrenoids (Ettl 1983). However, Pröschold *et al*. (2001) revised *Chloromonas* classification based on a phylogenetic analysis and demonstrated that the absence of pyrenoids was not significant for the classification. They transferred some species of *Chlamydomonas* to emended *Chloromonas* (Pröschold *et al*. 2001). In addition, Nakada *et al*. (2008) performed a comprehensive phylogenetic analysis based on 18S *r*DNA sequences of the Volvocales and identified numerous distinct clades in the order. They proposed names based on the PhyloCode for the clades. The clade *Chloromonadinia* is one

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of the names and it includes the type species of the genus *Chloromonas*, *Cr. reticulata* (Goroschankin) Gobi (Nakada *et al*. 2008). This clade originally included only *Chloromonas* revised by Pröschold *et al*. (2001). However, recent studies have revealed that some additional genera, such as *Chlainomonas* Christen (Novis *et al*. 2008), *Gloeomonas* Klebs (Nozaki *et al*. 2010, Nakada *et al*. 2015), *Ixipapillifera* Nakada (Nakada *et al*. 2016), as well as additional species of *Chloromonas* (Hoham *et al*. 2006, Matsuzaki *et al*., 2010, 2012, 2013, 2014, 2018, Muramoto *et al.* 2010, Remias *et al*. 2013, Barcytë *et al*. 2018a, b), are in the clade *Chloromonadinia*. The expansion of *Chloromonadinia* has presented a taxonomic problem in the genus *Chloromonas.* In recent molecular phylogenetic studies, the monophyly of *Chloromonas* as revised by Pröschold *et al*. (2001) is not supported (e.g., Matsuzaki *et al*. 2013, 2014, 2018, Yumoto *et al*. 2013, Nakada *et al*. 2016, Barcytë *et al*. 2018a).

We isolated a *Chlamydomonas*-like alga (NIES-4318) from an acidic bog in Nagano prefecture, Japan. The NIES-4318 strain produces a considerable amount of oil under acidic conditions (unpublished data). Another strain, NIES-4317, was isolated from a paddy field in Yamagata prefecture, Japan. The 18S *r*DNA sequence of the strain exhibited high similarity to that of SAG 12.72, the authentic strain of *Cd. chlorostellata* Flint & H. Ettl but labeled as *Cd. meslinii* Bourrelly in the Sammlung von Algenkulturen at the University of Göttingen (SAG). Fulnečková *et al*. (2012) and Nakada *et al*. (2015) showed that SAG 12.72 was allied in the *Chloromonadinia* clade. We found two additional strains, SAG 75.81 (authentic strain of *Cd. meslinii*) and SAG 19.88, labeled as *Cd. meslinii*, which were also closely related to the two Japanese strains. In this study, we performed a taxonomic analysis of the strains using the polyphasic approach (e.g., Pröschold & Leliaert 2007; Kawasaki *et al*. 2015) including light microscopy, molecular phylogenetic analysis of 18S rRNA, *atp*B, *psa*B and secondary structure analysis of the internal transcribed spacer (ITS) structures. Based on the polyphasic approach, we propose a novel genus, *Paludistella*, including four species, in the *Chloromonadinia* clade.

Materials and Methods

Strains and cultivation

The NIES-4317 and NIES-4318 strains were isolated from a paddy field and wet soil samples from a marsh, respectively (Table 1), by streaking on AF-6 1.5% agar medium (Kato 1982, modified according to Kasai et *al*. 2009). Both strains were deposited in the Microbial Culture Collection at the National Institute for Environmental Studies (NIES). Other strains were obtained from SAG (Table 1). Algal cultures were incubated in AF-6 at 25°C under a 12 h:12 h light: dark cycle with light provided by cool-white fluorescent lamps at approximately 150–200 µmol photons m^{-2} s^{-1}. AF-6 media were used at pH 4.0 and pH 6.6. The morphological observations were carried out to 1–2 weeks old of cultures meanwhile the oil droplets were able to be detected in old cultures (≥ 4 weeks).

Light microscopy

Light microscopy was carried out using a Leica DM 2500 microscope equipped with a Nomarski differential interference contrast filter (Leica, Wetzlar, Germany) and an MC 190 HD camera (Leica Microsystems, Tokyo).

Gene sequencing and phylogenetic analyses

Total DNA was extracted from all the strains using a FastDNA kit (MP Biomedicals, France). For molecular analyses, we used nucleotide sequences of the nuclear-encoded small subunit rRNA gene (18S *r*DNA), chloroplast-encoded ATP synthase beta subunit gene (*atp*B), P700 chlorophyll *a* apoprotein A2 gene (*psa*B), and internal transcribed spacer 2 (ITS-2) region of nuclear-encoded *r*DNA. Sequences were obtained using primer sets and conditions described previously (Hamby *et al*. 1988, Coleman *et al*. 1994, Nakayama *et al*. 1998, Nozaki *et al*. 1999, 2000, 2002 Nakazawa & Nozaki 2004, Pröschold *et al*. 2005, Nakada *et al*. 2007, 2010, Kawasaki *et al*. 2015) as well as four newly designed primers: MSL.18S-F1 (5′-TGGATGTGCTGGTGAAGTGT-3′; position 975-996 bp), MSL.ITS-R1 (5′-AACAACAGCATTTAAGCTACATCA-3′; position 401-376 bp), MSL.ITS-R (5′-TGGAGAGCCATATCCACACA-3′; position 922-903 bp), and MSL.ITS-R4 (5′-ACACCATCCCCATTGAAAAACTAAG-3′; position 419-394 bp). The positions of these primers were determined by alignment to SAG 75.81 sequence (accession number MK696131).

For the multigene phylogenetic analysis, we used the 18S *r*DNA, *atp*B, and *psaB* sequences of 29 operational taxonomic units including five strains examined in the present study (supplementary table). The sequence alignments were computed using MAFFT (Katoh *et al*. 2002) and checked using BioEdit (Hall 1999). The alignment data are available in supplemental material. The third positions of *atp*B and *psa*B codons were excluded and 3,384 bases

were used for the phylogenetic analysis. Homogeneity of base composition among the taxa was tested using 18S *r*DNA and each codon position of the protein-coding genes using the chi-squared test ($p < 0.05$) with PAUP* 4.0b10 (Swofford 2002). Bayesian Inference (BI) was performed using MrBayes 3.2.6 (Ronquist *et al*. 2012) with 1,000,000 generations of Markov Chain Monte Carlo iterations, discarding the first 25% as burn-in. The optimal substitution model GTR+I+G for each BI partition (18S *r*DNA, the first codon position, and the second codon position of *atp*B and *psa*B) was selected using PAUP* 4.0b10 and MrModeltest 2.3 (Nylander 2004). Maximum likelihood (ML) analysis was performed using IQ Tree 1.6.9 (Lua *et al*. 2012, Nguyen *et al*. 2015) with automatic ModelFinder and ultrafast bootstrap analysis based on 1,000 replications (Hoang *et al*. 2018). In addition, PAUP* 4.0b10 was used for maximum parsimony (MP) and MEGA X (Kumar *et al*. 2018) was used for minimum evolution (ME) analysis based on 1,000 bootstrap replications. Finally, the phylogenetic tree data were visualized using FigTree 1.4.3 (Rambaut 2012). The ITS-1 sequences analysis was initiated by performing the Multiple Sequence Alignment (MSA) of data set. However, there is a difficulty to compare the secondary structure reconstruction due to highly variable of ITS-1. Therefore, the analysis was excluded in this study. The secondary structure of ITS-2 sequences including the 25 last bases of the 5.8S rRNA and the first 25 bases of the LSU rRNA (Pröschold *et al*. 2018) were predicted using RNAstructure (Reuter & Mathews 2010) and Mfold (Zuker 2003) webservers. The comparative regions of ITS-2 among the strains examined in this study were determined by manual alignment to calculate the compensatory base changes (CBCs).

Preparation of resin embedded specimen for holotype

The 12-day cultures of SAG 19.88 and NIES-4318 strains in AF-6 medium were fixed with 1% OsO_4 and 2.5% glutaraldehyde followed by dehydration in an ethanol series (30–100%) diluted by cacodylate buffer. For the substitution using propylene oxide (PO), the following series were set: (1) PO:ethanol (1:1), (2) 100% PO, and (3) PO:Spurr's resin (1:1). Finally, fixed cells were infiltrated and then embedded with 100% resin (Spurr 1969, Yoshida *et al*. 2009, Matsuzaki *et al.* 2010). Cells-containing resin were transferred into capsuls and baked in oven (70^0C, 12h).

TABLE 1. Strains used in this study.

Species	Strain	Locality	Note
Paludistella meslinii	SAG 75.81	Ditch in Paris, France (Bourrelly 1951)	Labeled as *Cd. meslinii*, authentic strain of *Cd. meslinii*
P. chlorostellata	SAG 12.72	Tekoa acid soil from Southern Alps at Bealey, New Zealand (Flint & Ettl 1966)	Labeled as *Cd. meslinii*, authentic strain of *Cd. chlorostellata*
P. asymmetrica	SAG 19.88	Seedling bed at Mira, Portugal *	Labeled as *Cd. meslinii*
P. trianguloculus	NIES-4317	A fallow paddy field, Yamagata, Japan (38°17' 41''N, 140°22'24'' E)	
	NIES-4318	Ikenotaira marsh, Nagano, Japan (36°24'30.6"N 138°26'04.9"E)	

* Based on the SAG website (https://www.uni-goettingen.de/de/184982.html).

Results

Light microscopy

The strains examined in the present study, SAG 75.81 (Fig. 1), SAG 12.72 (Fig. 2), SAG 19.88 (Fig. 3), NIES-4317 (Fig. 4), and NIES-4318 (Fig. 5), possessed similar morphological features but exhibited some differences as summarized in Table 2. They were unicellular flagellates with two equal flagella (Figs. 1H, 2H, 3H, 4H, 5H) emerging from the anterior poles of the cells. The flagella were 1.0–1.5 times as long as the cells. The vegetative cells were circular in optical transverse section (Figs. 1I, 2I, 3I, 4I, 5I) and had rounded anterior and posterior ends (Figs. 1A–C, 2A–C, 3A–C, 4A–C, 5A–C). The young vegetative cells ranged from cylindrical to elliptical (Figs. 1A, 2A, 4A, 5A) and those of SAG 19.88 were frequently asymmetrical (Fig. 3A). The mature vegetative cells were typically elliptical (Figs. 1B, 4B, 5B) but were broad elliptical in some cases, particularly in SAG 12.72 and SAG 19.88 (Figs. 2B, 3B).

The vegetative cells were covered by moderately thick, smooth cell walls (Figs. 1–5). The papillae at the anterior poles of the cells were hemispherical to conical with rounded anterior ends (Figs. 1F, 2F, 3F, 4F, 5F). The chloroplasts were cup-shaped in young vegetative cells (Figs. 1A, 2A, 3A, 4A, 5A), but had asteroid forms with deep, narrow, radial incisions forming irregular lobes in mature vegetative cells (Figs. 1B–C, 2B–C, 3B–C, 4B–C, 5B–C). Pyrenoids were spherical, positioned laterally in young vegetative cells in some cases (Figs. 1A, 2A, 3A, 4A, 5A) or centrally in mature vegetative cells (Figs. 1B, 1I2, 2B, 2I2, 3B, 3I2, 4B, 4I2, 5B, I2). The starch plates covering the pyrenoids were small and typically globular (Figs. 1G, 2G, 3G, 4G, 5G). A pale red stigma was located on the anterior third to quarter of the cell (Figs. 1C2, 2C, 3C, 4A1, 5B). Stigma shapes were varied among the strains: narrow filiform in SAG 75.81 (Fig. 1E), oblong in SAG 12.72 (Fig. 2E), and triangular to elliptical in NIES-4317 (Fig. 4E) and NIES-4318 (Fig. 5E). The shapes of stigma were somewhat variable, ranging from oblong to small elliptical in SAG 19.88 (Fig. 3E). A spherical nucleus was anterior to the pyrenoid (Figs. 1B, 2B, 3B, 4B). Two contractile vacuoles were located near the base of the flagella (Figs. 1C2, 2A2, B, I1, 3B, 4B, I1, 5A2, B, I1). In old cultures, cells were sometimes filled with colorless to orange oil droplets (Figs. 6A–D). Asexual reproduction was accomplished through the formation of two or four zoospores (Figs 1D, 2D, 3D, 4D, 5D), but the formation of eight zoospores was sometimes observed in SAG 12.72 (Fig. 2D3). All strains showed unusual cell division, in which one to three buds were formed from a cell (Fig. 7). Although we did not observe the entire course of division, the division was similar to "protocytotomy" reported in some members of the Volvocales including *Chloromonas* (Masyuk & Demchenko 2001, Hoham *et al*. 2006, Demchenko *et al*. 2012, Chelebieva *et al*. 2018). Akinetes, spherical cells with thick smooth cell walls and no flagella and bearing numerous oil droplets were observed in some instances in old cultures of SAG 19.88 (Figs. 6E, F).

Cultures were green in color. All strains excluding SAG 75.81 grew well in AF-6 medium at both pH 4.0 and pH 6.6. SAG 12.72 and SAG 19.88 exhibited better growth in AF-6 at pH 6.6, while NIES-4317 and NIES-4318 grew better in AF-6 at pH 4.0 (data not shown). Morphological features of the strains were similar in both media. SAG 75.81 grew in AF-6 at pH 6.6, but not in AF-6 at pH 4.0 in the present study.

TABLE 2. Morphological comparison among *Paludistella* species.

Species	*P. meslinii*	*P. chlorostellata*	*P. asymmetrica*	*P. triangulocculus*	
Strain	SAG 75.81	SAG 12.72	SAG 19.88	NIES-4317	NIES-4318
Cell form	Cylindrical to ellipsoidal	Cylindrical to broad ellipsoidal	Cylindrical to broad ellipsoidal, frequently asymmetrical in young cells	Cylindrical to ellipsoidal	Cylindrical to ellipsoidal
Cell size:					
Length (μm)	13–21	12–21	13–20	11–19	10–19
Width (μm)	6–15	6–15	8–18	5–13	6–15
Stigma form	Filiform	Oblong	Oblong to small elliptical	Triangular to small elliptical	Triangular to small elliptical
Zoospores	2, 4	2, 4, 8	2, 4	2, 4	2, 4
Akinete	Unknown	Unknown	Present	Unknown	Unknown

Molecular phylogenetic analysis

In the molecular phylogenetic tree based on 18S *r*DNA, *atp*B, and *psa*B, the SAG 75.81, SAG 12.72, SAG 19.88, NIES-4317, and NIES-4318 strains formed a robust clade [posterior probability (PP) = 1.0, bootstrap proportion (BP) in all methods = 100%] in the clade *Chloromonadinia* (Fig. 8). The clade (*Paludistella*) was sister to *Cr. pseudoplatyrhyncha* (Pascher) Silva and they formed a robust clade (PP = 1.0, BPs = 90%–100%). They formed a clade with the snow algae (SA) clade *sensu* Matsuzaki *et al*. (2013), *Cr. asteroidea* (CA) clade *sensu* Matsuzaki *et al*. (2013), and *Ixipapillifera* (PP = 1.0, BPs ≤ 72%). The *Cr. reticulata* (CR) clade *sensu* Matsuzaki *et al*. (2013), including the type species of *Chloromonas*, *Cr. reticulata*, formed a robust clade (PP = 1.0, BPs = 99%–100%) with *Gloeomonas* and some other species of *Chloromonas*. In the clade composed of the strains examined in the present study (*Paludistella*), SAG 75.81

was situated at the base (PP = 1.0, BPs = 99%–100%). The subclade composed of SAG 12.72 and SAG 19.88, and the subclade composed of NIES-4317 and NIES-4318 were recovered with moderately high statistical supports (PPs = 0.99, BPs = 80%–90%).

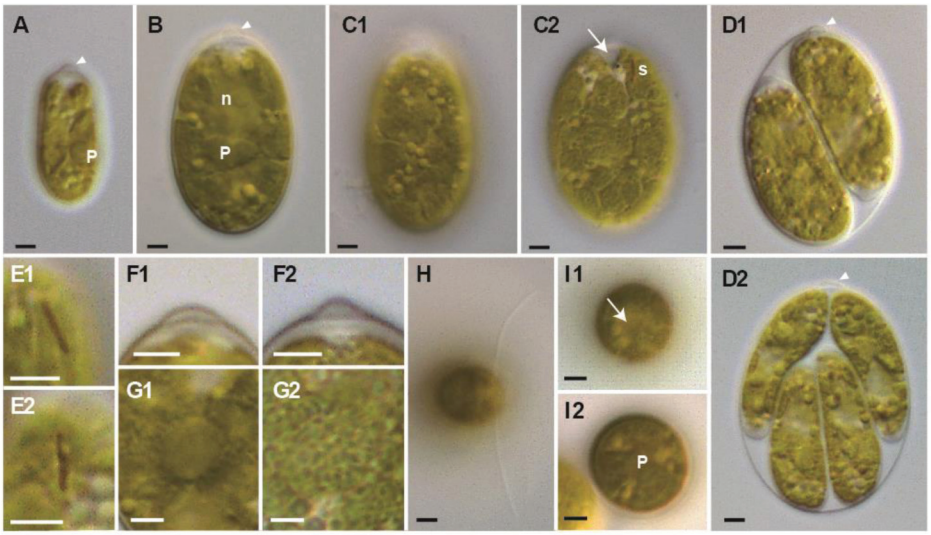


FIGURE 1. Light microscopy of *Paludistella meslinii* (SAG 75.81). A young cell (A), optical section (B) and cell surface (C) of mature cells (B and C1 are the same cell), zoosporangium including two (D1) or four (D2) daughter cells, filiform stigma (E), hemispherical to conical papilla (F), optical section (G1) and surface (G2) of a pyrenoid, two equal flagella (H), top view with contractile vacuoles (I1), and optical cross section (I2) of cells. Arrowheads indicate the papillae and arrows indicate contractile vacuoles. n = nucleus, p = pyrenoid, s = stigma. Scale bar = 2 µm.

The comparison of secondary structure of ITS-2

We compared the secondary structures of the nuclear *r*DNA ITS-2 region to verify the species delimitation among the strains examined in the present study (e.g., Coleman 2003). The predicted secondary structures possessed four helices: I to IV. Most of the helices were comparable, excluding helix III of SAG 75.81, which consist of three sub-helices and only the first sub-helix was comparable to those of other strains (Fig. 9). The predicted secondary structure of SAG 75.81 showed the GGU motif characteristic of green algae in the RNA processing site at the 5' site of helix III. However, the other examined strains apparently have the atypical motif in which GGU replaced by GAU (Fig.9). In the comparative region of ITS-2, we observed one to twelve CBCs among the strains examined in the study except in the NIES-4317 and NIES-4318 pair, which possessed three hemi-CBCs but no CBCs (Fig. 10).

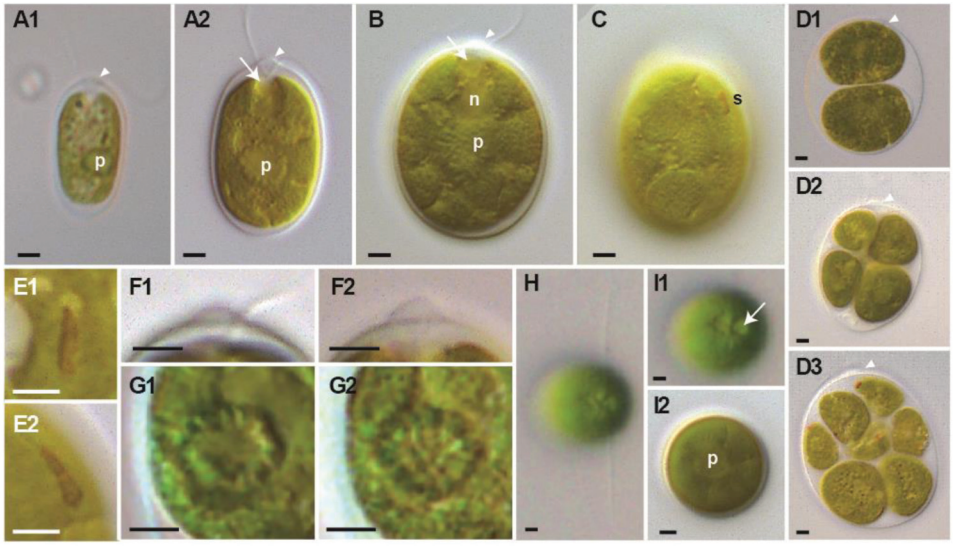


FIGURE 2. Light microscopy of *Paludistella chlorostellata* (SAG 12.72). Young cells (A), optical section (B) and cell surface (C) of a mature cell, zoosporangium including two (D1), four (D2), or eight (D3) daughter cells, oblong stigmata (E), hemispherical to conical papillae (F), optical section (G1) and surface (G2) of a pyrenoid, two equal flagella (H), top view with contractile vacuoles (I1) and optical cross section (I2) of cells. For abbreviations, see the legends in Figure 1. Scale bar = 2 µm.

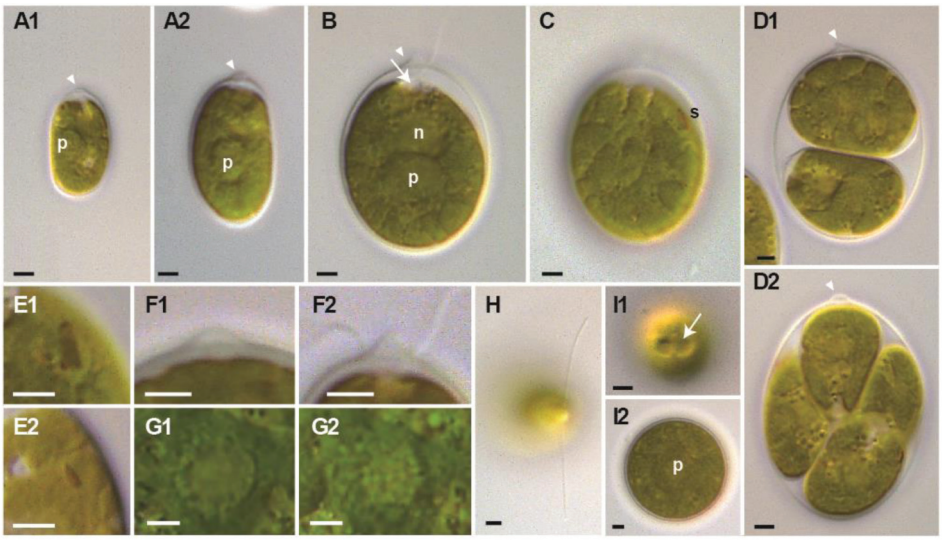


FIGURE 3. Light microscopy of *Paludistella asymmetrica* (SAG 19.88). Young cells (A), optical section (B) and cell surface (C) of a mature cell, zoosporangium including two (D)1 or four (D2) daughter cells, oblong to small elliptical stigmata (E), hemispherical to conical papilla (F), optical section (G1) and surface (G2) of the pyrenoids, two equal flagella (H), top view (I1) and optical cross section (I2) of cells. For abbreviations, see the legends in Figure 1. Scale bar = 2 µm.

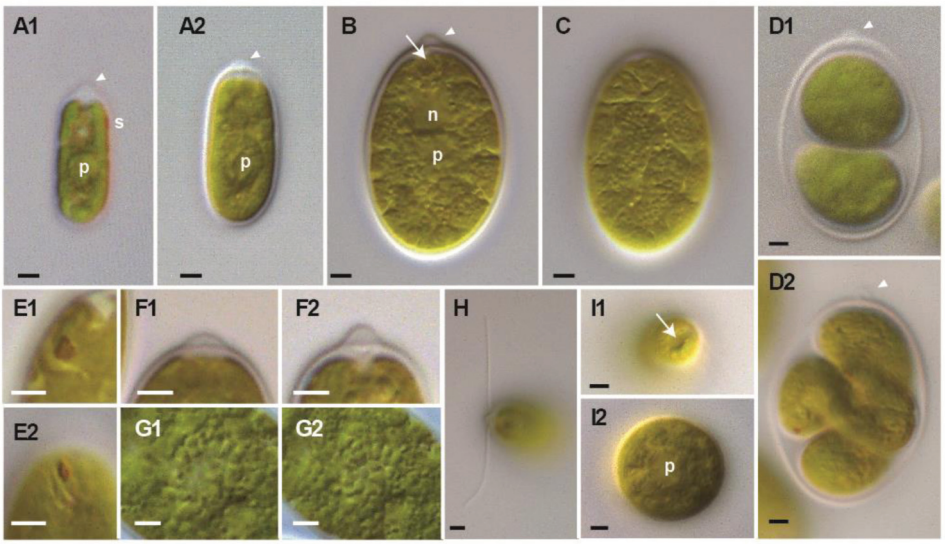


FIGURE 4. Light microscopy of *Paludistella trianguloculus* (NIES-4317). Young cells (A), optical section (B) and cell surface (C) of a mature cell, zoosporangium including two (D1) or four (D2) daughter cells, triangular to elliptical stigmata (E), hemispherical to conical papilla (F), optical section (G1) and surface (G2) of the pyrenoids, two equal flagella (H), top view with contractile vacuole (I1) and optical cross section (I2) of cells. For abbreviations, see the legends in Figure 1. Scale bar = 2 µm.

Discussion

Generic concept

The present study reveals a novel and distinct subclade in the clade *Chloromonadinia*. The morphological features of the subclade, such as unicellular flagellates with two equal flagella, cell walls, and chloroplasts with pyrenoids, are consistent with the traditional concept of the genus *Chlamydomonas* (Ettl 1983). However, presently, it is clear that *Chlamydomonas* is polyphyletic and the strains examined in the present study cannot be classified into *Chlamydomonas* because they are distantly related to the type species of the genus, *Cd. reinhardtii*. Pröschold *et al*. (2001) revised the genus *Chloromonas* based on the results of a phylogenetic analysis and transferred some species of *Chlamydomonas* to the emended *Chloromonas*. The strains in the present study can be classified into *Chloromonas sensu* Pröschold *et al*. (2001). However, some studies have revealed that other genera, such as *Chlainomonas*, *Gloeomonas*, and *Ixipapillifera*,

are included in *Chloromonas sensu* Pröschold *et al*. (2001) (equivalent to the clade *Chloromonadinia* in Nakada *et al.* 2008) (Novis *et al*. 2008, Nozaki *et al*. 2010, Nakada *et al*. 2015, 2016). Furthermore, the name of *Gloeomonas* Klebs 1886 is published earlier than *Chloromonas* Gobi 1899–1900. Therefore, *Chloromonas sensu* Pröschold *et al*. (2001) is polyphyletic, and *Chloromonas* should be limited for the CR clade that includes the type species of the genus.

Based on the molecular phylogenetic analysis, the subclade found in the present study is independent in the clade *Chloromonadinia* and its close relatives are not clear (except for *Cr*. *pseudoplatyrhyncha*; see below). However, the CR clade *sensu* Matsuzaki *et al*. (2013) (core *Chloromonas sensu* Barcytë *et al*. 2018a) containing the type species of *Chloromonas*, *Cr. reticulata*, formed a robust clade with *Gloeomonas* and some other species of "*Chloromonas*" in the phylogenetic tree based on three genes (Fig. 8). Therefore, the strains examined in the present study cannot be classified into *Chloromonas*.

In addition, the positions of pyrenoids are valid for use in distinguishing the strains in the present study from the species in the CR clade. All strains in the present study have asteroid chloroplasts with central pyrenoids ("asteroid gelappt" in *Euchlamydomonas* type; Ettl 1983) in mature cells. The members of the CR clade mostly lack pyrenoids but some species such as *Cr*. *chlorococcoides* (H. Ettl & Schwarz) Matsuzaki, Hara & Nozaki and *Cr. typhlos* (Gerloff) Matsuzaki, Hara & Nozaki have lateral pyrenoids (*Chlamydella* type) (Ettl 1983, Pröschold *et al*. 2001, Matsuzaki *et al.* 2012, Barcytë *et al*. 2018a, b). In addition, the strains in the present study are distinguishable from another large subclades (SA clade in Matsuzaki *et al*. 2013, clade 2 in Barcytë *et al*. 2018a) of *Chloromonas sensu* Pröschold *et al*. (2001) based on morphological features. All members of SA clade have no typical pyrenoids and no distinct papillae (Ettl 1983, Matsuzaki *et al.* 2014, 2018, Muramoto *et al*. 2010). The combinations of the asteroid chloroplasts with central pyrenoids and the hemispherical to conical papillae are also not observed in other species of the clade *Chloromonadinia* (Ettl 1983, Pröschold *et al*. 2001, Matsuzaki *et al.* 2010, 2013, Nakada *et al*. 2015). Therefore, we propose the new genus, *Paludistella*, for the strains examined in the present study.

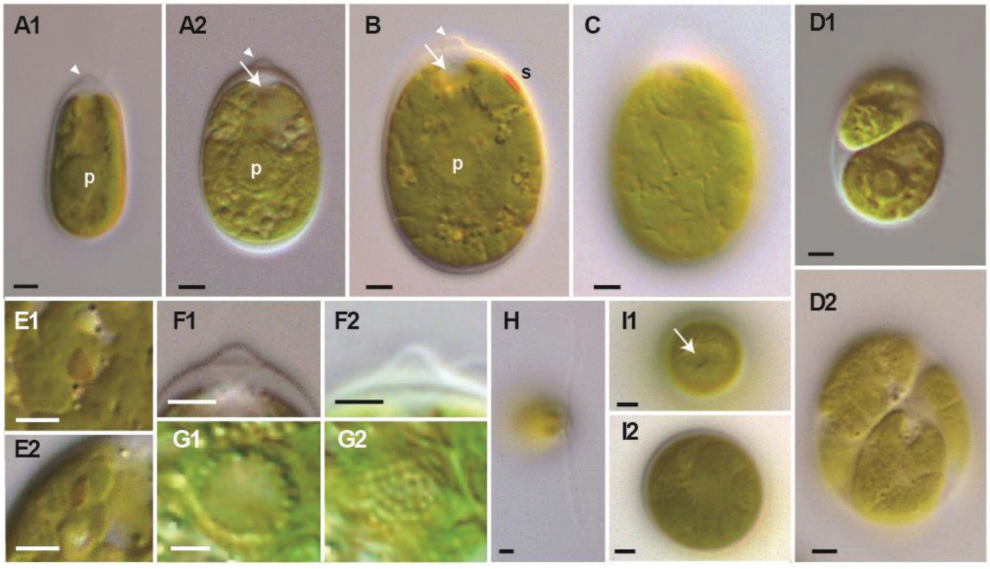


FIGURE 5. Light microscopy of *Paludistella trianguloculus* (NIES-4318). Young cells (A), optical section (B) and cell surface (C) of a mature cell, zoosporangium including two (D1) or four (D2) daughter cells, triangular to elliptical stigmata (E), hemispherical to conical papilla (F), optical section (G1) and surface (G2) of a pyrenoid, equal two flagella (H), top view with contractile vacuole (I1) and optical cross section (I2) of cells. For abbreviations, see the legends in Figure 1. Scale bar = 2 μm.

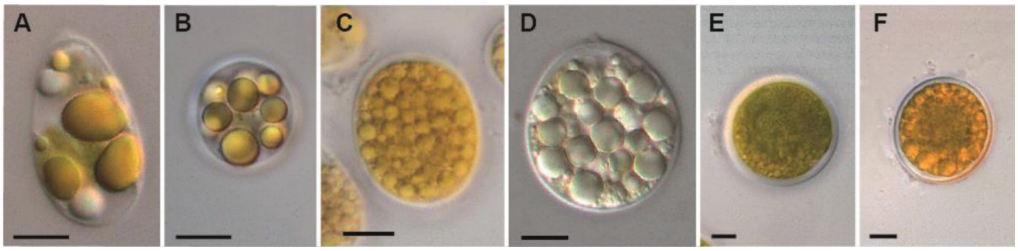


FIGURE 6. Light microscopy of the old cells storing oil droplets in *Paludistella meslinii* (SAG 75.81) (A), *P. chlorostellata* (SAG 12.72) (B), *P. trianguloculus* NIES-4317 (C) and NIES-4318 (D). The akinetes of *P. asymmetrica* (SAG 19.88) (E, F). Scale bar = 5 μm.

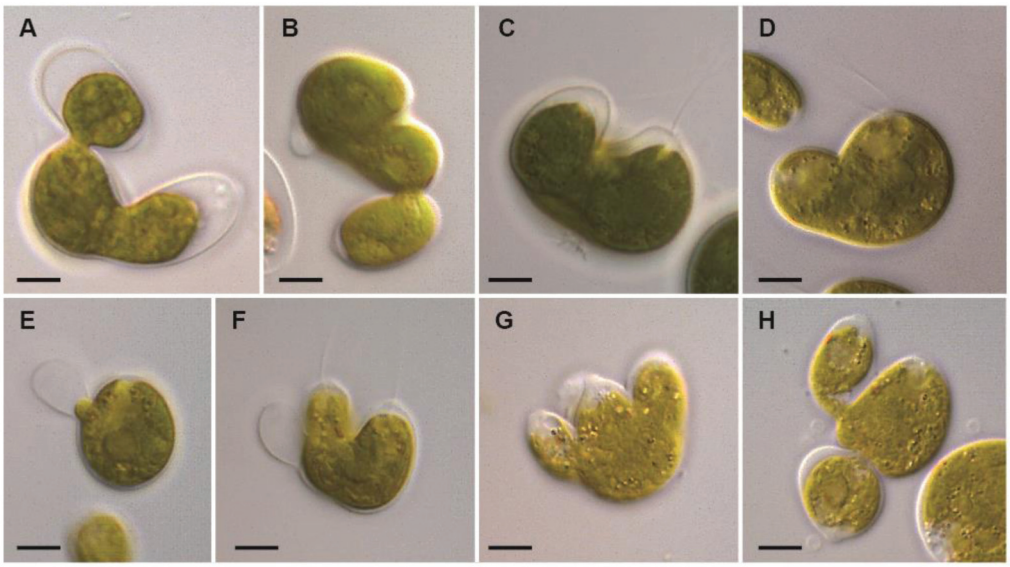


FIGURE 7. Light microscopy of the unusual cell division in *Paludistella. P. meslinii* (SAG 75.81) (A), *P. chlorostellata* (SAG 12.72) (B), *P. asymmetrica* (SAG 19.88) (C), *P. trianguloculus* (NIES-4317) (D), *P. trianguloculus* (NIES–4318) (E–H). Scale bar = 5 µm.

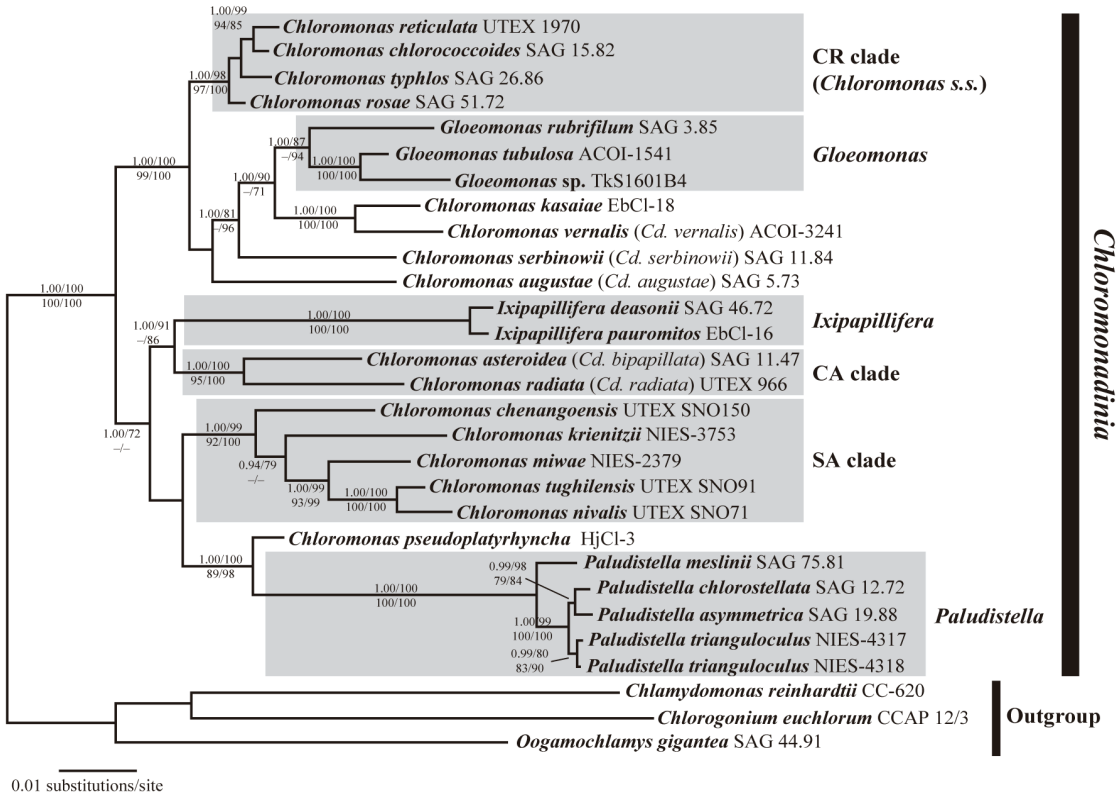


FIGURE 8. Bayesian phylogenetic tree of *Chloromonadinia* based on 18S *r*DNA, *atp*B, and *psa*B. Numbers at nodes indicate posterior probabilities (≥ 0.95, top left) and bootstrap proportions (≥ 70%) for ML (top right), MP (bottom left) and ME (bottom right) analyses.

Paludistella H. Susanti, Mas. Yoshida, Nakayama, Nakada & M.M. Watanabe *gen. nov*.

Type species: *Paludistella meslinii* (Bourr.) *comb. nov*.

Etymology: The genus name *Paludistella* refers to the habitat of numerous members of the genus and the stellate form of chloroplasts (*palus* in Latin means swamp or marsh, *stella* in Latin means star; feminine).

Description: Vegetative cells range from cylindrical to broad elliptical with rounded ends. Cell walls are moderately thick, with an anterior hemispherical to conical papilla from which two equal flagella emerge. Greenish asteroid

chloroplasts bearing numerous radial lobes are connected centrally in mature cells. Central pyrenoids are covered with numerous small starch grains. Stigma are filiform to elliptical, and are positioned at the anterior one third to quarter of cell. Nuclei positioned in the anterior cavity of the chloroplast. Two contractile vacuoles are at the apical tip of cell. Reproduced asexually via the formation of mainly two or four zoospores.

Diagnosis: Cell with a hemispherical to conical papilla and an asteroid chloroplast with central pyrenoid. The young cells are frequently cylindrical, but mature cells are elliptical to broad elliptical. *Paludistella* is also separable phylogenetically from other genera.

Based on a phylogenetic perspective, *Cr. pseudoplatyrhyncha* is a potential member of *Paludistella* because the alga is sister to *Paludistella* in the phylogenetic tree. However, *Cr. pseudoplatyrhyncha* is different from *Paludistella* species based on the shapes of papillae and the pyrenoid features. *Cr. pseudoplatyrhyncha* has a papilla with a flattened or slightly concave top face and multiple atypical pyrenoids without associated starch plates distributed in the interior regions of the lobes of the chloroplasts (Matsuzaki *et al*. 2010). It seems more appropriate to assign this alga to another new genus. However, the taxonomic revision of this alga should be considered only when further taxonomic information on the clade *Chloromonadinia* is available.

The members of *Paludistella* have asteroid chloroplasts with central pyrenoids. The feature is also observed in other species in the clade *Chloromonadinia*, which offers evolutionary insights about the clade. Such a chloroplast type is reported in *Cr. augustae* (Skuja) Pröschold, Marin, Schlösser & Melkonian and the CA clade *sensu* Matsuzaki *et al*. (2013) such as *Cr. radiata* (Deason & Bold) Pröschold, Marin, Schlösser & Melkonian (Ettl 1983, Pröschold *et al*. 2001, Takahashi *et al*. 2018). In the clade *Chloromonadinia*, *Cr. augustae*, CA clade, and *Paludistella* are distantly related to each other. The distribution of the feature suggests that the asteroid chloroplast with a central pyrenoid is a symplesiomorphy in the clade *Chloromonadinia.*

The *Paludistella* strains were collected from ditches, marshes, or soil (Table 1). Notably, SAG 12.72 and NIES-4318 were isolated from acidic habitats, and all strains (excluding SAG 75.81) grew well in the AF-6 medium at pH 4.0. In addition, all strains accumulated considerable amounts of oil droplets within their cells. Therefore, the algae have potential applications in the production of oil under acidic conditions.

Species taxonomy

Among the strains examined in the present study, there are one to twelve CBCs in ITS-2 except in the NIES-4317 and NIES-4318 pair. Although the strains are very similar morphologically, some differences, such as in the shapes of stigma are present. Therefore, molecular and morphological data support the separation of the strains (excluding NIES-4317 and NIES-4318) into different species.

Some *Chlamydomonas* taxa that possess the asteroid chloroplast with a central pyrenoid and the hemispherical papilla have been reported without DNA sequence data, such as *Cd. corrosa* Pascher & Jahoda, *Cd. fimbriata* H. Ettl, *Cd. metapyrenigera* Skuja, *Cd. nygaardii* H. Ettl, *Cd. subangulosa* Fritsch & John (Ettl 1983). However, such species are different from the strains examined in the present study based on cell shape, size, and stigma features. Therefore, we classify the five strains in *Paludistella* into four species including two new species as follows.

Paludistella meslinii (Bourr.) H. Susanti, Mas. Yoshida, Nakayama, Nakada & M.M. Watanabe *comb. nov*.

Basionym: Chlamydomonas meslinii Bourr. (1951), Hydrobiologia **3**: 258, fig. 52

Strain: SAG 75.81; authentic

Emended description: Vegetative cells 13–21 μm in length, 6–15 μm in width, cylindrical to ellipsoidal (Figs. 1A–C). Flagellum about one cell length. Cell with a hemispherical to conical papilla (Figs. 1F), two apical contractile vacuoles (Figs. 1C2, I1), an anterior nucleus (Fig. 1B), and an asteroid chloroplast with a central pyrenoid covered by small globular starch plates (Figs. 1B, C, G, I2). Stigma filiform, very narrow (Figs. 1C2, E). Asexual reproduction via the formation of two or four zoospores (Fig. 1D). Sexual reproduction not observed.

Emended diagnosis: The narrow filiform stigma is characteristic in this species and is not found in other *Paludistella* species. Diagnostic DNA sequence: 18S *r*DNA and ITS (accession number: MK696131).

Taxonomic remarks: The morphological features of SAG 75.81, the authentic strain of this species was consistent with those in Bourrelly (1951) and Ettl (1976) excluding flagellum length . The cell size in the original description (20–22 × 15 μm) is equivalent to the maximum size in the present observation.

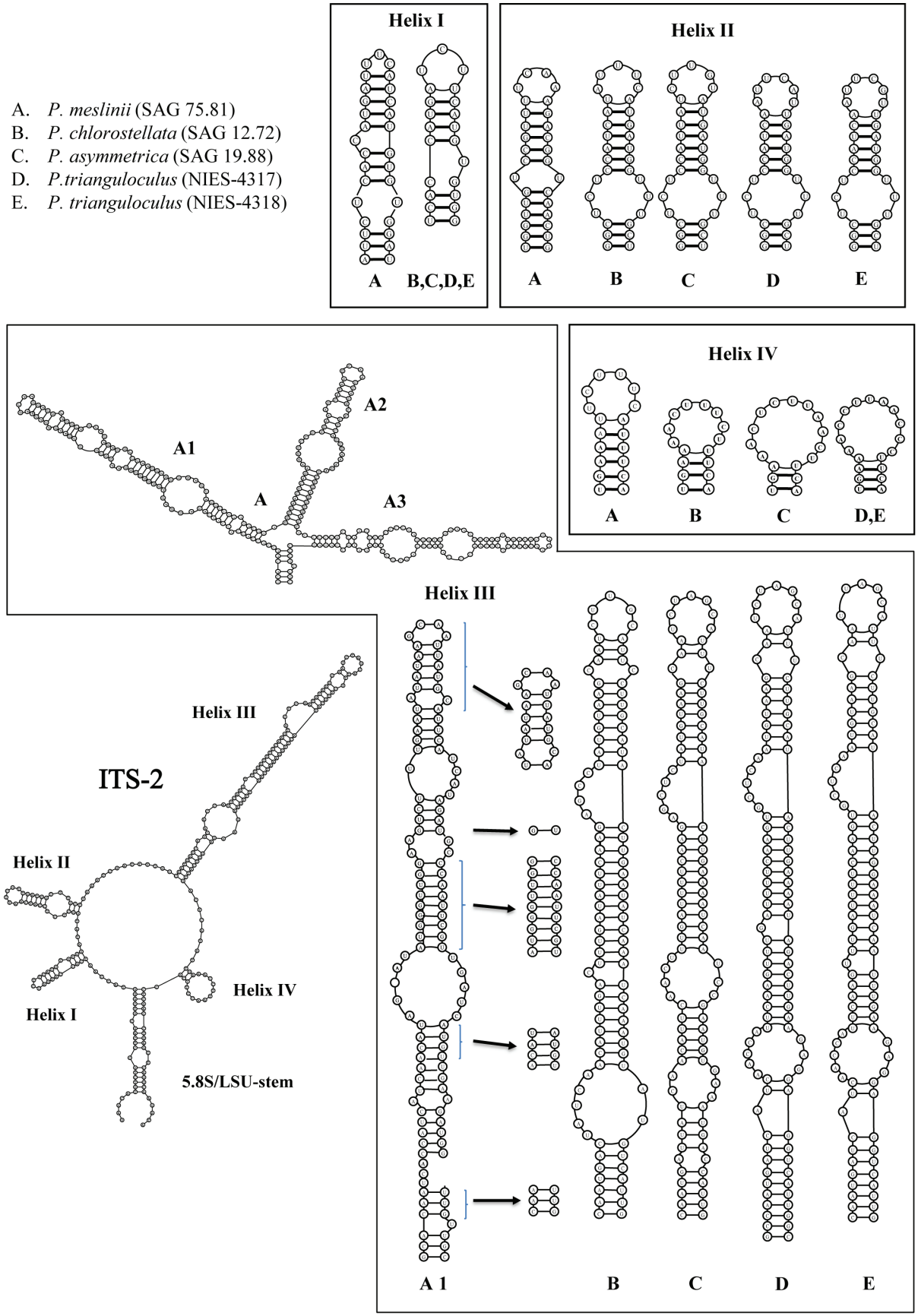
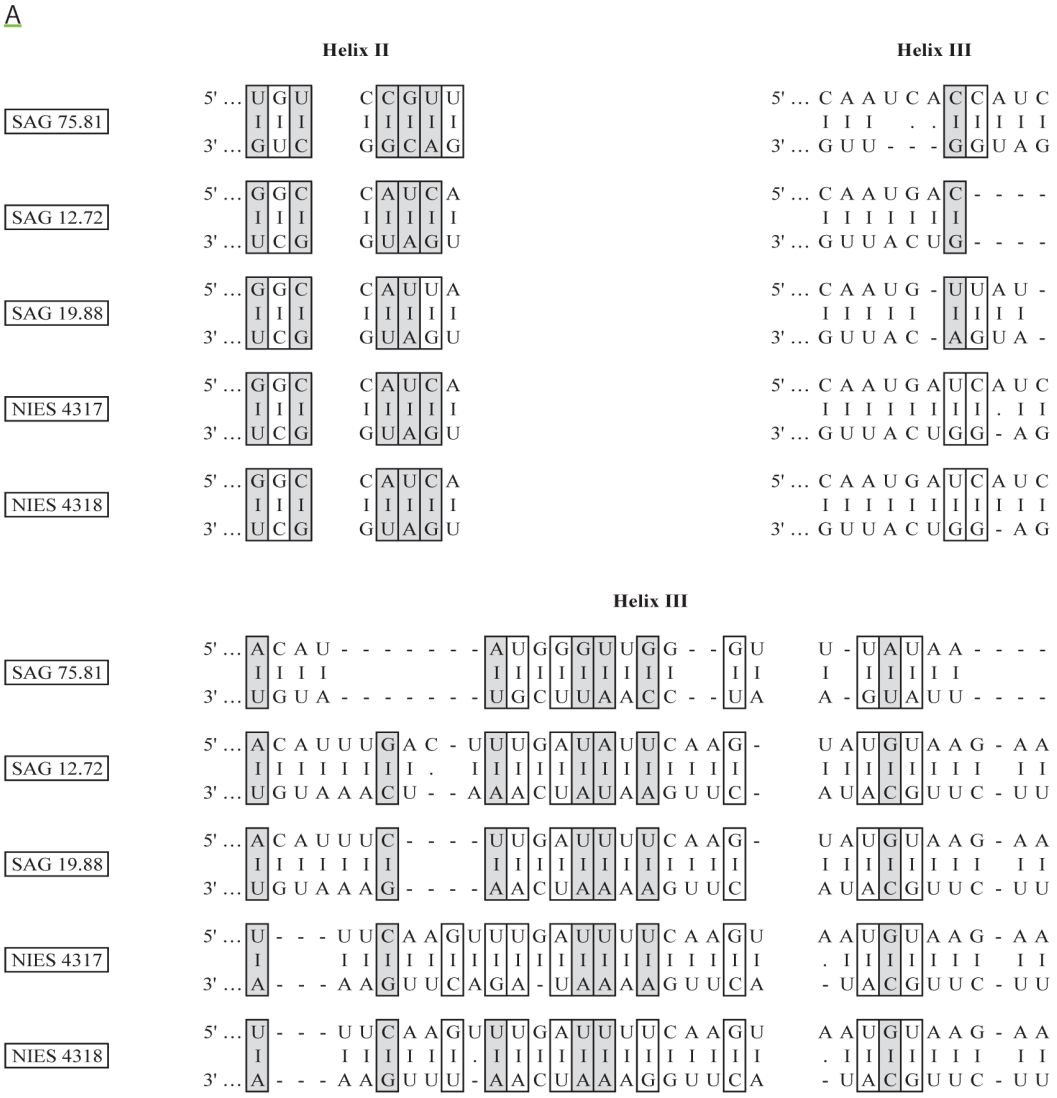


FIGURE 9. Secondary structure of ITS-2 *r*DNA sequences among the species of *Paludistella*. The helix three of SAG 75.81 consists of three sub-helices and the comparable positions were found only in sub-helix A1.



B

	P. chlorostellata (SAG 12.72)	*P. asymmetrica* (SAG 19.88)	*P. trianguloculus* (NIES-4317)	*P. trianguloculus* (NIES-4318)
P. meslinii (SAG 75.81)	11/6	10/8	12/7	12/7
P. chlorostellata (SAG 12.72)		3/1	3/2	3/1
P. asymmetrica (SAG 19.88)			1/4	1/4
P. trianguloculus (NIES-4317)				0/3

FIGURE 10. (**A**) The predicted secondary structures of ITS-2 *r*DNA helices II and III within the comparative positions in *Paludistella* strains. The positions marked black boxes and empty boxes indicate the presence of CBC and hemi-CBC respectively. (**B**) The numbers of CBCs/hemi-CBCs among the ITS-2 *r*DNA sequences of *Paludistella* strains.

Paludistella chlorostellata (Flint & H. Ettl) H. Susanti, Mas. Yoshida, Nakayama, Nakada & M.M. Watanabe *comb. nov*.

Basionym: *Chlamydomonas chlorostellata* Flint & H. Ettl (1966), New Zealand J. Bot. **4**: 420–423, fig. 2. (excluding var. *gracillima*)

Strain: SAG 12.72 (= CCAP 11/93); authenthic

Emended description: Vegetative cells 12–21 µm in length, 6–15 µm in width, cylindrical to broad ellipsoidal (Figs. 2A–C). Flagellum is about one and a half times the cell length. Cell with a hemispherical to conical papilla (Fig. 2F), two apical contractile vacuoles (Figs. 2A2, B, I1), an anterior nucleus (Fig. 2B), and an asteroid chloroplast with a

central pyrenoid covered with small globular starch plates (Figs. 2B, C, G, I2). Stigma oblong (Fig. 2E). Asexual reproduction via the formation of two, four, or eight zoospores (Fig. 2D). Sexual reproduction not observed.

Emended diagnosis: The mature cells are generaly broad elliptical. The stigma is oblong. The formation of eight zoospores is sometimes observed. Diagnostic DNA sequence: 18S *r*DNA and ITS (accession number: MK696129).

Taxonomic remarks: The morphological features of SAG 75.81, the authentic strain of this species, were consistent with those in Flint & Ettl (1966) and Ettl (1976) excluding cell size and stigma features. The cell size in the original description (18–24 × 11–27 µm) is slightly larger than in the present observation. The stigma in the original paper is wider and situated more posteriorly than in the present study. Ettl & Gärtner (2014) treated *Cd. chlorostellata* as a synonym of *Cd. meslinii* because of some morphological similarities. However, SAG 12.72 possesses a largely different ITS-2 helix III and eleven CBCs in the comparable positions to SAG 75.81, and these two strains are different based on the shape of cells and stigma. Therefore, we treat them as different species. *Cd. chlorostellata* contains a single variety, *Cd. chlorostellata* var. *gracillima* H. Ettl (Ettl 1983). However, based on the 18S *r*DNA sequence, SAG 25.87, the authentic strain of *Cd. chlorostellata* var. *gracillima*, was distantly related to SAG 12.72 but closely related to some strains of CR clade. Therefore, this variety was excluded from *Paludistella* (unpublished).

Paludistella asymmetrica H. Susanti, Mas. Yoshida, Nakayama, Nakada & M.M. Watanabe *sp. nov.*

Holotype: The SAG 19.88 strain specimen embedded in a resin block is deposited at TNS (National Museum of Nature and Science, Tsukuba, Japan) as TNS-AL-58967.

Etymology: The species epithet *asymmetrica* refers to the unusual asymmetrical shape of the young cell.

Strain: SAG 19.88 authentic

Description: Vegetative cells 13–20 µm in length, 8–18 µm in width, cylindrical to broad ellipsoidal, but frequently asymmetrical in young cells (Figs. 3A–C). Flagellum is about one and a half times the cell length. Cell with a hemispherical to conical papilla (Fig. 3F), two apical contractile vacuoles (Figs. 3B, I1), an anterior nucleus (Fig. 3B), and an asteroid chloroplast with a central pyrenoid covered with small globular starch plates (Figs. 3B, C, G, I2). Stigma oblong to small elliptical (Fig. 3E). Asexual reproduction via the formation of two or four zoospores (Fig. 3D). Spherical akinetes with thick cell wall sometimes produced in old culture (Figs. 6E, F). Sexual reproduction not observed.

Diagnosis: The young cells are frequently asymmetrical. The mature cells are generaly broad elliptical as *P. chlorostellata.* The stigma is sometimes small elliptical. Akinetes are formed. Diagnostic DNA sequence: 18S *r*DNA and ITS (accession number: MK696130).

Taxonomic remarks: Although the SAG 19.88 strain is referred to as *Cd. meslinii*, the present study indicates that both are different species based on morphological and molecular features. The strain is sister to *P. chlorostellata* (SAG 12.72) in the molecular phylogenetic tree. However, the presence of three CBCs between both strains and morphological differences support the separation into different species (Wolf *et al*. 2013).

Paludistella trianguloculus H. Susanti, Mas. Yoshida, Nakayama, Nakada & M.M. Watanabe *sp. nov.*

Holotype: The NIES-4318 and the paratype NIES-4317 were permanently cryopreserved in liquid nitrogen at Microbial Culture Collection NIES, Japan. The isotype NIES-4318 and the paratype NIES-4317 were conserved as TNS-AL-58966 and TNS-AL-58965 respectively.

Etymology: The species epithet, *trianguloculus*, refers to the unique shape of the stigma (eyespot) in this species. It is a combination of *triangularis* (= triangular in Latin) and *oculus* (= eye in Latin)

Strains: NIES-4317, NIES-4318

Description: Vegetative cells 10–19 µm in length, 5–15 µm in width, cylindrical to ellipsoidal (Figs. 4A–C, 5A–C). Flagellum is about one cell length. Cell with a hemispherical papilla (Figs. 4F, 5F), two apical contractile vacuoles (Figs. 4B, I1, 5A2, B, I1), an anterior nucleus (Fig. 4B), and an asteroid chloroplast with a central pyrenoid covered with small globular starch plates (Figs. 4B, C, G, I2, 5B, C, G, I2). Stigma small, triangular to elliptical (Figs. 4E, 5E). Asexual reproduction via the formation of two or four zoospores (Figs. 4D, 5D). Sexual reproduction not observed.

Diagnosis: The stigma is small, frequently triangular. Diagnostic DNA sequence: 18S *r*DNA and ITS (accession numbers: MK696132, MK696133).

Taxonomic remarks: The NIES-4317 and NIES-4318 strains exhibited no CBCs in ITS-2. In addition, since we could not find distinct morphological differences between the two strains, we consider them similar and the new species, *P. trianguloculus*.

Conclusion

Chlamydomonas meslinii and its relatives were reassessed as members of a novel genus, *Paludistella*, following revision and emergence of two novel species based on morphological comparisons, phylogenetic analysis of multiple genes (18S *r*DNA, *atp*B and *psa*B), and comparison of ITS-2 secondary structures. Further studies on ultrastructural features are required for the morphological comparison of *Paludistella* relatives. In addition, to obtain invaluable information on the potential bioproducts of this oil-rich algae, further biochemical analyses are required.

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