Taxonomical Study of *Paludistella trianguloculus* gen. et sp. nov. (Volvocales, Chlorophyceae) from Acidic Wetland with Its Relatives and The Biomass Production

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CONTENTS

Abstract

We introduce *Paludistella trianguloculus* gen. et sp. nov. NIES-4318 isolated from the acidic wetland in Nagano prefecture, Japan. The molecular phylogeny of multigene (18S *r*DNA, *atp*B and *psa*B) and morphological analyses especially in combination of the asteroid chloroplast with a central pyrenoid and the hemispherical papilla, demonstrated that NIES-4318 and its relatives were independent from the *Chloromonadinia* clade *sensu* Nakada *et al*. In addition, some morphological features supported by the number of compensatory base changes (CBCs) and hemi-CBCs detected in the predicted secondary structures of ITS-2 region delimited *P. trianguloculus* from its relatives. The polyphasic approaches revealed that NIES-4318 represents a new species in a novel genera *Paludistella*. Some morphological features of NIES-4318 and its relatives including cell colonization, "protocytotomy-like" cell division and oily cell are presented.

In this study, *P. trianguloculus* NIES-4318 was cultivated using a medium enriched with a *Sphagnum* soil extract pH 4.0 in comparison to the classical media AF-6 used for freshwater algae. The biomass study reported that *Paludistella trianguloculus* NIES-4318 produced biomass of up to 700 mg/L and oil up to 43% in acidic condition with 67% composed of unsaturated fatty acid from total lipid. This strain released 145 mg/L external organic material and 23% w/w exopolysaccharide. In the biorefinery viewpoint, this strain has shown the ability to change the culture pH from acidic pH 4.0 to a neutral pH after 4 weeks. The cultivation experiments showed the merits of this strain for oil production, external organic material production as byproduct and acidity refinement in the aquatic environment. Based on this experiment, the results showed that the substances inside the peat-water extract has a positive impact on algal biomass production and has a potential for paludiculture application.

General Introduction

Wetland is an interesting field to be studied. As a form of the natural landscape, wetland is found world-wide, from tropical to tundra climate, except for Antarctica. It provides a range of services for ecosystems such as nutrient storages and chemical cycling (Wetzel, 1996; EPA, 2002), and habitats for many useful plants, animals, and microbes including algae (Anderson *et al*., 2013; Bodelier & Dedysh, 2013; Vymazal, 1995). In wetland that consists of accumulation of degraded plant materials (peat), algae communities are found among habitats dominated by chlorophytes and acidophilic diatoms (EPA, 2002). These wetland algae can grow at a low pH environment and utilize the organic material from wet soil or peat as nutrients.

An acidic wetland is widely distributed in the world, especially in temperate to boreal zones in Northern Hemisphere and tropical zone in Southeast Asia (Hu *et al*., 2017). Acidic wetland is an important carbon sink in ecosystems (Vymazal, 1995) because the low pH in these environments (due to the accumulation of organic acids) holds the decomposition of organic matter stored as peat. From the viewpoint of application, such low pH makes it difficult for the usage of water and area in the wetland (e.g., White *et al*., 1997; Sumawijaya, 2006). Although the understanding of the ecosystems in a wetland is required for the sustainable development of such environments, our knowledge is limited. Even in the wetland at low pH, some microalgae grow well and act as producers supporting such ecosystems. The studies of these microalgae are important to understand the wetland.

In this study, *Paludistella trianguloculus* NIES-4318 (Volvocales, Chlorophyceae) was chosen as unique algae from an acidic wetland, isolated from Japanese wetland at Nagano Prefecture. The detailed studies on algae have not been described in many wetland literatures, but many studies are focused on ecological, botanical and limnological viewpoints. The taxonomical study and biomass study of this strain will be able to reduce the limited information about wetland algae.

A taxonomical study of *P. trianguloculus* NIES-4318, a *Chlamydomonas*-like algae, and its relatives were carried out by a polyphasic approach. Morphological variations have been specifically distinguished by comprehensive observation of morphological features such as cell shape and size, flagella, papilla, chloroplast, stigma, cell division, etc. Molecular phylogenetic relationships were examined by multigene analysis using 18S *r*DNA (e.g Nakayama *et al*., 1998; Proschold *et al*., 2001) and particular chloroplast genes (*atp*B

and *psa*B) without *rbcL* gene (Nozaki *et al*., 2002). In addition, ITS *r*DNA comparison was carried out from predicted secondary structures of ITS-2 (e.g Proschold *et al*., 2005; Nakada *et al*., 2010 a,b; Matsuzaki *et al*., 2012; Kawasaki *et al*., 2015) to support the separation of species within genus *Paludistella*.

The strain of *P. trianguloculus* NIES-4318 was isolated from the site dominated by *Sphagnum* spp. These plants are known to produce phenolics (Naumova *et al*., 2013) and carbohydrate compounds besides the other important biomass components such as lipids (Baas *et al*., 2000), trace elements (Orru *et al*., 2011) and amino acids, that are available in peat after long term biomass degradation. *Sphagnum* acid, sphagnorubin, and another anthocyanin belong to the phenolics compounds, while monosaccharides, uronic acid and a sphagnan (pectin-like substance) are the carbohydrates that usually found in *Sphagnum* biomass (Verhoeven & Liefveld, 1997). Such biomass composition from degraded *Sphagnum*, notably glucose and uronic acid, are considerably important as carbon sources for the microbes (Khodse *et al*., 2010; Kostka *et al*., 2016) such as bacteria to be converted into various organic acids such as α- ketoglutaric acid and pyruvic acid by an uronic acid oxidase (Riov, 1975). The conversion of carbon sources into various organic acids also possibly occurred in wetland's edaphic algae. In addition, the phenolic compounds are more complex organic compounds and not dealing with the simplest phenols which are highly toxic to living organisms. The free phenolic compounds in *Sphagnum* peat are responsible for practical applications such as bactericidal or any kind of protection agent from pathogenic organisms (Naumova *et al*., 2013).

In this study, *P. trianguloculus* NIES-4318 was cultivated using a medium enriched with a *Sphagnum* soil extract pH 4.0 in comparison to the classical medium AF-6 used for freshwater algae. Biomass performance (such as global biomass production, growth rate, lipid production, fatty acid profiles, and exopolysaccharide production) was followed and compared for these two media. In the present study, the experimental procedure of soil extract utilization will be explained and the impact of this wetland soil extract to *P. trianguloculus* NIES-4318 biomass performance with potential generalization on the Volvocales (Chlorophyceae) will be discussed.

CHAPTER 1: Taxonomical study of *Paludistella triangulocculus* NIES-4318 and its relatives

1.1. Introduction

Chlamydomonas (*Cd.*) Ehrenberg (Volvocales, Chlorophyceae), one of the largest genera of green microalgae and includes 400–600 species, was defined as the unicellular green algae with two equal flagella, a cell wall, chloroplast(s) possessing pyrenoid(s), and no 'distinct' features (Ettl 1976, 1983). However, molecular phylogenetic studies showed that *Chlamydomonas* in this sense is apparently polyphyletic (e.g Buchheim *et al*. 1990, 1996, Nozaki *et al*. 2000, Pröschold *et al*. 2001, Nakada *et al*. 2008, 2019) and currently delimited to include only the clade containing the conserved type species, *Cd. reinhardtii* Dangeard (Pröschold *et al*. 2001, Pröschold & Silva 2007, Wiersema *et al*. 2015) and other limited species (Nakada *et al*. 2010, 2019, Pröschold *et al*. 2018). The revision have been performed based on this situation supported with the emerging of some *Chlamydomonas*-like new species. 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* Ehrenberg (Demchenko *et al*. 2012), *Dangeardinia* Tempère, *Ixipapillifera* Nakada, *Rhysamphichloris* Nakada (Nakada *et al*. 2016), and *Edaphochlamys* Pröschold & Darienko (Pröschold *et al*. 2018). However, a great number of species still remain in *Chlamydomonas* in the traditional sense.

The genus *Chloromonas* (*Cr*.) Gobi traditionally included the *Chlamydomonas*-like algae without pyrenoid (Ettl 1970, 1983). However, Pröschold *et al*. (2001) revised *Chloromonas* based on the phylogenetic analysis and showed that the absence of pyrenoid is not significant for the classification. They transferred some species of *Chlamydomonas* to emended *Chloromonas* (Pröschold *et al*. 2001). Nakada *et al*. (2008) performed the comprehensive phylogenetic analysis based on the 18S rDNA sequences of the Volvocales and recognized many distinct clades in the order. They proposed *Chloromonadinia* based on PhyloCode for these clades and include the type species of the genus *Chloromonas, Cr. reticulata* (Goroschankin) Gobi (Nakada *et al*. 2008) and others *Chloromonas* revised by Pröschold *et al*. (2001). However, recent studies showed 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 adittional species of *Chloromonas* (Hoham *et al*. 2006, Matsuzaki *et al*., 2010, 2012, 2013, 2014, 2015, 2018,

Muramoto *et al.* 2010, Remias *et al*. 2013, Barcytė *et al*. 2018a, b), in this clade (Fig. 1). Therefore, the monophyly of *Chloromonas* 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, Barcytė *et al*. 2018a) based on molecular phylogenetic studies.

We isolated *Chlamydomonas*-like alga (NIES-4318) from the acidic bog in Nagano prefecture, Japan. The NIES-4318 produced a considerable amount of oil in moderately acidic condition (pH 4.0). Another Japanese strain (NIES-4317) was closely related to this strain, isolated from the paddy field in Yamagata prefecture, Japan. The 18S *r*DNA sequence of these strains showed the high similarity to that of strain SAG 75.81, the authentic strain of *Cd. meslinii* Bourrelly. Nakada *et al*. (2015) showed the phylogenetic tree in that SAG 75.81 was included in the clade *Chloromonadinia.* We found that two additional strains, SAG 12.72 (the authentic strain of *Cd. chlorostellata* Flint & Ettl) and SAG 19.88, identified as *Cd. meslinii* in the Sammlung von Algenkulturen at the University of Göttingen (SAG), were also closely related to these two Japanese strains. We studied the taxonomy of these strains using a polyphasic approach (e.g. Kawasaki *et al*. 2015) including light microscopy, molecular phylogenies, and the analysis of internal transcribed spacer (ITS) structures. Based on the polyphasic approach, we propose a new genus, *Paludistella*, including four species in the clade *Chloromonadinia.*

1.2. Material and Methods

1.2.1. Sample collection and cultivation

In October 2016, *P. triangulocullus* NIES-4318 was collected from Ikeno Taira Marsh, a wetland area of Nagano Prefecture in Japan. Wet soil sample enriched with AF-6 liquid media and incubated for 7-10 days. Then, the concentrate transferred into a new flask for isolation by 1.5% agar AF-6 (Kato 1982, modified according to Kasai *et al*. 2009) plating method in order to obtain a single colony. Unialgal cultures were established by serial dilution into AF-6 liquid media then incubated in test tubes at 25 °C under a white fluorescence lamp irradiance with 12h light (100-150 μmol m^{-2} s^{-1}):12h dark cycle. Other strains were obtained from Tsuruoka, Keio, Algae Collection (TKAC) deposited as NIES-3417 and from the Sammlung von Algenkulturen at the University of Göttingen (SAG) (Table 1). The AF-6 media at pH 4.0 and pH 6.6 were used for this taxonomical study.

1.2.2. Light microscopy

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

1.2.3. Gene sequencing and phylogenetic analyses

The total DNA was extracted from all strains using FastDNA kit (MP Biomedicals, France). We used nucleotide sequences of nuclear-encoded small subunit *r*RNA gene (18S *r*DNA), chloroplast-encoded ATP synthase beta subunit gene (*atp*B) and P700 chloroplyll *a* apoprotein A2 gene (*psa*B) and internal transcribed spacer 2 (ITS-2) region of nuclear-encoded *r*DNA for molecular analysis. Sequences were obtained using primer sets described previously for ITS (Coleman *et al*. 1994, Kawasaki *et al*. 2015), 18 *r*DNA (Nakayama *et al*. 1998), *atp*B (Nozaki *et al*. 1999) and *psa*B (Nozaki *et al*. 2000, 2002). Four newly designed primers were also used: MSL.18S-F1 (5′ -TGGATGTGCTGGTGAAGTGT-3′ ; position 975-996 bp), MSL.ITS-R1 (5′ -AACAACAGCATTTAAGCTACATCA-3′ ; position 401-376 bp), MSL.ITS-R3 (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). The condition 95^0C/2 min, 46^0C/2 min, 66 C/ 3 min) × 31^0 , 72^0C/ 15 min, 4^0C/ ∞ was used for direct PCR , whereas the PCR condition for sequencing was 96^0C/1m, (96^0C/10s, 50^0C/5s, 60^0C/ 4 min) × 25, 4^0C/∞.

The total 3,384 bases of 18S *r*DNA, *atp*B and *psaB* sequences from 29 OTUs were used for the multigenes phylogenetic analysis including five strains examined in this study (Table 1.2). The sequence alignments were computed using MAFFT (Katoh *et al*. 2002) and checked using BioEdit (Hall 1999). The third position of *atp*B and *psa*B codons were excluded based on the homogeneity test to 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) performed using MrBayes 3.2.6 (Ronquist *et al*. 2012) with 1,000,000 generations of Marcov

Chain Monte Carlo (MCMC) iterations, discarding the first 25% as burn-in. The best substitutional 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 by PAUP* 4.0b10 and MrModeltest 2.3 (Nylander 2004). Maximum likelyhood (ML) analysis was performed using IQ Tree 1.6.9 (Lua *et al*. 2012, Nguyen *et al*. 2015) with authomatic ModelFinder and ultrafast bootstrap analysis based on 1,000 replications (Hoang *et al*. 2018). In addition, PAUP* 4.0b10 and MEGA X (Kumar *et al*. 2018) were used for maximum parsimony (MP) and minimum evolution analysis (ME) based on 1,000 bootstrap replications respectively. Finally, the phylogenetic tree data was visualized using FigTree 1.4.3 (Rambaut 2012). The secondary structure of ITS-2 sequence was predicted using RNAstructure webserver developed by Reuter & Mathews (2010).

1.2.4. Preparation of specimen embedded into resin block

The 2 ml cultures (12 days old) of strain SAG 19.88, NIES-4317 and NIES-4318 in AF-6 medium were collected and centrifugated. After discarding the supernatans, cultures were fixed with 4% glutaraldehide in 0.025M cacodylate buffer (CA) and mixed by gently pipeting then centrifused. The 3 ml cacodylate buffer 0.05M were added into pellets of cultures and wait for 15 minutes, this step was repeated several times. 1% OsO_4 in 0.025M CA buffer were added into pellets and gently shaked followed by dehydration in ethanol series (30%, 50%, 70%, 90%, 95%, 100%). The ethanol was substituted with 3 ml propylene oxide and wait for 15 minutes (Yoshida *et al*. 2009, Matsuzaki *et al*. 2010). Finally, 1 ml Spurr's resin (Spurr 1969) was applied to the fixed material.

1.3. Results

1.3.1. Strain locality

Paludistella trianguloculus NIES-4318 originated from Japanese wetland (Fig. 1.2) collected in the early winter season. This strain isolated from wet soil sample which was overgrown by *Sphagnum* spp. The location is called Ikenotaira Marsh, a national park in Nagano Prefecture

inhabited by the alpine trees, various grass species, mosses such as *Sphagnum* spp., etc. The altitude of this place was 2,000 m above sea level.

1.3.2. Light microscopy of morphological features

General descriptions of *Paludistella* gen. nov.

The strain *P. triangulocolus* NIES-4318 and its relatives (NIES-4317, SAG 75.81, SAG 12.71, SAG 19.88) possessed similar morphological features but showed some differences as summarized in Table 1.3.

They were basically unicellular with two equal flagella (Figs 1.3H, 1.4H, 1.5H, 1.6H, 1.7H) emerging from the anterior pole of the cell and two contractile vacuoles were located near the bases of the flagella (Figs 1.3I1, 1.4I1, 1.6I1). The length of flagellum was 1.0–1.5 times as long as cell. The vegetative cells were circular in optical transverse section (Figs 1,3I, 1.4I, 1.5I, 1.6I, 1.7I) and possessed rounded anterior and posterior ends were found during observation (Figs 1.3A–C, 1.4A–C, 1.5A–C, 1.6A–C, 1.7A–C). The young vegetative cells are cylindrical to elliptical (Figs 1.3A, 1.4A, 1.5A, 1.6A), and those of SAG 19.88 were frequently asymmetrical dorsoventrally (Fig. 1.7A). The mature vegetative cells were usually elliptical (Figs 1.3B–C, 1.4B–C, 1.5B–C), but sometimes broad elliptical especially in SAG 12.72 and SAG 19.88 (Figs 1.6B–C, 1.7B–C). The vegetative cell was covered by thin smooth cell wall (Figs 1.3–1.7).

Unique features of *Paludistella* gen. nov.

The combination of papilla, chloroplast, starch grains and stigma shape are unique in this new genera. In addition, pyrenoid position is also contribute to remark this uniqueness. The papilla at the anterior tip of the cells was hemisperical to conical (Figs 1.3F, 1.4F, 1.5F, 1.6F, 1.7F). The chloroplast was cup shaped in young vegetative cells (Figs 3A, 4A, 5A, 6A, 7A), but asteroid form with deep narrow radial incisions forming irregular lobes in mature vegetative cells (Figs 1.3B-C, 1.4B-C, 1.5B-C, 1.6B-C, 1.7B-C). A pyrenoid was spherical, sometimes situated laterally in young vegetative cells (Figs 1.3A, 1.4A, 1.6A, 1.7A), but centrally in mature vegetative cells (Figs 1.3B, 1.4B, 1.5B, 1.6B, 1.7B). The starch grains covering the pyrenoid were small, and usually globular (Figs 1.3G, 1.5G, 1.6G, 1.7G), but sometimes oblong in SAG 19.88 (data not shown) and NIES-4317 (Fig. 1.4G). A pale red stigma was

situated on the anterior third to quarter of the cell (Figs 1.3A, 1.4B, 1.6C, 1.7C). The shape of stigma was various among the strains; long filiform in SAG 75.81 (Fig. 1.5E), oblong in SAG 12.72 (Fig. 1.6E), triangular to elliptical in NIES-4317 (Fig. 1.4E) and NIES-4318 (Fig. 1.3E) or somewhat variable, oblong to small elliptical in SAG 19.88 (Fig. 1.7E). A spherical nucleus was anterior to the pyrenoid (Figs1. 3B, 1.4B, 1.5B, 1.6B, 1.7B).

Reproduction and colonization of *Paludistella* gen. nov.

Asexual reproduction was accomplished through the formation of two or four zoospores (Figs 1.3D, 1.4D, 1.5D, 1.6D, 1.7D), but the formation of eight zoospores was sometimes observed in SAG 12.71 (Fig. 1.6D3). All strains showed unusual dividing cells, in which one to four buddings from a cell (Fig. 1.8). Although we did not observe the full course of division, this division was similar to 'protocytotomy' reported in some members of the Volvocales including *Chloromonas* (Masyuk & Demchenko 2001, Hoham *et al*. 2006, Demcenko *et al*. 2012, Chelebieva *et al*. 2018).

Various types of colonization were observed such as a grape-like formation (Fig. 1.9A) which allowed the cells move in or out to the colony. The palmelloid forms were also shown in which cells covered by mucilage materials. The cells inside palmelloid arrange among daughter cell either irregular (Fig. 1.9B) to regular (Fig.1. 9C) formation.

The oily cells of *Paludistella* gen. nov.

In old cultures, cells were sometimes filled with colorless to orange oil droplets (Fig. 1.10). Akinetes, spherical cells with thick smooth cell wall and no flagella storing many oil droplets, were sometimes observed in the old culture of SAG19.88 (Fig. 1.10E–F).

1.3.3. Molecular phylogenetic analyses

In the molecular phylogenetic tree based on 18S *r*DNA, *atp*B and *psa*B, the strains NIES-4318 NIES-4317, SAG 75.81, SAG 12.72, and SAG 19.88 formed a robust clade [posterior probability (PP) = 1.0, bootstrap proportion (BP) in all methods = 100%] in the clade *Chloromonadinia* (Fig. 1.11). *Paludistella* clade 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 SA clade *sensu* Matsuzaki *et al*. (2013), CA clade *sensu* Matsuzaki *et al*. (2013)

and *Ixipapillifera* (PP = 1.0, BPs ≤ 72%). The 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 this study (*Paludistella*), SAG 75.81 was situated at the base (PP = 1.0, BPs = 99–100%). The subclade composed of SAG 12.71 and SAG 19.88, and the subclade composed of NIES-4317 and NIES-4318 were recovered with moderatly high statistical supports (PPs = 0.99, BPs = 80–90%).

1.3.4. 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 this 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. 1.12). 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. 1.12). 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. 1.13).

1.4. Discussion

1.4.1. Genus taxonomy

The Polyphyletic of Chloromonadinia clade

Present study showed a new distinct subclade in the clade *Chloromonadinia*. The morphological features of this subclade, such as unicellular flagellate with two equal flagella, cell wall, and chloroplast with a pyrenoid, are congruent with the traditional concept of the genus *Chlamydomonas* (Ettl 1976, 1983). However, now it is clear that *Chlamydomonas* in this sense is polyphyletic and the strains examined in this 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 phylogenetic

analysis and transferred some species of *Chlamydomonas* to emended *Chloromonas*. The strains in this study can be classified into *Chloromonas sensu* Pröschold *et al*. (2001). However, following studies indicated that some 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). Therefore, *Chloromonas sensu* Pröschold *et al*. (2001) is not monophyletic.

The unique features of *Paludistella* gen. nov.

The position of pyrenoid is valid to distinguish the strains in this study from the species in the CR clade. All strains in this study have the asteroid chloroplast with a central pyrenoid ('asteroid gelappt' in *Euchlamydomonas* type; Ettl 1976, 1983) in mature cells. The members of the CR clade are mostly lack pyrenoids but some species such as *Cr. chlorococcoides* (Ettl & Schwarz) Matsuzaki, Hara & Nozaki and *Cr. typhlos* (Gerloff) Matsuzaki, Hara & Nozaki have a lateral pyrenoid (*Chlamydella* type) (Ettl 1976, 1983, Pröschold *et al*. 2001, Matsuzaki *et al.* 2012, Barcytė *et al*. 2018a, b). Furthermore, the strains in this study are also distinguishable from another large subclade (SA clade in Matsuzaki *et al*. 2013, clade 2 in Barcytė *et al*. 2018a) of *Chloromonas sensu* Pröschold *et al*. (2001) in morphological features. All members of SA clade have no typical pyrenoid and no distinct papilla (Ettl 1976, 1983, Matsuzaki *et al.* 2014, 2015, 2018, Muramoto *et al*. 2010). The combination of the asteroid chloroplast with a central pyrenoid and the hemispherical to conical papilla is also not found in other species of the clade *Chloromonadinia* (Ettl 1976, 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 this study.

Taxonomy of *Paludistella* gen, nov.

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

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

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

Description: Vegetative cells cylindrical to nearly spherical with rounded ends. Cell wall thin, with an anterior hemispherical to conical papila from which two equal flagella emerge. Greenish asteroid chloroplast bearing many radial lobes connected centrally in mature cells. Central pyrenoid covered by numerous small starch grains. Stigma filiformis to eliptical positioned at the anterior one third to quarter of cell. Nucleus positioned in the anterior cavity of chloroplast. Two contractile vacuoles at the apical tip of cell. Asexual reproduction by formation of mainly two or four zoospores.

Paludistella gen, nov. is independent clade within *Chloromonadinia*

In the molecular phylogenetic analysis, the subclade found in this study is independent in the clade *Chloromonadinia* and the close relative of this subclade is not clear (except for *Cr. pseudoplatyrhyncha*; see below). However, the CR clade *sensu* Matsuzaki *et al*. (2013) (core *Chloromonas sensu* Barcytė *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. 1.11). Therefore, the strains examined in this study cannot be classified into *Chloromonas*.

Chloromonas pseudoplatyrhyncha is possible to classify as the member of *Paludistella* because this alga is sister to *Paludistella* in the phylogenetic tree. However, *Cr. pseudoplatyrhyncha* is different from the species of *Paludistella* in the shape of papilla and the feature of pyrenoid. *Cr. pseudoplatyrhyncha* has a papilla with a flattened or slightly concave top face and the multiple atypical pyrenoids without associated starch grains distributed in the interior regions of the lobes of the chloroplasts (Matsuzaki *et al*. 2010). This alga seems to be better to be classified into another new genus. However, the taxonomic revision of this alga should be considered until further taxonomic information in the clade *Chloromonadinia* is available.

Symplesiomorphy of asteroid chloroplast within *Chloromonadinia* clade

The members of *Paludistella* have the asteroid chloroplast with a central pyrenoid. This feature is also found in some other species in the clade *Chloromonadinia* and this evidens provides an evolutional insight about this clade. This type of chloroplast 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 1976, 1983, Pröschold *et al*. 2001, Takahashi *et al*. 2018). In the clade *Chloromonadinia*, *Cr. augustae*, CA clade and *Paludistella* are distantly related each other. The distribution of this feature suggest that the asteroid chloroplast with a central pyrenoid is a symplesiomorphy in the clade *Chloromonadinia.*

1.4.2. Species taxonomy

Among the strains examined in this study, there are one to twelve CBCs in ITS-2 except for the pair of NIES-4317 and NIES-4318. Although these strains are very similar each other morphologically, some differences such as the shape of stigma are present. Therefore, the separation of these strains (except for NIES-4317 and NIES-4318) into different species is supported by molecular and morphological data.

Some taxa of *Chlamydomonas* possessing 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* Ettl, *Cd. gerloffii* Ettl, *Cd. metapyrenigera* Skuja, *Cd. nygaardii* Ettl, *Cd. subangulosa* Fritsch & John (Ettl 1976, 1983). However, these species are apparently different from the strains examined in this study in the cell shape, size and the features of stigma. Therefore, we classify the five strains in *Paludistella* into four species including two new species as folows.

***Paludistella triangulocul*us** Susanti, Yoshida, Nakayama, Nakada, & 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 *triangulocul*us refers the unique shape of stigma (eyespot) in this species. It was combined from *triangularis* (= triangular, Latin) and *oculus* (= eye, Latin)

Authentic strain: NIES-4318

Description: Vegetative cells 10–19 µm in length, 5–15 µm in width, cylindrical to ellipsoidal (Figs 1.3A-C, 1.4A-C). Flagella is about one time of cell length. Cell with a hemispherical to conical papilla (Figs 1.3F, 1.4F), two apical contractile vacuoles (Figs 1.3I1, 1.4I1), an anterior

nucleus (Figs 1.3B, 1.4B), and an asteroid chloroplast with a central pyrenoid covered by small globular to oblong starch grains (Figs 1.3B, C, G, 1.4B, C, G). Stigma small, triangular to elliptical (Figs 1.3E, 1.4E). Asexual reproduction via the formation of two or four zoospores (Figs 1.3D, 1.4D). Sexual reproduction not observed.

Taxonomic remarks: The strains NIES-4317 and NIES-4318 showed no CBCs in ITS-2. In addition, because we could not find distinct differences between these two strains, we treat them as the same new species, *P. triangluloculus*.

Paludistella meslinii (Bourrelly) Susanti, Yoshida, Nakayama, Nakada, & Watanabe *comb. nov*.

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

Authentic strain: SAG 75.81

Emended description: Vegetative cells 13–21 µm in length, 6–15 µm in width, cylindrical to ellipsoidal (Fig. 1.5A-1.5C). Flagella is about one time of cell length. Cell with a hemispherical to conical papilla (Fig. 1.5F), two apical contractile vacuoles, an anterior nucleus (Fig. 1.5B), and an asteroid chloroplast with a central pyrenoid covered by small globular strach grains (Fig. 1.5B, C, G). Stigma filiform, very narrow (Fig. 1.5E). Asexual reproduction via the formation of two or four zoospores (Fig. 1.5D). 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 were well congruent with those in Bourrelly (1951). The cell size in the original description (20–22 × 15 µm) is equivalent to the maximum size in the present observation. The narrow filiform stigma is characteristic for this species and not found in other species of *Paludistella*.

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

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

Authentic strain: SAG 12.72 (= CCAP 11/93)

Emended description: Vegetative cells 12–21 µm in length, 6–15 µm in width, cylindrical to broad ellipsoidal (Fig. 1.6A–1.6C). Flagella is about one and a half times of cell length. Cell with a hemispherical to conical papilla (Fig. 1.6F), two apical contractile vacuoles (Fig. 1.6I1), an anterior nucleus (Fig. 1.6B), and an asteroid chloroplast with a central pyrenoid covered by small globular strach grains (Figs. 1.6B, C, G). Stigma oblong (Fig. 1.6E). Asexual reproduction via the formation of two, four or eight zoospores (Fig. 1.6D). 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 basically congruent with those in Flint & Ettl (1966) except for the cell size and the features of stigma. The cell size in the original description (18–24 × 11–27 µm) is slightly larger than that in the present observation. The stigma in the original paper is wider and situated more posterior than those in this study. Ettl & Gartner (2014) treated *Cd. chlorostellata* as a synonym of *Cd. meslinii* because of some morphological similarities. However, SAG 12.72 possesses completely different ITS-2 helix III and at least three CBCs in other helices comparison to SAG 75.81 and these two strains are different in the shape of cells and stigma. Therefore, we treat them as different species. *Chamydomonas chlorostellata* contains a single variety, *Cd. chlorostellata* var. *gracillima* Ettl (Ettl 1976, 1983). However, the 18S *r*DNA sequence of SAG 25.87, the authentic strain of *Cd. chlorostellata* var. *gracillima* was distantly related to that of SAG 12.72 and did not included in *Paludistella* (unpublished data).

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

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

Etymology: The species epithet *asymmetrica* refer the unusual asymmetric shape of the young cell.

Authentic strain: SAG 19.88 authentic

Description: Vegetative cells 13–20 µm in length, 8–18 µm in width, cylindrical to broad ellipsoidal (Fig. 1.7A-7C). Flagella is about one and a half times of cell length. Cell with a hemispherical to conical papilla (Fig. 1.7F), two apical contractile vacuoles (Fig. 1.7I1), an anterior nucleus (Fig. 1.7B), and an asteroid chloroplast with a central pyrenoid covered by small globular strach grains (Fig. 1.7B, C, G). Stigma oblong to small elliptical (Fig. 1.7E). Asexual reproduction via the formation of two or four zoospores (Fig. 7D). Spherical akinetes with thick cell wall sometimes produced in old culture (Fig. 1.8C). Sexual reproduction not observed.

Taxonomic remarks: Although the strain SAG 19.88 is refered to *Cd. meslinii*, the present study indicates that they are different species based on morphological and molecular features. This strain is sister to *P. chlorostellata* (SAG 12.72) in the molecular phylogenetic tree. However, the presence of two CBCs between them and morphological differences, such as the asymmetrical cells in SAG 19.88, support the separation of both as different species.

Figures

Chloromonas	*Gloeomonas*	*Ixipapillifera*	*Chlainomonas*	*Paludistella*
Chloromonas reticulata (Goroschankin) Gobi	*Gloeomonas kupfferi, G.anomalipyrenoides, G. lateperforata*	*Ixipapillifera deasonii, I. pauromitos, I.sacculiformis*	*Chlainomonas* sp. DL06	*P. meslinii, P. chlorostellata, P. asymmetrica, P. trianguloculus*
Ellipsoid or ovoid, a hemispherical papilla, cup or urn -shaped choroplast with irregular perforations and incisions on surface, pyrenoid absent, D or rod-shape stigma, nucleus in central L: 11-20µm W: 5-15µm	Broadly ellipsoidal to nearly spherical, papilla low and dome-shaped in front view, deep-cup shaped chloroplast with atypical pyrenoid, circular to short elliptical stigma, nucleus in central, 2 remoted flagellar bases Cell diameter: 11-25µm	Elipsoidal to spherical with both ends rounded, angular trapezoidal papilla narrow x in top view, amphichloris-type chloroplast with one or several typical pyrenoids , small circular stigma, nucleus in central or posterior L: 13-24µm W: 5-14µm	Spherical to ellipsoidal, Quadriflagellata (2 pairs flagellar), Cell relatively wide > 20µm	ellipsoidal-cylindrical, hemispherical papila, cup-shaped chloroplast with narrow radial incisions, typical pyrenoid, elongated stigma (filiformis to eliptical), nucleus in anterior L: 10-21µm W: 5-18µm
(Matsuzaki *et al.*, 2012)	(Nozaki *et al.*,2010; Nakada *et al.*, 2015)	(Nakada *et al.*, 2016)	(Novis *et al.*, 2008)	

Figure 1.1. Morphological comparison among genera within *Choromonadinia* clade represented by *Chloromonas*, *Gloeomonas*, *Ixipapilifera*, *Chlainomonas* and new determined genera *Paludistella* in this study.

Figure 1.2. Locality of *P. trianguloculus* NIES-4318. Map of location (A) at Ikenotaira Marsh Nagano, Japan (B) and specific habitat of NIES-4318, wet soil covered by *Sphagnum* spp. (C).

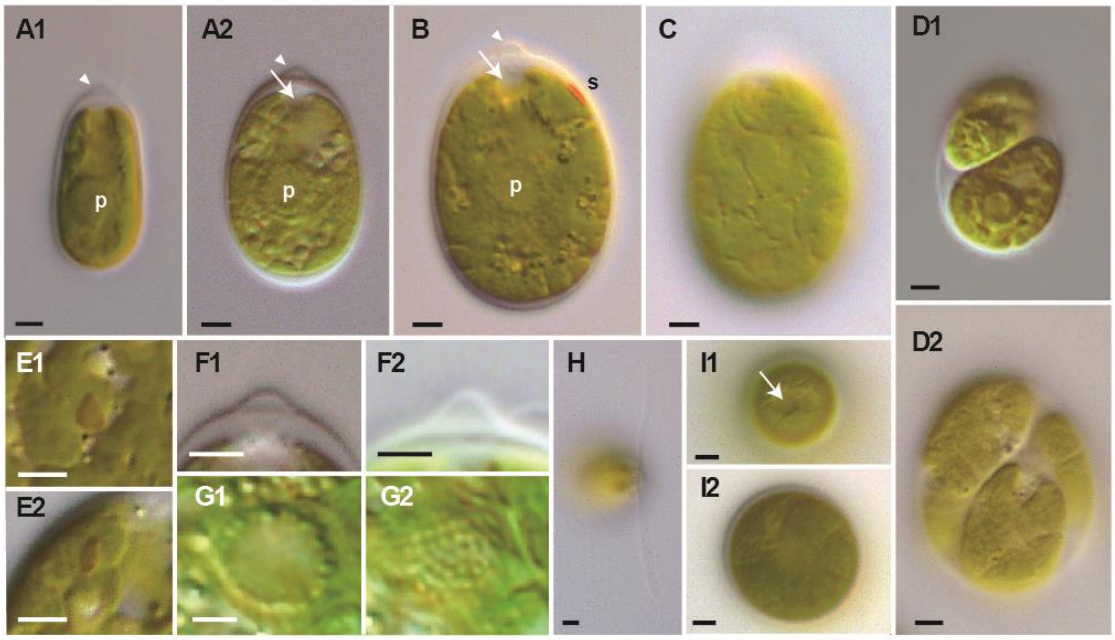


Figure 1.3. Light microscopy of *Paludistella trianguloculus* (NIES-4318). Young cells (A), optical section (B) and cell surface (C) of a mature cell, zoosporangium including two (D1) or four (D2) daughter cells, triangular to eliptical stigmata (E), hemispherical to conical papilla (F), optical section (G1) and surface (G2) of a pyrenoid, equal two flagella (H), top view with contractile vacuole (I1) and optical cross section (I2) of cells. . n = nucleus, p = pyrenoid, s = stigma. Scale bar = 2 µm.

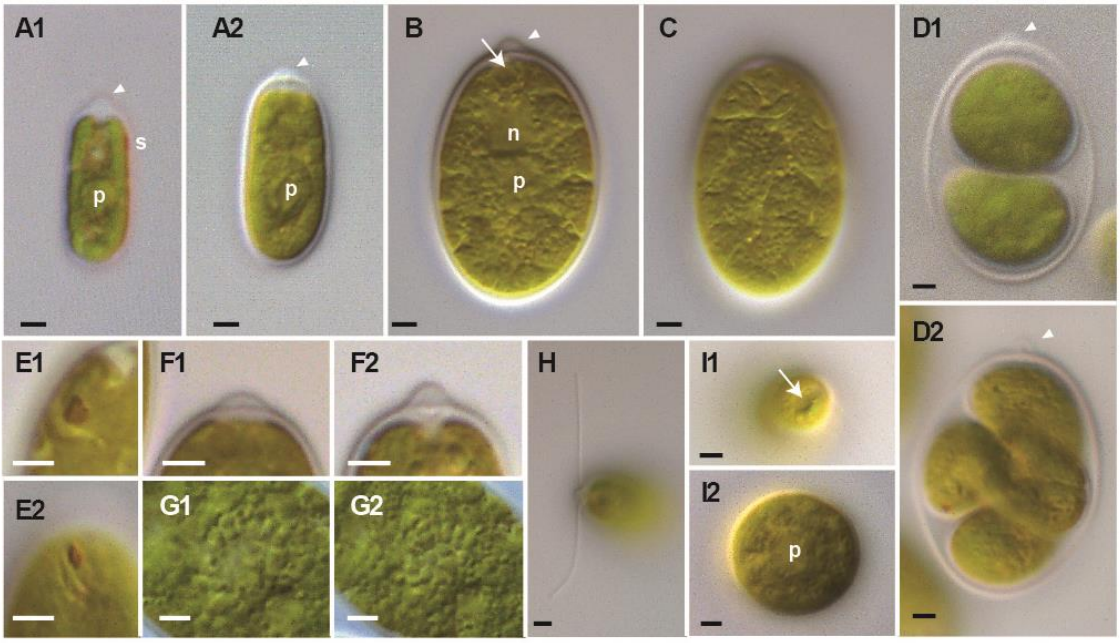


Figure 1.4. Light microscopy of *Paludistella trianguloculus* (NIES-4317). Young cells (A), optical section (B) and cell surface (C) of a mature cell, zoosporangium including two (D1) or four (D2) daughter cells, triangular to eliptical stigmata (E), hemispherical to conical papilla (F), optical section (G1) and surface (G2) of the pyrenoids, equal two flagella (H), top view with contractile vacuole (I1) and optical cross section (I2) of cells. For abbreviations, see the legend to Figure 1. Scale bar = 2 µm.

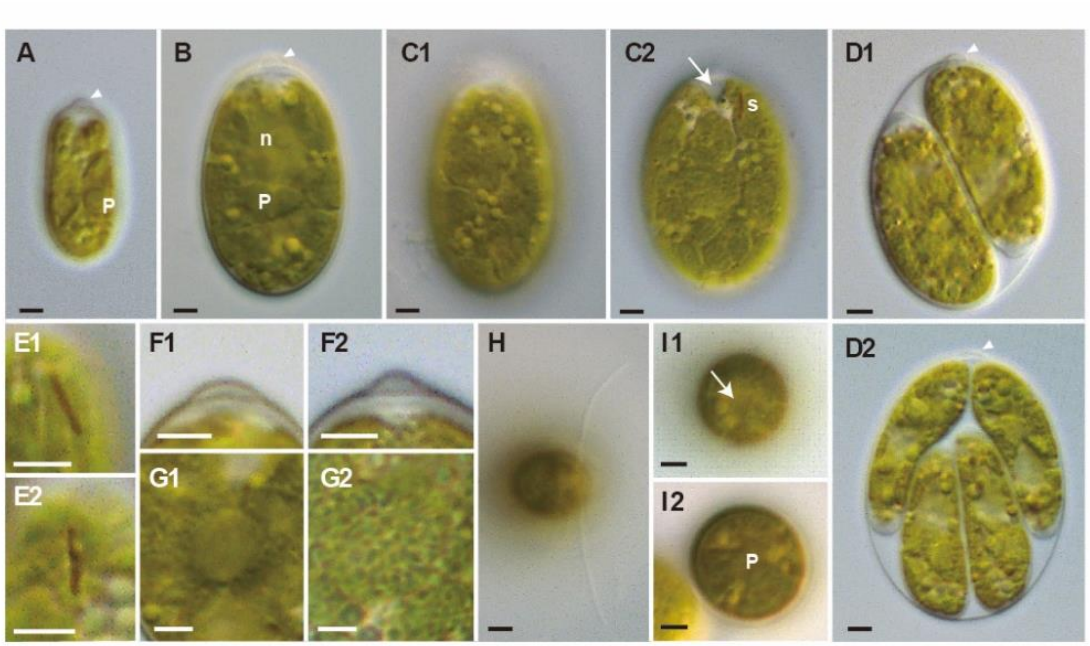


Figure 1.5. Light microscopy of *Paludistella meslinii* (SAG 75.81). A young cell (A), optical section (B) and cell surface (C) of mature cells (B and C1 are the same cell), zoosporangium including two (D1) or four (D2) daughter cells, filiform stigmata (E), hemispherical to conical papilla (F), optical section (G1) and surface (G2) of a pyrenoid, equal two flagella (H), top view with contractile vacuoles (I1) and optical cross section (I2) of cells. Arrowheads indicate the papillae and arrows indicate contractile vacuoles. For abbreviations, see the legend to Figure 1. Scale bar = 2 µm.

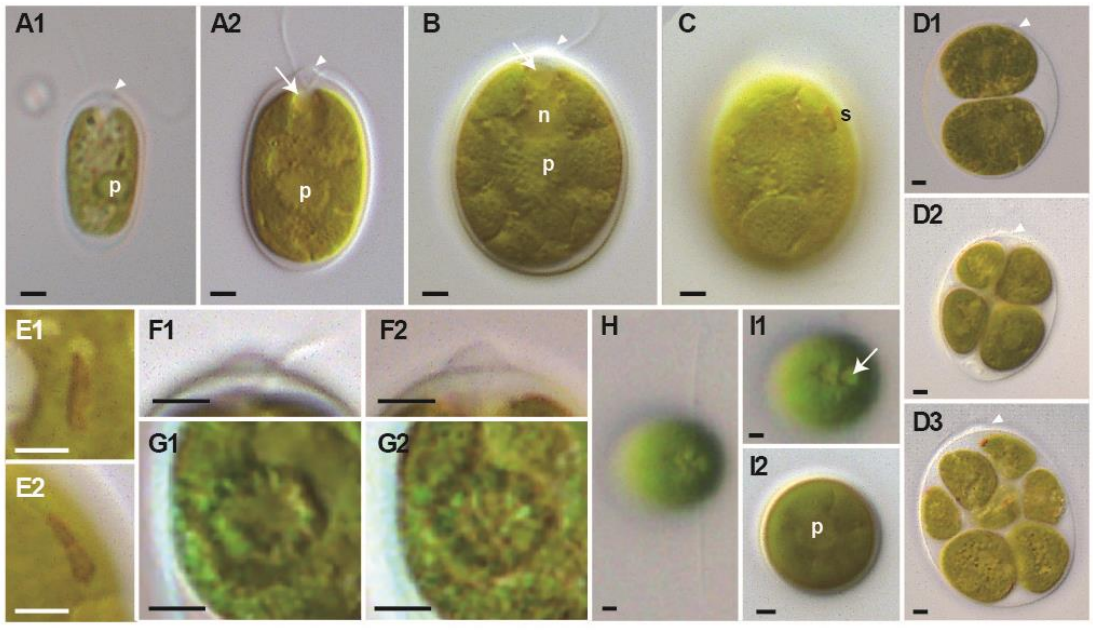


Figure 1.6. Light microscopy of *Paludistella chlorostellata* (SAG 12.72). Young cells (A), optical section (B) and cell surface (C) of a mature cell, zoosporangium including two (D1), four (D2) or eight (D3) daughter cells, oblong stigmata (E), hemispherical to conical papillae (F), optical section (G1) and surface (G2) of a pyrenoid, equal two flagella (H), top view with contractile vacuoles (I1) and optical cross section (I2) of cells. For abbreviations, see the legend to Figure 1. Scale bar = 2 µm.

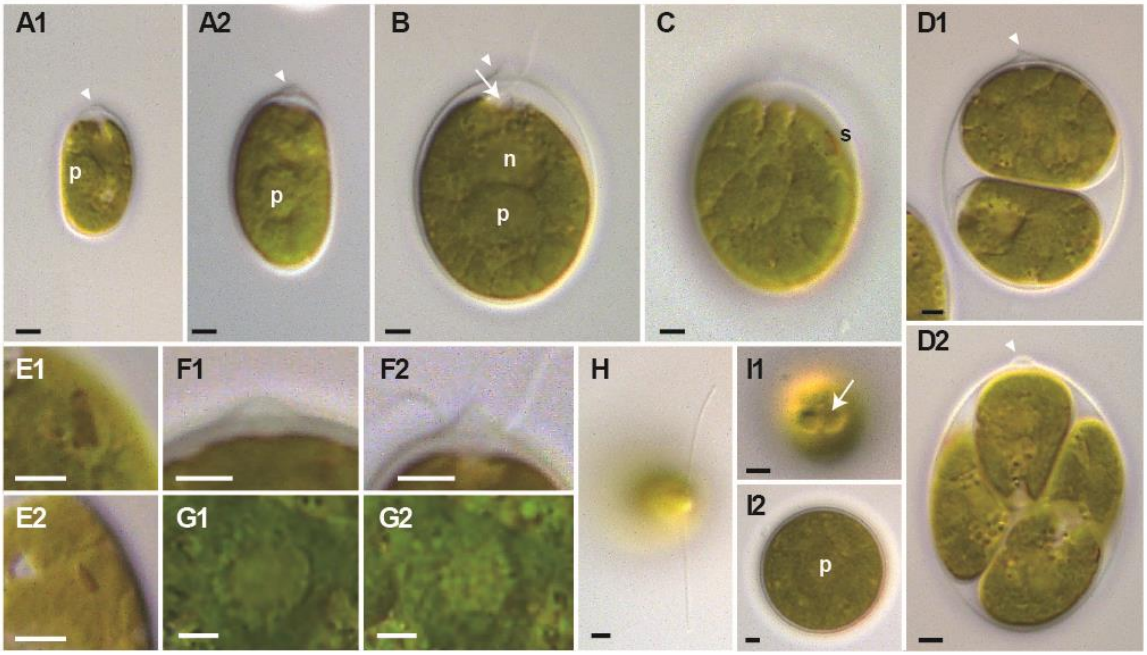


Figure 1.7. Light microscopy of *Paludistella asymmetrica* (SAG 19.88). Young cells (A), optical section (B) and cell surface (C) of a mature cell, zoosporangium including two (D)1 or four (D2) daughter cells, oblong to small eliptical stigmata (E), hemispherical to conical papilla (F), optical section (G1) and surface (G2) of the pyrenoids, equal two flagella (H), top view (I1) and optical cross section (I2) of cells. For abbreviations, see the legend to Figure 1. Scale bar = 2 µm.

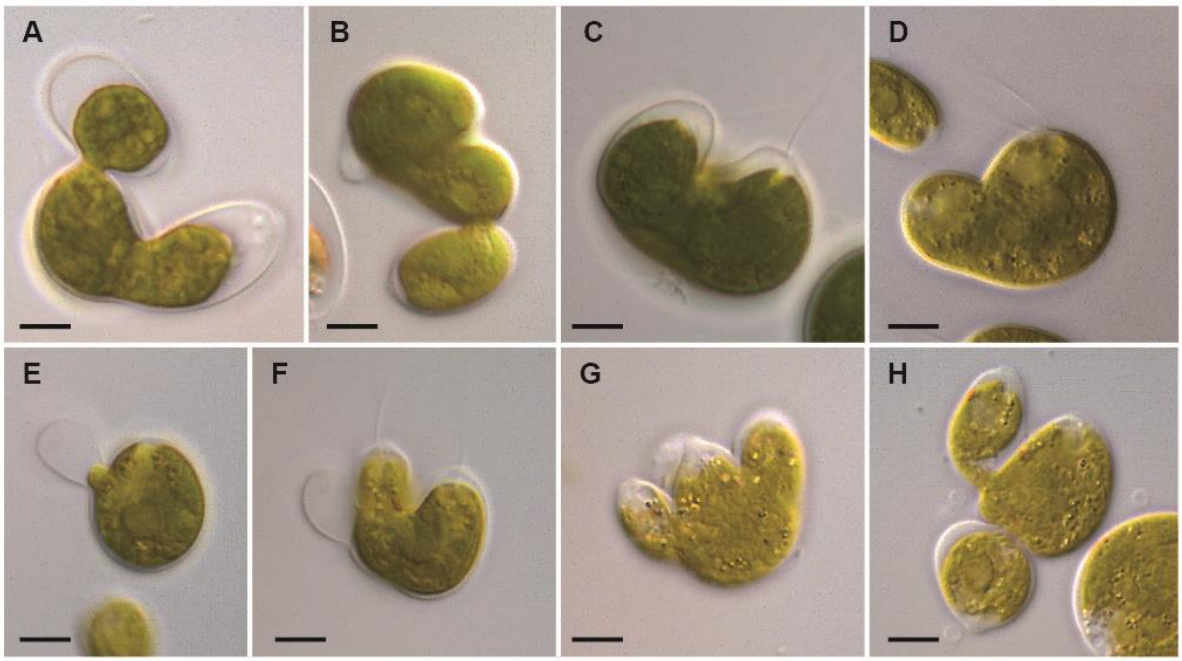


Figure 1.8. Light microscopy of the unusual cell divisions of *Paludistella. P. meslinii* (SAG 75.81) (A), *P. chlorostellata* (SAG 12.72) (B), *P. asymmetrica* (SAG 19.88) (C), *P. trianguloculus* (NIES-4317) (D), *P. trianguloculus* (NIES–4318) (E–H). Scale bars = 5 µm.

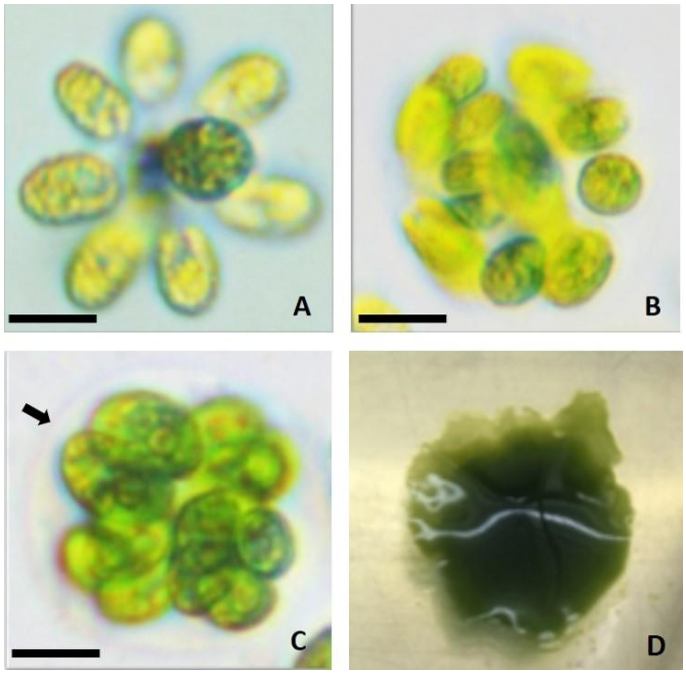


Figure 1.9. Various colonizations on *Paludistella* clade. A grape-like formation (A) Palmelloid (B) Daughter cells inside a palmeloid (C) Colony on agar plate (D). Scale bars = 10 µm.

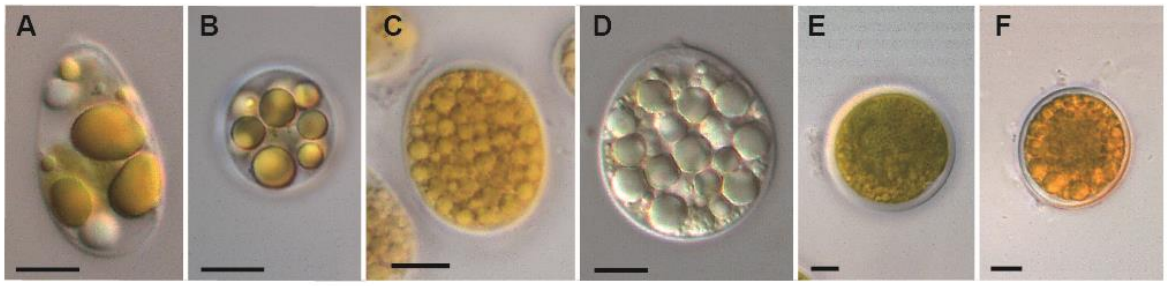


Figure 1.10. Light microscopy of the old cells storing oil droplets of *Paludistella meslinii* (SAG 75.81) (A), *P. chlorostellata* (SAG 12.72) (B), *P. trianguloculus* NIES-4317 (C) and NIES-4318 (D). The akinetes of *P. asymmetrica* (SAG 19.88) (E, F). Scale bars = 5 µm.

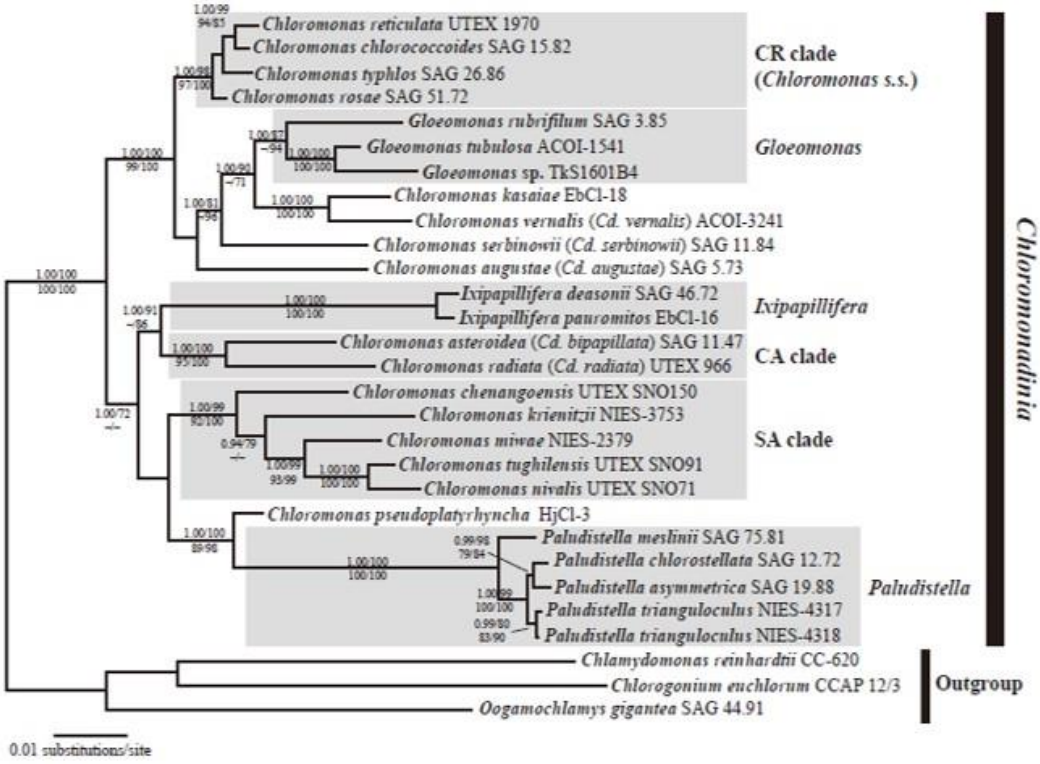


Figure 1.11**.** Bayesian phylogenetic tree of *Chloromonadinia* based on 18S *r*DNA, *atp*B and *psa*B. Numbers at nodes indicate posterior probabilities (≥ 0.95, top left) and bootstrap proportions (≥ 70%) for ML (top right), MP (bottom left) and ME (bottom right) analyses.

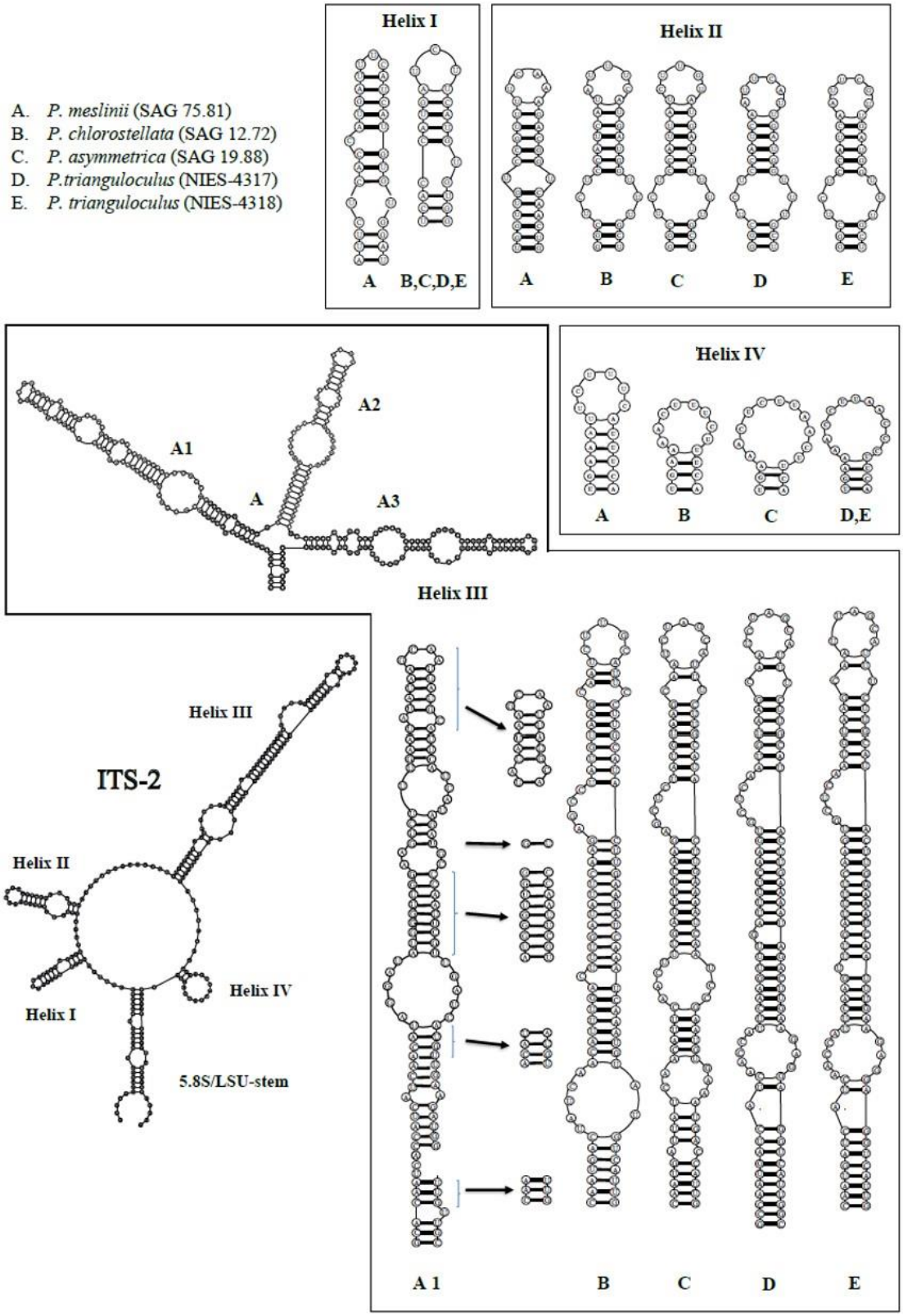
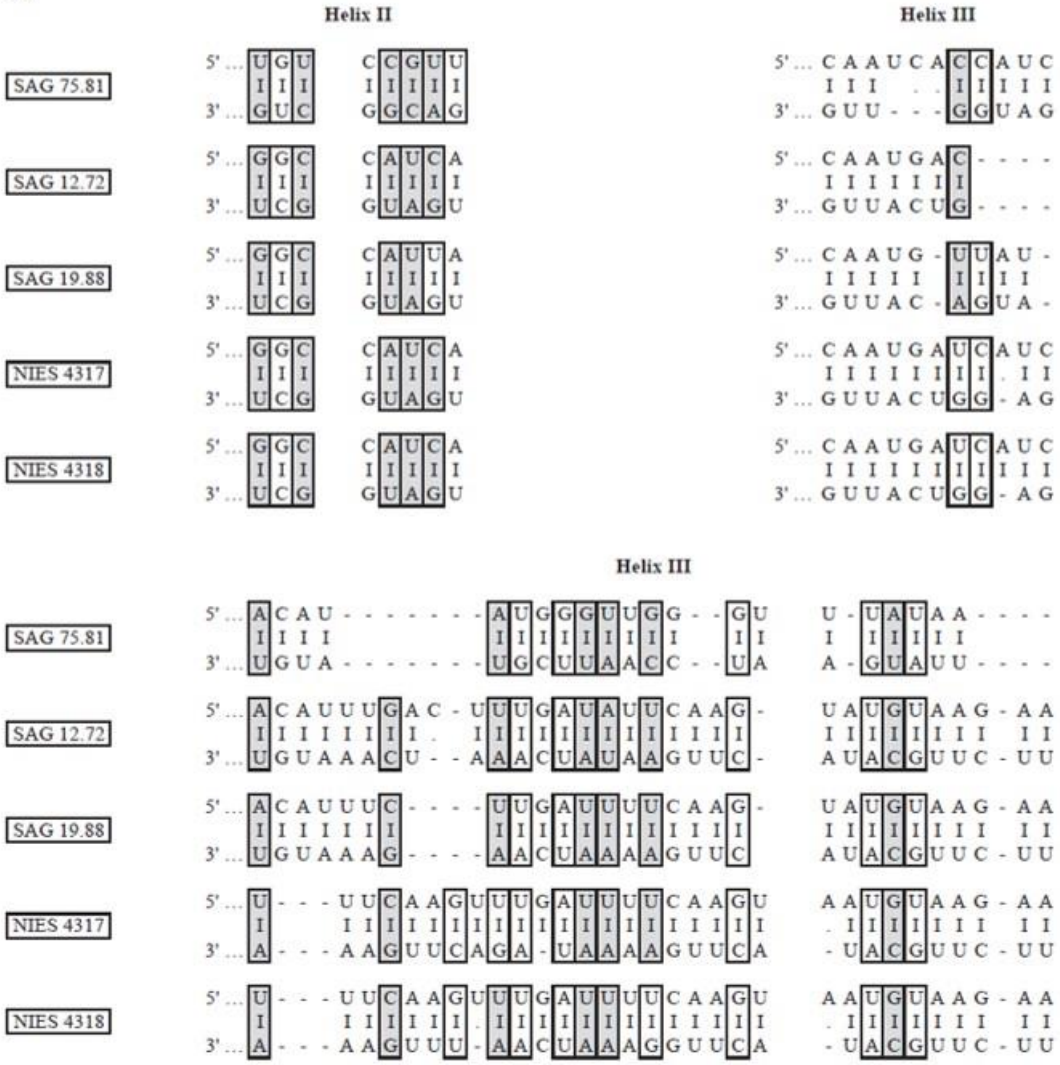


Figure 1.12. Secondary structure of ITS-2 *r*DNA sequences among the species of *Paludistella*. The helix III of SAG 75.81 consist of three sub-helices (A1, A2, and A3) and the comparable positions were found only in sub-helix A1.

A



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 1.13. (A) The predicted secondary structures of ITS-2 *r*DNA helices I, II, III and IV of *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.

Tables

Table 1.1. Strains used in this study.

Species	Strain	Locality	
Paludistella meslinii	SAG 75.81	Ditch in Paris, France (Bourrelly 1951)	Deposited 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)	Deposited as *Cd. meslinii*, authentic strain of *Cd. Chlorostellata*
P. asymmetrica	SAG 19.88	Seedling bed at Mira, Portugal *	Deposited as *Cd. meslinii*
P. trianguloculus	NIES-4317	A fallow paddy field, Yamagata, Japan (38°17' 41''N, 140°22'24'' E)	Formerly deposited as strain TKAC 3013
	NIES-4318	Ikenotaira marsh, Nagano, Japan (36°24'30.6"N 138°26'04.9"E)	

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

Table 1.2. Species, strains, and genes used in the phylogenetic analyses. The sequences of accession numbers for *Paludistella* 18S *r*DNA include the ITS sequences.

Genus/clade Species (Strain)	Accession number 18S *r*RNA (Strain)	 *atp*B (Strain)	 *psa*B (Strain)
Paludistella			
Paludistella chlorostellata (SAG 12.72)	MK696129	MK650458	MK650453
Paludistella asymmetrica (SAG 19.88)	MK696130	MK650459	MK650454
Paludistella meslinii (SAG 75.81)	MK696131	MK650460	MK650455
Paludistella trianguloculus (NIES-4318)	MK696132	MK650461	MK650456
Paludistella trianguloculus (NIES-4317)	MK696133	MK650462	MK650457
CR clade (*Chloromonas* s.s.)			
Chloromonas chlorococcoides (SAG 15.82)	AJ410449	AB624580	AB624595
Chloromonas reticulata (UTEX 1970)	U70791	AB084312	AB084346–7
Chloromonas rosae (SAG 51.72 = UTEX 1337)	AB624565 (SAG 51.72)	AB084315 (UTEX 1337)	AB084350–1 (UTEX 1337)
Chloromonas typhlos (SAG 26.86 = UTEX 1969)	AB624566 (SAG 26.86)	AB084307 (UTEX 1969)	AB084341 (UTEX 1969)
Gloeomonas			
Gloeomonas rubrifilum (SAG 3.85)	AJ410455	AB504758	AB504770

Gloeomonas tubulosa (ACOI-1541)	AB971360	LC437937 (unpublished)	LC437941 (unpublished)
Gloeomonas sp. (TkS1601B4 [= NIES-4283])	LC437935 (unpublished)	LC437936 (unpublished)	LC437940 (unpublished)
Ixipapillifera			
Ixipapillifera deasonii (SAG 46.72)	AJ410446	AB101503	AB101514
Ixipapillifera pauromitos (EbCl-16 = NIES-3707)	LC057290 (EbCl-16)	LC360487 (NIES-3707)	LC360491 (NIES-3707)
CA clade			
Chloromonas asteroidea (SAG 11-47)	U70783	AB084808	AB084342
Chloromonas radiata (UTEX 966)	U57697	AB084311	AB084345
SA clade			
Chloromonas chenangoensis (UTEX SNO150)	AB906341	AB906360	AB906371
Chloromonas krienitzii (NIES-3753)	LC012712	LC012720	LC012728
Chloromonas miwae (NIES-2379)	AB906350	AB906369	AB906380
Chloromonas tughillensis (UTEX SNO91)	AB906348	AB906367	AB906378
Chloromonas nivalis (UTEX SNO71)	LC360465	LC360484	LC360488
Other *Chloromonadinia* species			
Chloromonas augustae (SAG 5.73)	AJ410452	AB504757	AB504769
Chloromonas kasaiae (EbCl-8 [= NBRC 109389 = NIES-2862])	AB734109	AB734110	AB734111
Chloromonas pseudoplatyrhyncha (HjCl-3 [= NIES-2563])	AB548689	AB548690	AB548691
Chloromonas serbinowii (SAG 11.84)	Unpublished	AB084317	AB084354
Chloromonas vernalis (ACOI-3241)	AB971361	LC437939 (unpublished)	LC437943 (unpublished)
Outgroup			
Chlamydomonas reinhardtii (CC-620, CC-503)	JX888471 (CC-620)	FJ423446 (CC-503)	FJ423446 (CC-503)
Chlorogonium euchlorum (CCAP 12/3)	AB278604	AB084328	AB084369
Oogamochlamys gigantea (SAG 44.91)	AJ410465	KT625412	KT625412

Table 1.3. Morphological comparison among *Paludistella* species.

Species	*P. meslinii*	*P. chlorostellata*	*P. asymmetrica*	*P. trianguloculus*	
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) Width (μm)	 13–21 6–15	 12–21 6–15	 13–20 8–18	 10–19 6–15	 11–19 5–13
Stigma form	Filiform	Oblong	Oblong to small elliptical	Triangular to small elliptical	Triangular to small elliptical
Akinete	Unknown	Unknown	Present	Unknown	Unknown

CHAPTER 2: Biomass study of *Paludistella trianguloculus* NIES-4318

2.1. Introduction

Research of soil algae began in the 19th century resulting in the discovery of some important algae such as *Chlorella* (Beijerinck, 1893), C*hlamydomonas* (Ehrenberg, 1833), *Coccomyxa*, and *Scenedesmus*. These findings lead investigation of other soil algae including from wetland to be utilized for further application.

The current situation has shown that algae are potentially commercially valuable owing to their bioproducts accumulation derived from biomass which consists mainly of primary metabolites such as carbohydrate, lipid or oil, and protein. Moreover, they have been established as commercial sources of high-value chemicals such as β-carotene, astaxanthin, DHA, EPA, pigments (Borowitzka, 2013), extracellular metabolites for drugs and natural antioxidants (Liu *et al*., 2016), and so on. In addition, the current issue of renewable energy and food security brings out the algae as an important source of oil for biofuel (Chisti, 2007; Scott *et al*., 2010; Yoshida *et al*., 2012; Khan *et al*., 2017) and edible oil (Huang *et al*., 2016).

Algal oil accumulation is derived from lipid production expressed by the percentage of lipid yield per dry biomass (Wang et al., 2009). In this situation, many cellular components such as starch, proteins, pigments and cell walls contribute to dry weight and changeable during the fluctuation in growth condition. A wide range study of lipid production and induction has been performed related to algal biomass utilization (Sharma *et al*., 2012). However, in many cases, algal biomass production and lipid biosynthesis compete under optimal growth conditions (Tan & Lee, 2016). Therefore, high lipid productivity of fast-growing algae is a focus for species selection.

Despite not superior in oil productivity, the green algae widely used as a source of biomass and oil production because they are distributed in various habitats, easily to be isolated, and relatively fast-growing in comparison with other taxonomic groups under laboratory conditions (Hu *et al*., 2008). In this study, my attention was paid to a green algae, *P. trianguloculus* NIES-4318 collected from the soil of Japanese wetland, having developed oil droplets shown in Fig. 10D. Considering the origin of habitat for *P. trianguloculus* NIES-4318, the growth performance by using *Sphagnum* peat soil extract will be evaluated and compared

with a conventional medium for microalgae, AF-6 medium. The finding of new nutrition source makes possible to overcome the limitation on algal lipid production.

As valuable lipid, algal oil is eagerly produced mainly by nitrogen depletion conditions (Breuer *et al*., 2012; Perez-Garcia & Bashan, 2015). In addition, acidity also contributes to stimulating lipid biosynthesis because many enzymes are highly pH-dependent (Gerloff-Elias *et al*., 2005). The NIES-4318 strain expected to grow well under neutral and acidic conditions of AF-6 medium and accumulated biomass in certain cultivation periods. In this study, a moderate acidic condition (pH 4.0) was applied for both AF-6 and peat soil extract media due to the liquid medium of *Sphagnum* soil extract detected in this pH value. The AF-6 medium at pH 6.6 was used as a control medium.

The peat soil extract contains decomposed plant material such as polysaccharides, fatty acids, amino acids, phenolic compounds, vitamins, and various trace elements (Verhoeven & Liefveld, 1997; Baas *et al*., 2000; Orru *et al*., 2011; Naumova *et al*., 2013). Distribution of peat in the world has shown the availability of this resource to be used for applications in agreement with responsible peatland management. Water from peat extract consists of dissolved organic materials mainly humic and fulvic acid. Both substances are known to have an important role in crop productivities by enhancing the nutrients uptake, increasing stress tolerance, and so on (Jardin, 2015; Canellas *et al*., 2015). However, there is a poor report about the utilization of all these nutrients source for algal biomass production. The substances are expected to give a positive impact on algal biomass production. This research is aimed to evaluate the utilization of peat-extract water for biomass and oil production of novel algae under acidic culture conditions.

2.2. Methods

2.2.1. Algal media determination

Algae were cultivated by using two kinds of media, the artificial freshwater media (AF-6) and peat water media extracted from peat soil containing *Sphagnum* biomass from Estonia. In this study, the medium which contains the peat water from Estonia was named as Estonia peat water (EsPW). Saturated EsPW was obtained by addition of 1 L destilated water into 20 g peat soil. Two times autoclave was carried out for sterilization of peat water medium.

AF-6 media at pH 4.0 and pH 6.6 were used during experimental periods, derived from the standard recipe as provided in table 2.1. The acidity was adjusted by 1M HCl and 1M NaOH to obtain AF-6 media at pH 4.0 and pH 6.6, respectively.

2.2.2. Culture condition

The experiments were carried out in 500 ml Erlenmeyer flasks containing 200 ml culture kept under continuous light (100–150 µmol m^{-2} s^{-1}) and temperature at 25˚C. The initial culture was prepared and used as a seed for experiments after it reached the exponential growth phase. *Paludistella trianguloculus* NIES-4318 was grown under three different media treatments AF-6 at pH 4.0, AF-6 at pH 6.6, and EsPW media. These experiments were run in triplicate. The flask was placed in a Double-Shaker NR-150 incubator (Taitec) with speed at 121 m^{-1}.

2.2.3. Growth measurement

Daily measurement of algal growth was carried out using a multiplate reader SH-9000 Lab (Corona Electric Co., Ltd.) at 660 nm of wave-length. Approximatelly 1 ml culture of each treatment was used and measured by using IWAKI polystyrene microplate 24 well (Asahi Glass co., Ltd., Japan).

2.2.4. Biomass measurement

Biomass was collected every 7 days into a 15 ml flask. The dried biomass was obtained from 10 ml culture filtered by glass microfiber filters Whatman GF/C diameter 47 mm (GE Healthcare Life Science, UK) and dried with oven, temperature 60^0C for 24 hours. Biomass measured gravimetrically and calculated as gram per liter algal media.

2.2.5. Lipid production and visualization

Lipid production measurement was carried out by Blight and Dyer method and calculated as percentage of dry lipid (gram) per dry biomass (gram).

The fluorescence microscopy observation of lipid droplet was conducted using an Olympus IX71 equipped by Olympus U-RFL-T lamp. Samples were initially stained using Nile-Red.

2.2.6. Fatty acid esterification and determination

The crude oil samples were diluted in 1 ml chloroform, 5 mg was used for analysis. The 200 µl internal standard metil tricosanoic acid (C 23:0) 1mg/ ml hexane then added into the samples by using microsyringe and dried. 0.5 ml of NaOH 0.5 M (in methanol) was added into samples and incubated for 10 minutes under temperature 100°C. Then, 0.7 ml BF3 0.5 M were added and incubated for 7 minutes under temperature 100°C following by adding 2 ml hexane and 3 ml saturated NaCl respectively, and centrifuged (2500 rpm, 5 minutes). Finally, each 500µl sample was placed into GC-MS vials and 500 µl hexane was added.

For GC-MS analysis, the peak area of spectra is used by calculating the area under the peak data and determine the contribution of individual fatty acids as a percentage of total fatty acids by comparison with internal standard (Fisk *et al*., 2014). The equation as follows,

$$\%\ \text{area interest of fatty acid} = \frac{\text{area interest of fatty acid}}{\text{total area of fatty acids}} \times 100\ \%$$

2.2.7. TOC, pH & organic elemental (C, H, N only) determination

Total organic carbon was determined using TOC-L Shimatzu device. TOC value obtained from the difference between total carbon and total inorrganic carbon for each samples.The organic elemental analysis performed by CHN analyzer (PerkinElmer, USA) and the acidity of culture media was measured by pH/ion meter F-53 (Horiba, Japan). The total carbon, hidrogen and nitrogen within AF-6 media were calculated and percentaged based on the media compositions listed in table 2.1.

2.2.8. Exopolysaccharide (EPS) determination

Exopolisaccharide was separated from biomass by centrifugation (15.000g, 20 minutes) and extracted by Velea *et al*. (2011) method with some modifications. The aqueous solution containing EPS are concentrated to one fourth volume through evaporation at 60^0C. The polysaccharides inside concentrates were precipitated by cold ethanol (1:2 v/v) and incubated under 4°C overnight. The precipitated EPS then was measured gravimetrically. The EPS production was calculated as percentage (%) of EPS production compared to the dry biomass production (w/w).

2.2.9. Statistic approaches

The average and deviation standard of all data were calculated, and the significancy was analyzed by using analysis of variance (ANOVA) single factor with number of samples, n = 3.

2.3. Results

2.3.1. The growth and biomass production

The present study shown the growth comparison in *P. triangulocullus* NIES-4318 by culturing under three different media, the media compositions are listed in Table 1. Daily growth of cells was measured as optical density (OD) increased by times. The growth was measured until 15 days which was experiencing the exponential growth stage. The maximum growth rate reach on the 13^{th} day and 11^{th} day for AF-6 at pH 4.0 and EsPW treatments respectively (Fig. 2.1).

The growth and biomass production (0.71 ± 0.08 g/L) of *P. triangulocullus* NIES-4318 shown higher in normal conditions than those of an acidic one. In the acidic condition, algae in AF-6 media grew slightly faster than those in EsPW media for 15 days (Fig. 2.1). Meanwhile, the biomass continued to increase after 15 days then biomass in EsPW media (0.63 ± 0.05 g/L) slightly higher than those in AF-6 pH 4.0 media (0.6 ± 0.16 g/L) after 4 weeks (Fig. 2.2). In this situation, some metabolites such as lipid and other organic material probably produced higher by EsPW treatment than AF-6. However, based on statistical analysis the total biomass production was not different significantly (P-value = 0.541) at the end of the cultivation period (Table 2.2).

In this study, organic elemental analysis were determined as percentages (%) of total carbon, hydrogen and nitrogen (Table 2.3) by standard CNH analysis method. Carbon, hidrogen and nitrogen are essencial organic carbon to be evaluated. In this study, the composition of these three elements different between AF-6 media and EspW media.

In the culture condition of this study, AF-6 media provided 15% nitrogen and 1.1% carbon meanwhile EsPW provided less than 1% nitrogen and relatively high carbon content (39.1%) (Tabel 2.3). Normally, the required nitrogen in culture of Chlorophyceae was found to be 6.5-8.3% (Vymazal, 1995).

2.3.2. The lipid production and fatty acid profiles

The comparable conditions for lipid accumulation in *P. trianguloculus* NIES-4318 was observed in this study. The results showed that *P. trianguloculus* NIES-4318 produced lipid and after 4 weeks, lipid production by using EsPW media (43.72% ± 3.71) was higher compared to that using AF-6 pH 4.0 (33.67% ± 3.86) and AF-6 pH 6.6 (28.54 ± 6.87). This situation was supported by morphological observation using Nile-Red staining (Fig. 2.3). Based on statistical analysis, the lipid production at the end of cultivation periods using EsPW media was significantly different within all treatments (P-value 0.029) (Table 2.2). The higest lipid production estimated reach 0.31 gram/L.

This study showed that the composition of fatty acids was slightly affected by the different treatments of media. Based on GC-MS analysis, the saturated and unsaturated fatty acids were recognized in all treatments. Unsaturated fatty acids were produced higher than saturated fatty acids and dominated by linoleic acid (C 18:2n6c) in all treatments followed by oleic acid (C 18:1n9c), palmitic acid (C 16:0) and α linolenic acid (C 18:3n3c). Oleic acid (C 18:1n9c) increased significantly when the algae fed by EsPW media meanwhile mild increasement of palmitic acid and vaccenic acid (C 18:1n7c) occurred. The important fatty acids (stearic acid, linoleic acid, and α linolenic acid) were changed by EsPW treatment in comparing to that AF-6 pH 4.0 and pH 6.6 treatments. In addition, the relatively long-chain fatty acids (C 22:0 and C 24:0) were produced in very small quantities in pH 4.0 treatment (table 2.4).

Total saturated and unsaturated fatty acids were increased as result of EsPW media treatment in comparison to that with AF-6 media. Under pH 4.0, mono-unsaturated fatty acids (MUFA) increased and poly-unsaturated fatty acids (PUFA) decreased than those under pH 6.6. The highest MUFA was achieved by EsPW media treatment meanwhile the highest PUFA reached under normal condition (Table 2.4).

2.3.3. The Exopolysaccharide production

The comparable conditions for EPS in *P. triangulocullus* NIES-4318 was observed in this study. The results showed that *P. triangulocullus* NIES-4318 produced EPS started from early week and increase gradualy by time. EPS production (% w/w) by using EsPW media (22.6 ± 0.02) was higher compared to that using AF-6 at pH 4.0 (15.5 ± 0.02) and AF-6 at pH 6.6 (16 ± 0.03) at the end of experiment period. This situation is in line with total organic carbon (TOC) result (table 2.5). We measured TOC released into media at the end of cultivation periods compared to the initial value of TOC in all media (AF-6 and EsPW). The soluble total organic carbon in all media was detected and the highest TOC produced by EsPW treatment. Based on statistical analysis, the EPS production using EsPW media was significantly different with those of using AF6 media (P-value 0.006) at the end of cultivation periods.

2.3.4. Acidity changes

This experiment was able to change the acidic condition of culture compared to the initial pH. The results showed that after 28 days the pH of media getting normal nearly pH 7.0 under all treatments (Table 2.5).

2.4. Discussion

2.4.1. The growth of *Paludistella triangulocullus* NIES-4318

The growth of *Paludistella triangulocullus* NIES-4318 under normal better than those in acidic condition and by using AF-6 at pH 4.0 is slightly better than EsPW media during 15 days exponential period. However, based on statistical analysis the results showed not significantly different. Acidity influences photosynthesis, growth and nutrients assimilation (Gensemer *et*

al., 1993) and many algae have an external range of pH value for optimum growth similar to those of in microbes (Slonczewski & Foster, 2011). For example, the optimum growth rates of *Chlamydomonas acidophila* were achieved at pH 2.6-3.0 and low growth rate in pH 4.0 under high light condition indicated an extremely stressful condition for this strain (Gerloff-Elias *et al*., 2005). The snow algae *Chloromonas* be able to perform an optimum growth at pH 5.0 (Hoham & Mohn 1985). Decrease growth and photosynthesis at a certain pH tend to be caused by internal acidification or alkalization meanwhile in some algae, they are able to perform constant growth rate and photosynthesis due to their capability to regulate the cytosolic pH value compatible with many protein's functions. When internal pH become too low, the unwanted proton will be prevented by exchanging extracellular K+ for intracellular protons. Many enzimes related to metabolism are able to alter their structures and activities due to pH change, exhibit the optima, minima and maxima growth. There are the physiological response systems involving the genes and proteins in the acid stress response, and including the membrane lipid composition modifications, enhancing pH homeostasis and numerous other changes. The uncharged form of organic acids may transported to intracellular and change the equilibrium of internal pH. This will drive buffering's system to maintain the intracellular condition (Slonczewski & Foster, 2011). However, the mechanism of organic acids from peat-extract water transported into NIES-4318's intracellular remains uncertain.

In this study, we expected that biomass was derived from the assimilation of carbon sources and photosynthetic during cultivation periods. Beside the phototropic mode of cultivation, the AF-6 media provided inorganic nutrients meanwhile EsPW media provided the organic acids as carbon sources for assimilation. The source of carbon including hydrogen and nitrogen in EsPW media derived from water-soluble humic substances of *Sphagnum* spp. biomass. Therefore, the algae are able to grow in the same culture condition by using different sources of carbon. This situation influenced the biochemical composition of algae during cultivation. However, the produced biomass apparently quantitatively equal (table 2.2).

The source of carbon in AF-6 media only from citric acid and fe-citrate (table 2.1). The carbon source in EsPW responsible promoted the growth of strain NIES-4318 even though the nitrogen concentration is low. Glucose and dissolved uronic acid from Estonia peat extract estimated to act as carbon sources in this study. Verhoeven & Liefveld, (1997) reported that uronic acid compound accumulated in *Sphagnum* biomass as a second major group of metabolites after phenolic compounds and compose 10-30% of the dry weight of the living

Sphagnum plant. Uronic acid is oxidized glucose and can be transported into cell based on the gradient concentration. Carbon and nitrogen were known responsible for cell growth and lipid accumulation (Prathima *et al*., 2013; Li *et al*., 2011; Yang *et al*., 2018). Uronic acid was also reported provides several essential functions for cation binding of carboxyl groups in calcification of coccoliths (Lee *et al*., 2016), for biofilm formation of *Amphora* sp. (Jin *et al*., 2017), and for negative surface charge of EPS in *Chlorella stigmatophora* (Liu *et al*., 2016).

Under the acidic condition, bicarbonate pool is absence in the water and CO_2 will become more available (Vymazal, 1995; Balkos *et al*., 2007). Therefore, some algae in the natural environment condition grow mainly in near terrestrial or actively move into CO_2 rich water (Gross, 2000). In the moderately acidic condition of this study, a few bicarbonate compounds possibly were remaining as dissolved inorganic carbon in AF-6 media for the algae growth. On the other hand, the EsPW medium provided carbon source higher than those in AF-6 but lack of nitrogen. In this situation, the carbon assimilation from photosynthesis and additional carbon source from peat water were converted into biomass production. NIES-4318 capable to grow under mixotrophic condition by utilize inorganic carbon and organic carbon source simultaneously in the presence of light. Mixotrophic mode of growth provide larger biomass and yield of valuable organic materials compared with photo-autotrophic growth, prolonged exponential growth phase, reduction of lost biomass from respiration during dark condition, and also provide environmental service by nutrient removal mechanism in biorefinery process (Perez-Garcia & Bashan, 2015). However, mixotrophic mode under acidic condition resulted a slightly low biomass production in comparison to that of photo-autotrophic growth. This probably due to the enzimes activities related to growth and biomass accumulation were influenced by low pH.

Nitrogen content is higher in AF-6 media meanwhile lack of nitrogen was detected in EsPW media (Table 2.3). Nitrogen sources in AF-6 media derived from $NaNO_3$ and NH_4NO_3. The nitrogen limitation in EsPW media leads to lipid production. The low nitrogen concentration and carbon source influenced the growth in EsPW media. This is in agreement with the previous study performed in *Nannochloropsis* sp. and *Chlorococcum* sp. (Yap *et al*., 2016), the marine diatom *Phaeodactylum tricornotum* (Yodsuwan *et al*., 2017; Tan & Lee, 2016), *Tetraselmis* sp. (Kim *et al*., 2016) and *Chlamydomonas reinhardtii* (Yang *et al*., 2015). Nitrogen is one of essential macronutrient responsible generally in macromolecular synthesis pathway (Yodsuwan *et al*., 2017), play an important role in physical property development of

cells (Yap *et al*., 2016), and the most critical nutrient affecting lipid metabolism in algae (Sharma *et al*., 2012).

Further study of biomass production of NIES-4318 is necessary to be conducted including effect of pH changes, temperature and nutrient composition changes.

2.4.2. Lipid Production of *Paludistella trianguloculus* NIES-4318

In this study, *Paludistella trianguloculus* NIES-4318 which was collected from acidic habitat accumulated a considerable amount of oil droplets within the cell in pH 4.0 of both media. Acidity and low nitrogen concentration impacted the lipid accumulation in EsPW media which was higher than those in AF-6 media. The possible reason for oil accumulation is decreasing lipid catabolism, which would have minimal impact on growth (Tan & Lee, 2016). Lipid accumulation in algae is also influenced by some stress conditions in nutrients and culture conditions. Therefore, NIES-4318 have the potential to apply for the production of oil in acidic condition. This experiment revealed an additional merit of acidic culture condition for oil production.

Typically, algal biomass production and lipid biosynthesis compete under optimal growth conditions (Tan & Lee, 2016). This study showed that the more biomass production, the less lipid accumulation, and vise versa. This condition normally occurred due to algae reduce proliferation and produce storage lipids in response to environmental stress or nutrients deficiency (Tan & Lee, 2016). Metabolite engineering is needed in order to enhance lipid production without sacrificing growth.

There is a diversity of fatty acid composition among microalgae (Kasai *et al*., 2005). Some fatty acid usually recognized in green algae and utilized as biomarkers such as palmitic acid, oleic acid and linoleic acid (Sahu *et al*., 2013). Microalgae mainly produced fatty acids C16 and C18 in carbon chain length, but some species can make up to C24 (Breuer *et al*., 2013; Lang *et al*., 2011). In this study, NIES-4318 produced fatty acid C24 only in acidic condition, meanwhile fatty acid C22 produced only under EsPW treatment. A 16- and 18-carbon fatty acids synthesized in algal chloroplast and produced as the precursor for chloroplast, cellular membrane, and neutral storage-lipid synthesis (Hu *et al*., 2008). Palmitic acid, oleic acid, and linoleic acid are commonly detected in green algae (Sahu *et al*., 2013). Fatty acid C18:1 increased significantly in *Scenedesmus obliquus* (Breuer *et al*., 2013) and C16:0 in

Chlamydomonas reinhardtii (Yang *et al*., 2018) when the nitrogen deprivation was applied. The major PUFA in green algae were C18:2 and C18:3 (Hu *et al*., 2008). Fatty acids C16 and C18 also suitable for biodiese.. Further study is necessary to be conducted to enhance the lipid accumulation for biodiesel production.

The same algae may be a source of different fatty acids, depending on the nutritive composition of culture media (Darki *et al*., 2017). Related with this study, total unsaturated fatty acid and MUFA produced higher by peat water (EsPW) treatment, and a polyunsaturated will be higher by culturing in AF-6 media. The enhancement of MUFA may induced by nitrogen limitation in culture (Lopez et al. 2000; Darki et al. 2017). This report showed that total MUFA was highest by EsPW media treatment, in which nitrogen concentration was lower than those of other treatments.

Nutrient compositions especially nitrogen concentration and carbon sources are the factors influenced fatty acid composition. Besides, acidity, salinity, and temperature are also able to influence the fatty acid composition. In this study, under the same pH 4.0 and different media (AF-6 and EsPW) showed different fatty acid composition. On the other hand, under same AF-6 media and different pH (4.0 and 6.6) also showed different fatty acid composition. In the natural condition, the habitats also affected the composition of fatty acid. The marine algae, snow algae, and alpine algae produce polyunsaturated fatty acids (PUFAs) for adaptation purposes.

2.4.3. Exopolysaccharide production of *Paludistella trianguloculus* NIES-4318

EPS production gradualy increased by cultivation age (Fig. 2.4). In the first week, the production reached very small and then enhanched as per biomass production. The culture under EsPW media treatment, in which the media lacked of nitrogen, showed highest EPS yield in comparing with those using AF-6 media. Culture age and media composition may influence the EPS production besides the culture condition and algae species. The stationary phase of growth caused by nitrogen depletion may also induce the EPS production (Bafana, 2013). Common composition of EPS in green algae is uronic acid besides other polysaccharides (Kumar *et al*., 2018). However, we have not analyzed the composition of EPS during performed the experiment.

EPS is also secreted by many Cyanobacteria and green algae. Katona *et al*.(2018) studied about EPS production of 20 *Chlamydomonas* strains and the highest EPS production

reached 2 g/L meanwhile Khangembam *et al.* (2016) reported *Anabaena sp*. strain produced 1,27 g/L and Trabelsi *et al*. (2013) reported for *Arthrospira platensis* reached 1.5 g/L EPS. In addition, Priatni *et al*. (2016) determined EPS from many Cyanobacteria and resulted 3-6 g/L. In this study, we calculated the EPS as per dry biomass production and the highest EPS production reached 22.6% w/w.

EPS have been produced by algae and other microbes in response to environmental condition such as protection from stress condition, biofilm formation, colony and bloom formation, symbiosis and movement (Kumar *et al*., 2018; Liu *et al*., 2018; Katona *et al*.,2018).

In application viewpoint, EPS are used in bioremediation, bioflocculant and soil aggregation and stabilization in agroindustry (Trabelsi *et al*., 2013; Bafana, 2013). In addition, current biomedical industry utilize EPS for many products such as tumor and virus inhibitors (Khangembam *et al*., 2016) and antidiabetic (Priatni *et al*., 2016).

NIES-4318 excreted for about 120-150 mg/L dissolved organic material into media. The initial value of total organic carbon (TOC) in EsPW media higher significantly rather than in AF-6 due to this media contain high organic material as a result of *Sphagnum* biomass degradation. The TOC increased at the end of cultivation periods for all treatment, proved by TOC value enhancement (ΔTOC) (table 2.2). The TOC increasement occured probably due to the production in external organic materials released by algae into media during cultivations. The slight difference of ΔTOC value was probably generated by the difference in chemical composition in media.

The total organic carbon represented the extracellular polymeric substances from microalgae that mainly consist of polysaccharides, proteins, nucleic acids, and lipids (Xiao & Zheng, 2016). The external organic carbon have the potential to be applied as a valuable byproduct of algae biomass. Such organic material is potentially applicable for the biomass production of heterotrophic organisms.

2.4.4. Acidity refinement by *Paludistella trianguloculus* NIES-4318

Paludistella trianguloculus NIES-4318 in this study was able to grow well in acidic condition and increased the pH of media nearly normal at the end of cultivation (table 2.5). The acidotolerant algae provide such mechanisms to deal with H^+ uptake in pH stress condition.

The acidotolerant species produce energy-demanding process and metabolic costs less than normal algae when they maintain a fairly constant or neutral cytosolic pH over a wide range of external pH value (Gross, 2000).

In the acidic aquatic environment, inorganic carbon (CO_2) was dissolved in the high concentration as compared to that of water with neutral pH (Gross, 2000). Typically, the algae consume CO_2 as result of photosynthesis and generated bases environment inside the cells, lead ion transport through the cell membrane. Extracellular uptake of H+ ions were performed to balance the ion gradient. This condition has caused increasement of pH value in culture media. The reduction of dissolved organic acid in media as nutrients for algae also be able to contribute the increasement of pH value. Therefore, the acidic aquatic environment refine to be a neutral condition.

There are some reports of potential value derived from the acidotolerant algae such as for heavy metal removal and biodiesel production (Abinandan *et al*., 2019), wastewater treatment and bioremediation (Varshney *et al*., 2015). The other potential application is addressed to the algricultural field in which the soil acidity is a problem on some crops. Since pH 4.0 within a range pH for soil in forest (McCauley *et al*., 2017), the *Paludistella trianguloculus* NIES-4318 have potential to be use for aquatic rehabilitation in forest as well as acidic refinement in agronomic field, mining area and acidified aquatic environment.

2.4.5. General potential of *Paludistella trianguloculus* NIES-4318 in paludiculture

Considering the capability of NIES-4318 to be cultivated under peat water media, paludiculture will be fit in term of application viewpoint. Paludiculture defines as biomass cultivation in wet and or rewetted conditions (Biancalani & Avagyan, 2014) by applying a productive use of wet peatland without sacrificing the land conservation strategies (Wichtmann *et al*., 2017). However, current report shows that algae, which is closely related phylogenetically to the plant (Madigan et al., 2012), not registered into the data base of potential paludiculture plants (Abel *et al*., 2013). Therefore, based on cultivation experiment in this study showed the posibility of NIES-4318 as the potential object for paludiculture.

Figures

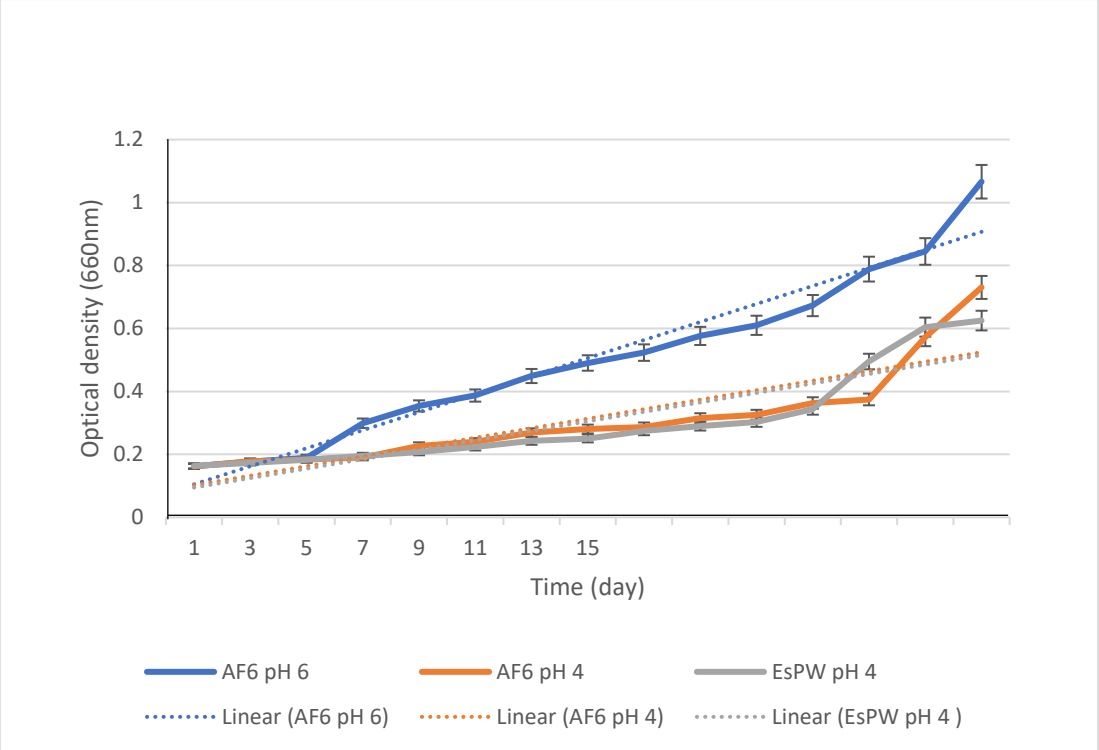


Figure 2.1. *Paludistella trianguloculus* NIES-4318 growth in cultivation experiment using three kinds of media (AF-6 pH 4.0, AF-6 pH 6.6, and EsPW media). Error bar shows standard deviation (SD). The linear line indicated by dotted line of three media used to compare the growth rate from the angle formed in initial point.

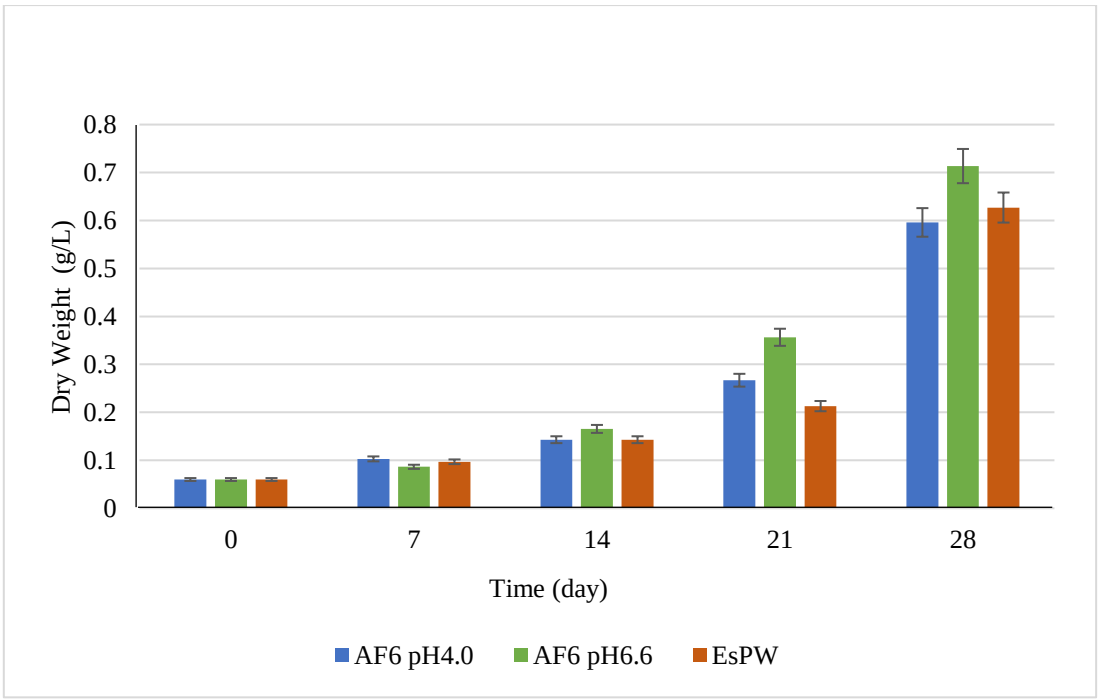


Figure 2.2. Biomass production of *Paludistella trianguloculus* NIES-4318 in cultivation experiment. Error bar shows standard deviation (SD).

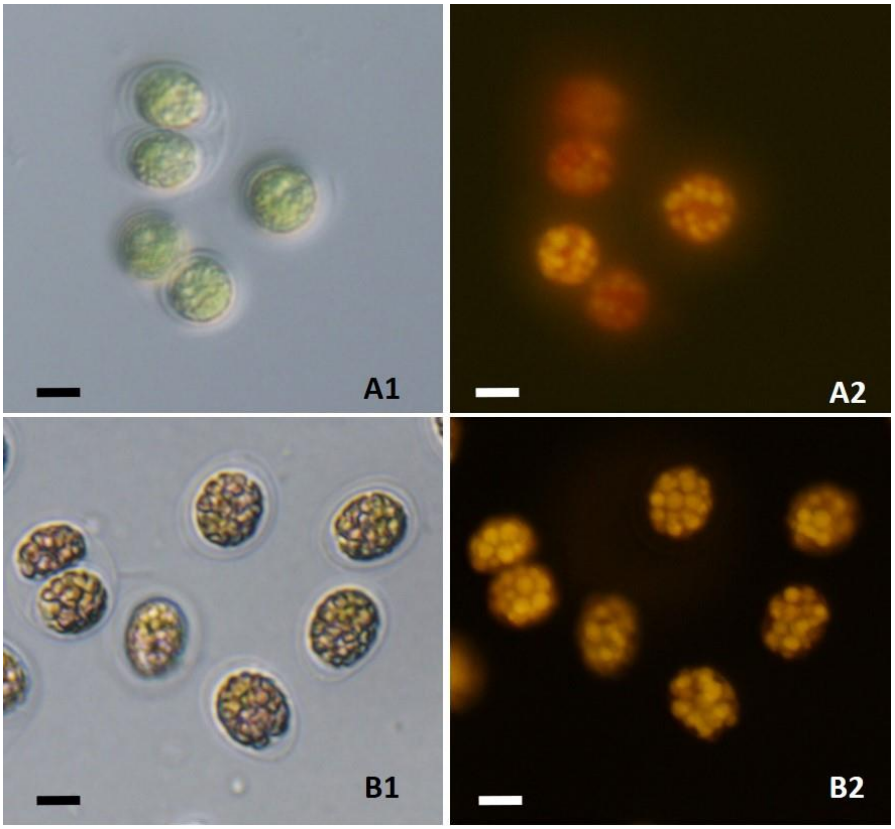


Figure 2.3. Oil droplets visualization by Nile Red staining at the end of cultivation experiment using AF-6 pH4 (A1-A2) and EsPW media (B1-B2)

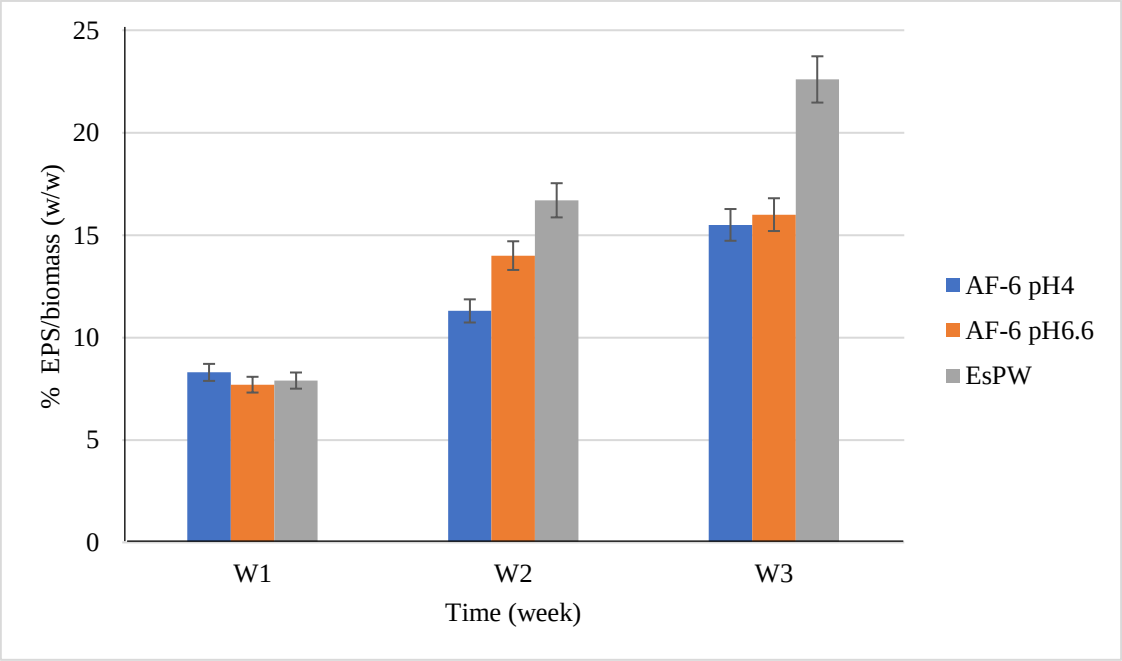


Figure 2.4. Exopolysaccharide (EPS) production of *Paludistella trianguloculus* NIES-4318 in cultivation experiment. Error bar shows standard deviation (SD).

Tables

Table 2.1. AF-6 media composition (Kato 1982; reproduced from NIES culture collection book)

$NaNO_3$	14 mg
NH_4NO_3	2.2 mg
$MgSO_4.7H_2O$	3 mg
KH_2PO_4	1 mg
K_2HPO_4	0.5 mg
$CaCl_2.2H_2O$	1 mg
$CaCO_3$	1 mg
Fe-citrate	0.2 mg
Citric acid	0.2 mg
Biotin	0.2 µl
Thiamin HCl	1 µl
Vitamin B6	0.1 µl
Vitamin B12	0.1 µl
Traces metals*)	0.5 ml
Distilled water	99.5 ml
MES	40 mg

*) Trace metals

$Na_2EDTA.\ 2H_2O$	100 mg
$FeCl_3.\ 6H_2O$	19.6 mg
$MnCl_2.\ 4H_2O$	3.6 mg
$ZnCl_2$ **)	1.04 mg
$CoCl_2.\ 6H_2O$	0.4 mg
$Na_2MoO_4.\ 2H_2O$	0.25 mg
Distilled water	100 ml

**) In this experiment 1.04 mg $ZnCl_2$ is replaced by 2.2 mg $ZnSO_4.\ 7H_2O$

Table 2.2. Summary of growth performance of NIES-4318 with statistical analysis

Parameter	AF-6 pH4.0	AF-6 pH6.6	EsPW	Anova P-value*
Biomass (g/L)	0.6 ± 0.16	0.71± 0.08	0.63 ± 0.05	0.541
Lipid (%)	33.67 ± 3.86	28.54 ± 6.87	43.72 ± 3.71	0.029

* ≤ 0.05 is considered significantly different.

Table 2.3. Comparison of AF-6 media and Estonia peat water composition

	AF-6	EsPW
Carbon (%)	1.1	39.1
Hydrogen (%)	1.5	3.7
Nitrogen (%)	15.0	0.9

Table 2.4. Fatty acid profile in the end of cultivation experiment using AF-6 and EsPW media. nd=not detected.

	%Area		
Fatty Acid	**AF6 pH4.0**	**AF6 pH6.6**	**EsPWB**
C 16:0 (palmitic acid)	17.3 ± 0.5	15.8 ± 0.5	18.6 ± 0.1
C 16:2n4	3.0 ± 0.1	6.9 ± 0.4	1.5 ± 0.1
C 18:0 (stearic acid)	1.1 ± 0.0		0.85 ± 0.0
C 18:1n9c (oleic acid)	12.67 ± 0.8	10.5 ± 0.06	23 ± 0.2
C 18:1n7c	9.9 ± 0.3	9.1 ± 0.3	11.1 ± 0.3
C 18:2n6c (linoleic acid)	24.87 ± 0.5	29 ± 0.8	22.1 ± 0.3
C 18:3n3c (α linolenic acid)	12.0 ± 0.5	10.7 ± 0.02	9.57 ± 0.2
C 22:0	nd	nd	0.9 ± 0.0
C 24:0	0.65 ± 0.0	nd	0.86 ± 0.0
undetected fatty acid	18.7	17.9	12.43
saturated fatty acid	18.83 ± 0.5	15.8 ± 0.5	20.3 ± 0.2
unsaturated fatty acid	62.47 ± 0.2	66.3 ± 1	67.27 ± 0.2
MUFA	22.6 ± 1.1	19.6 ± 0.2	34.1 ± 0.4
PUFA	39.87 ± 1.1	46.7 ± 1.1	33.17 ± 0.5

Table 2.5. Acidity and total organic carbon enhancement during cultivation period

	AF-6 pH4.0		**AF-6 pH6.6**		**EsPW**	
	before	**after**	**before**	**after**	**before**	**after**
pH	4	6.74 ± 1.1	6.6	7.52 ± 0.01	4.05 ± 0.2	6.99 ± 0.7
TOC (mg/L)	5.59 ± 1.93	136.22 ± 12.51	78.71	198.734	201.1 ± 9.5	346.74 ± 6.45
Δ TOC	130.63		120.024		145.64	

General Discussion

In this study, *Chlamydomonas*-like green alga, NIES-4318, was isolated from the acidic wetland in Nagano prefecture, Japan. This strain grew well in acidic condition and produced a considerable amount of oil. The 18S *r*DNA sequence of this strain showed a high similarity to those of strains NIES-4317, SAG 75.81, SAG 12.72 and SAG 19.88. The taxonomical study of these strains was performed using a polyphasic approach including light microscopy, molecular phylogenies and the analysis of internal transcribed spacer 2 (ITS-2) secondary structures.

In the light microscopy, NIES-4318 and four related strains possessed similar morphological features such as the hemispherical to conical papilla and asteroid chloroplast with a central pyrenoid but showed some differences such as in the shape of stigma (eyespot). In the multigene molecular phylogenetic analysis based on 18S *r*DNA, *atp*B and *psa*B, these strains formed a robust subclade in the clade *Chloromonadinia*, which is one of the clades recognized in the Volvocales (Chlorophyceae). In the clade *Chloromonadinia*, the type species of known genera (e.g. *Chloromonas*) were not closely related to the subclade including NIES-4318. In addition, the combination of the hemispherical to conical papilla and asteroid chloroplast with a central pyrenoid was not found in the other members of the clade *Chloromonadinia.* Therefore, a new genus, *Paludistella* gen. nov., was proposed for the subclade including NIES-4318. Within the clade *Paludistella*, SAG 75.81 was situated at the base and two subclades, composed of NIES-4317 and NIES-4318, and SAG 12.71 and SAG 19.88, were recognized. The predicted secondary structures of ITS-2 possessed four helices and most of these helices were comparable to each other, except for the helix III of SAG 75.81 that was longer and consist of three subhelices. In the comparative region of ITS-2, one to twelve compensatory base changes (CBCs; used widely to detect the species delimitation) were found among strains except for the pair of NIES-4317 and NIES-4318, which possessed three hemi-CBCs but no CBCs. Based on this polyphasic approach, the five strains as *P. meslinii* comb. nov. (SAG 75.81), *P. chlorostelata* comb. nov. (SAG 12.72), *P. asymmetrica* sp. nov. (SAG 19.88) and *P. trianguloculus* sp. nov. (NIES-4317 and NIES-4318) were classified. Interestingly, all species of *Paludistella*, except for *P. meslinii*, grew well in pH 4.0 and accumulate a considerable amount of oil.

The preliminary experiment reported that *Paludistella trianguloculus* NIES-4318 grew well in the AF-6 medium at pH 4.0 as well as pH 6.6. Due to the strain was originally isolated from acidic peat swamp in a Japanese wetland, therefore we use the media produced from peat material to study for the biomass production and application in a wetland. The chemical composition of peat water is unique, including unusual chemicals such as phenolics. In this study, *P. trianguloculus* NIES-4318 was cultivated under pH 4.0 using Estonia peat soil extract water (EsPW) compared with AF-6, a common medium for freshwater microalgae, to know the biomass production of this alga.

EsPW medium contained a smaller amount of nitrogen but larger amount of carbon in comparison to AF-6 medium. *P. trianguloculus* NIES-4318 grew wall in both AF-6 and EsPW media. Algal biomass production at the end of cultivation periods showed no significantly different between AF-6 and EsPW media. On the other hand, the oil production using the EsPW medium was statistically higher than that using AF-6 medium. In addition, the composition of fatty acid was different in AF-6 and EsPW media; larger oleic acid and smaller linoleic acid were detected in the EsPW medium. These differences are probably generated by the difference of chemical composition of media. Because the starvation of nitrogen is widely known to enhance lipid production in various algae, the production of larger amount of oil in the EsPW medium may be caused by the small amount of nitrogen. In addition, acidic culture condition in this study contributed to enhance the lipid production in comparison with normal culture condition. Therefore, there is an additional merit of algal cultivation under acidic condition for oil production.

At the end of cultivation, pH of both AF-6 and EsPW media increased to neutral (6.74–6.99). The increase of pH through the cultivation may be useful to the application of water in wetland. EPS production reached 15.5–22.6% w/w, and 130–145 mg/Liter of the soluble total organic carbon in the media are produced. The increase of soluble total organic carbon in media was probably due to the production of external organic materials released by algae into media during cultivations. Such organic material has the potential to use applications such as the cultivation of heterotrophic organisms for biomass production.

This study brings us to the discovery of a new genus, *Paludistella* gen. nov., including the new species, *P. trianguloculus* living in the acidic wetland. The cultivation experiments showed the merits of this strain for oil production, external organic material production as byproduct and acidity refinement in aquatic environment. This experiment also revealed the

benefit of peat water substances on biomass production and applicable for paludiculture system, a biomass cultivation in wet and rewetted condition. Further research to enhance algal biomass productivity is necessary to be conducted as well as research to increase algal oil productivity.

Conclusion

Study of *Paludistella trianguloculus* NIES-4318 involve taxonomical study of this strain with the relatives, and biomass study. From the taxonomical study, we propose new genera *Paludistella* gen. nov. and two novel species including *Paludistella trianguloculus* NIES-4318. The taxonomical study was performed 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 these oil-rich algae, the biomass study was performed. The results showed the merits of this strain for oil production, external organic material production as byproduct, and acidity refinement in acidic environment. Such organic material is applicable in cultivation of heterotrophic organism for biomass production. Further research to enhance the biomass productivity in this strain is necessary to be conducted as important as research to increase algal oil productivity. Based on the cultivation experiment, we concluded that the substances of peat-water extract have a positive impact on algal biomass production and applicable for paludiculture.

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