

Taxonomic re-examination of two NIES strains of “*Chlamydomonas*” within the *Reticulata* group of the genus *Chloromonas* (Volvocales, Chlorophyceae)

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Two strains, NIES-2215 and NIES-2243, have been labeled “*Chlamydomonas* (*Cd.*) *gerloffii*” and “*Cd. subangulosa*,” respectively. Although these two strains were previously suggested to belong to the *Reticulata* group of the genus *Chloromonas* (*Cr.*), detailed taxonomic examinations are lacking for both strains. Here we taxonomically re-examined the two strains based on light and transmission electron microscopy and molecular phylogenetic analysis using a combined dataset from 18S and 28S ribosomal RNA gene sequences and genes encoding ATP synthase β -subunit, P700 chlorophyll a-apoprotein A1 and P700 chlorophyll a-apoprotein A2. The “*Cd. gerloffii*” strain NIES-2215 differed from all other species of the *Reticulata* group in terms of phylogeny and ultrastructure of the pyrenoid and eyespot; it was morphologically identified as *Cd. gerloffii*. Thus, we propose *Cr. difformis* Tomo. Makino, Matsuzaki & Nozaki *nom. nov.*, to encompass *Cd. gerloffii*, because of presence of the previous valid description of *Cr. gerloffii* for a different species. The “*Cd. subangulosa*” strain NIES-2243 was significantly different in morphology from the original description of *Cd. subangulosa*. It was identified as *Cr. typhlos* because NIES-2243 and *Cr. typhlos* strain SAG 26.86 formed a small robust clade with essentially the same morphological characteristics except for the presence or absence of an eyespot in the chloroplast.

Key words: *Chlamydomonas gerloffii*, *Chlamydomonas subangulosa*, *Chloromonas difformis nom. nov.*, *Chloromonas typhlos*, molecular phylogeny, morphology, taxonomy

INTRODUCTION

Chloromonas (*Cr.*) is a genus of unicellular green algae with the type species *Cr. reticulata* (Goroschankin) Gobi (Pröschold *et al.*, 2001). This genus was previously distinguished from *Chlamydomonas* (*Cd.*) by the absence of pyrenoids in the chloroplast (e.g. Ettl, 1983). Based on phylogenetic analysis of 18S ribosomal RNA (*rRNA*) genes and light microscopy, Pröschold *et al.*, (2001) classified a clade composed of five pyrenoid-containing “*Chlamydomonas*” strains and two pyrenoid-lacking “*Chloromonas*” strains as a single species, *Cr. reticulata*. Later, Matsuzaki *et al.* (2012) re-examined nine strains of “*Cr. reticulata*” *sensu* Pröschold *et al.*

(2001), based on light and electron microscopy and phylogenetic analysis of multiple DNA regions. They found enough morphological and genetic differences among the “*Cr. reticulata*” strains to divide them into four species: *Cr. reticulata*; *Cr. rosae* (H. & O. Ettl) H. Ettl; *Cr. chlorococcoides* (H. Ettl & K. Schwartz) Matsuzaki, Y. Hara & Nozaki; and *Cr. typhlos* (Gerloff) Matsuzaki, Y. Hara & Nozaki. They further classified these species as the “*Reticulata* group.”

Subsequently, based on comprehensive analysis of 18S *rRNA* gene sequences, Yumoto *et al.* (2013) suggested that the *Reticulata* group includes the following three additional “*Chlamydomonas*” strains deposited in the Microbial Culture Collection at the National Institute for Environmental Studies (MCC-NIES) (Kawachi *et al.*, 2013; http://mcc.nies.go.jp/index_en.html): *Cd. gerloffii* H. Ettl strain NIES-2215, *Cd. subangulosa* F.E. Fritsch & R.P. John strain

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NIES-2243, and *Cd. subtilis* Pringsheim strain NIES-2244. Although *Cd. subtilis* strain NIES-2244 was resolved to be a mislabeled strain of *Cd. gerloffii* (our unpublished data), the other two strains are identical to those listed in the Culture Collection of Algae and Protozoa (CCAP; <https://www.ccap.ac.uk>) as authentic strains of *Cd. gerloffii* and *Cd. subangulosa* (George 1976; Kawachi *et al.*, 2013). However, these two strains have not been evaluated by electron microscopy or other phylogenetic methods except in a recent study by Barcytė *et al.* (2018a), who examined the secondary structure of the internal transcribed spacer region-2 of nuclear *rDNA* (nuclear *rDNA* ITS2) in *Cd. gerloffii* strain CCAP 11/72 (=NIES-2215; Kawachi *et al.*, 2013).

The present study was undertaken to taxonomically re-examine these two strains of “*Chlamydomonas*” based on light and electron microscopy and phylogenetic analysis of five DNA regions. The results indicated that *Cd. gerloffii* should be reclassified into the genus *Chloromonas* whereas *Cd. subangulosa* strain NIES-2243 should be classified as *Cr. typhlos*. The morphology, phylogeny and taxonomy of *Cr. difformis* Tomo. Makino, Matsuzaki & Nozaki *nom. nov.* and *Cr. typhlos* are described in this report.

MATERIALS AND METHODS

Cultures

Nine strains of four species in the *Reticulata* group (Matsuzaki *et al.*, 2012) were obtained from the Sammlung von Algenkulturen at the University of Göttingen (SAG; <https://www.uni-goettingen.de/en/www.uni-goettingen.de/de/184982.html>): *Cr. reticulata* strains SAG 29.83, SAG 32.86 and SAG 26.90; *Cr. rosae* strain SAG 51.72; *Cr. chlorococcoides* strains SAG 15.82, SAG 16.82, SAG 12.96 and SAG 72.81; and *Cr. typhlos* strain SAG 26.86. “*Cd. gerloffii*” strain NIES-2215 and “*Cd. subangulosa*” strain NIES-2243 were provided by MCC-NIES. The original strain of “*Cd. subangulosa*” (CCAP 11/28) was directly sent from CCAP. The cultures were grown in screw-cap tubes (18×150 mm) containing approximately 10 ml AF-6 medium (Kato 1982; Kawachi *et al.*, 2013) at 20°C under 110–150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light from fluorescent lamps under a 14 h light: 10 h dark photoperiod.

Light microscopy

Light microscopy was performed using BX-53 and

BX-60 microscopes (Olympus, Tokyo, Japan) equipped with Nomarski differential interference optics. Vegetative cells in 6- to 9-day-old cultures were observed during the light photoperiod. Cell divisions were observed during the light or dark photoperiod.

Transmission electron microscopy

Six- to 9-day-old cultures were fixed during the light photoperiod for observation by transmission electron microscopy (TEM). Fixation and observation were performed as described by Matsuzaki *et al.*, (2012).

Gene sequencing

New sequences were determined by direct sequencing of polymerase chain reaction products as described previously (Nozaki *et al.*, 2000, 2002; Matsuzaki *et al.*, 2012, 2014) for genes encoding ATP synthase β -subunit (*atpB*), P700 chlorophyll a-apoprotein A1 (*psaA*) and P700 chlorophyll a-apoprotein A2 (*psaB*) in “*Cd. gerloffii*” strain NIES-2215 and “*Cd. subangulosa*” strain NIES-2243, and for 28S *rRNA* sequences in two outgroup species (Table 1) except for *psaB* gene of NIES-2215. The “*Cd. gerloffii*” strain NIES-2215 *psaB* gene was sequenced by using cDNA that was constructed using RNA (see below) with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Sequences of nuclear *rDNA* ITS2 of NIES-2244 and CCAP 11/28 were determined as described previously (Matsuzaki *et al.*, 2012).

For next-generation sequencing, RNA was extracted during the light photoperiod based on the methods of Featherston *et al.* (2018), and cDNA library was constructed using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). cDNA was sequenced using the MiSeq platform (Illumina, San Diego, CA, USA) with the 500-cycle MiSeq Reagent Kit v2 to construct a *de novo* assembled database via Trinity (Grabherr *et al.*, 2011). For 11 strains of ingroup taxa (Table 1), 28S *rRNA* sequences were extracted from the database.

Phylogenetic analysis

The 14 strains analyzed here (Table 1) were composed of 11 strains belonging to *Reticulata* group and three species served as the outgroup. The outgroup strains were selected following Matsuzaki *et*

Table 1 List of taxa/strains included in the phylogenetic analyses of *Reticulata* group (Fig. 1) and DDBJ/EMBL/GenBank accession numbers of 18S *r*RNA, 28S *r*RNA, *atpB*, *psaA* and *psaB* sequences

Taxa	Strain	Accession Number				
		18S <i>r</i> RNA	28S <i>r</i> RNA	<i>atpB</i>	<i>psaA</i>	<i>psaB</i>
Ingroup						
<i>Cr. chlorococcoides</i>	SAG 12.96	AJ410451, AB624556	LC438810 [†]	AB624581	AB624584	AB624596
	SAG 15.82	AJ410449, AB624555	LC438801 [†] , AB906359	AB624580	AB624583	AB624595
	SAG 16.82	AJ410450, AB624557	LC438809 [†]	AB624582	AB624585	AB624597
	SAG 72.81	U70785, AB624558	LC438806 [†] , DQ015725	AB084309	AB624586	AB084343
<i>Cr. reticulata</i>	SAG 26.90	AF517090, AB624563	LC438803 [†]	AB084316	AB624589	AB084352-3
	SAG 29.83 (=UTEX 1970)	U70791, AB624560	LC438808 [†] , AF395508	AB084312	AB624587	AB084346-7
	SAG 32.86	AF517091, AB624561	LC438805 [†]	AB084314	AB624588	AB084349
<i>Cr. rosae</i>	SAG 51.72 (=UTEX 1337)	AB624565	LC438804 [†] , KC196731	AB084315	AB624590	AB084350-1
<i>Cr. typhlos</i>	SAG 26.86 (=UTEX 1969)	AB624566	LC438802 [†] , LC360479, AF395497	AB084307	AB624591	AB084341
<i>Cr. difformis</i>	NIES-2215	AB701536	LC438811 [†]	LC438815 [†]	LC438818 [†]	LC438814 [†]
<i>Cr. typhlos</i>	NIES-2243	AB701538	LC438807 [†]	LC438816 [†]	LC438817 [†]	LC438813 [†]
Outgroup						
<i>Cr. augustae</i>	SAG 5.73	AJ410452	LC360474.1	AB504757	AB624592	AB504769
<i>Cr. serbinowii</i>	UTEX 492	U70795, AB624568	LC360478 [†]	AB084317	AB624594	AB084354
<i>Gloeomonas anomalipyrenoides</i>	NIES-447	AB504776, AB624567	LC437681 [†]	AB084313	AB624593	AB084348

[†] Sequenced in the present study

al. (2012). The new nucleotide sequences are available in DDBJ/EMBL/GenBank (Table 1).

The 18S *r*RNA, 28S *r*RNA, *atpB*, *psaA* and *psaB* sequences were aligned using ClustalW (Thompson *et al.*, 1994). The 18S *r*RNA sequence data were found to correspond to positions 127–1626 of the 18S *r*RNA sequence of *Volvox carteri* F. Stein strain UTEX 1885 (Rausch *et al.*, 1989). The 28S *r*RNA sequence data correspond to positions 279–2095 of the 28S *r*DNA sequence of *Oryza sativa* Linnaeus (Takaiwa *et al.*, 1985). The analyzed *atpB*, *psaA* and *psaB* exons correspond to positions 199–1326, 580–1365 and 271–1155 of the *Chlorella vulgaris* Beijerinck *atpB*, *psaA* and *psaB* genes, respectively (Wakasugi *et al.*, 1997). Because the combined sequences from 18S *r*RNA, 28S *r*RNA *atpB*, *psaA*

and *psaB* were the same between SAG 15.82 and SAG 16.82, they were treated as a single operational taxonomic unit (OTU).

The combined 6,115-bp data matrix for the 18S *r*RNA, 28S *r*RNA, *atpB*, *psaA* and *psaB* sequences from the 13 OTUs (available from TreeBASE: <http://www.treebase.org/treebase-web/home.html>; study ID: S23720) was subjected to Bayesian inference (BI), maximum likelihood (ML), maximum parsimony (MP) and neighbor-joining (NJ) analyses. The substitution model for each BI partition was HKY + G for 18S *r*RNA; GTR + I + G for 28S *r*RNA; GTR + I + G for the first codon position of the plastid genes *atpB*, *psaA* and *psaB*; F81 + I + G for the second codon position of the plastid genes; or GTR + G for the third codon position of the plastid genes, select-

ed using MrModeltest 2.3 (Nylander, 2004). BI was performed using MrBayes 3.2.6 (Ronquist *et al.*, 2012), as described previously (Nakada *et al.*, 2008), with 1,000,000 generations of Markov chain Monte Carlo iterations and discarding the first 25% as burn-in. The ML analysis was conducted using PAUP* 4.0b10 (Swofford, 2002) with the GTR + I + G model selected by Modeltest 3.7 (Posada & Crandall, 1998), to estimate the bootstrap values (BVs; Felsenstein, 1985) based on 1000 heuristic search replications using the tree-bisection-reconnection (TBR) branch-swapping algorithm. Bootstrap values of the MP analysis were also obtained using PAUP* 4.0b10 based on 1000 replicates of the MP analysis and randomly adding 10 replicates from a heuristic search using the TBR branch-swapping algorithm, and NJ analysis, with ML distances deter-

mined using the appropriate substitution model.

RESULTS AND DISCUSSION

Molecular phylogenetic analysis

The BI tree constructed using the dataset composed of 18S *r*RNA, 28S *r*RNA, *atpB*, *psaA* and *psaB* gene sequences is shown in Figure 1.

The *Reticulata* group was composed of two clades. One clade contained “*Cd. subangulosa*” strain NIES-2243 and *Cr. typhlos* strain SAG 26.86, and its monophyly was strongly supported by 1.00 posterior probability in BI and 100% BV in the ML, MP and NJ analyses. The other clade was composed of four other species, supported by 51–71% BV, and was subdivided into two lineages. One lineage is a robust monophyletic group (supported by 1.00 posterior probability and 80–87% BV) composed of the

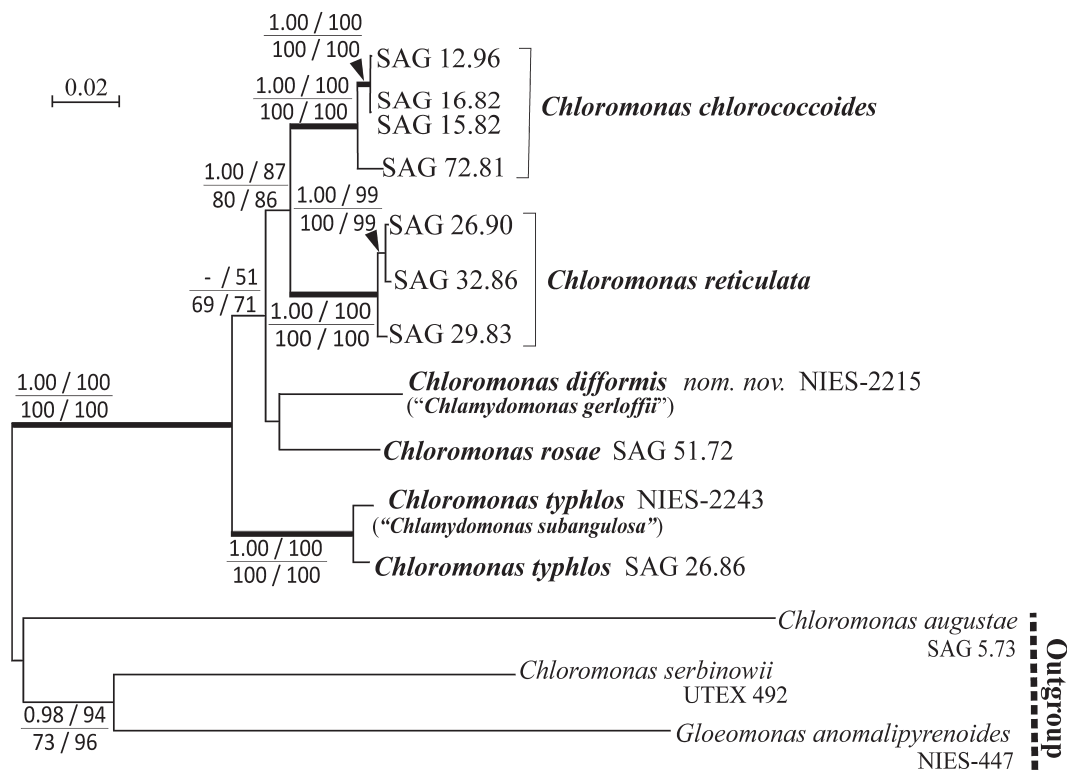


Fig. 1 Phylogenetic positions of “*Chlamydomonas (Cd.) gerloffii*” strain NIES-2215 and “*Cd. subangulosa*” strain NIES-2243 within *Reticulata* group of the genus *Chloromonas* (Table 1). The tree was constructed by Bayesian inference based on 6,115 base pairs from combined 18S and 28S *r*RNA, and *atpB*, *psaA* and *psaB* genes. Corresponding posterior probabilities (PP; 0.95 or more) are shown at top left. Numbers shown in top right, bottom left and bottom right indicate bootstrap values (50% or more) from maximum likelihood, maximum parsimony and neighbor-joining analyses respectively. Branch lengths are proportional to the evolutionary distances which are indicated by bar top left to the tree.

four *Cr. chlorococcoides* strains and the three *Cr. reticulata* strains. The other lineage was composed of “*Cd. gerloffii*” strain NIES-2215 and *Cr. rosae*, although its monophyly was only found in BI tree with low support values (<0.95 posterior probability). Thus, it was not clear which species or group is the sister group to the clade composed of *Cr. chlorococcoides* and *Cr. reticulata*. However, it is clear from the present tree that “*Cd. gerloffii*” strain NIES-2215 was not closely related to any other included species in the *Reticulata* group, and “*Cd. subangulosa*” strain NIES-2243 was sister to *Cr. typhlos* strain SAG 26.86.

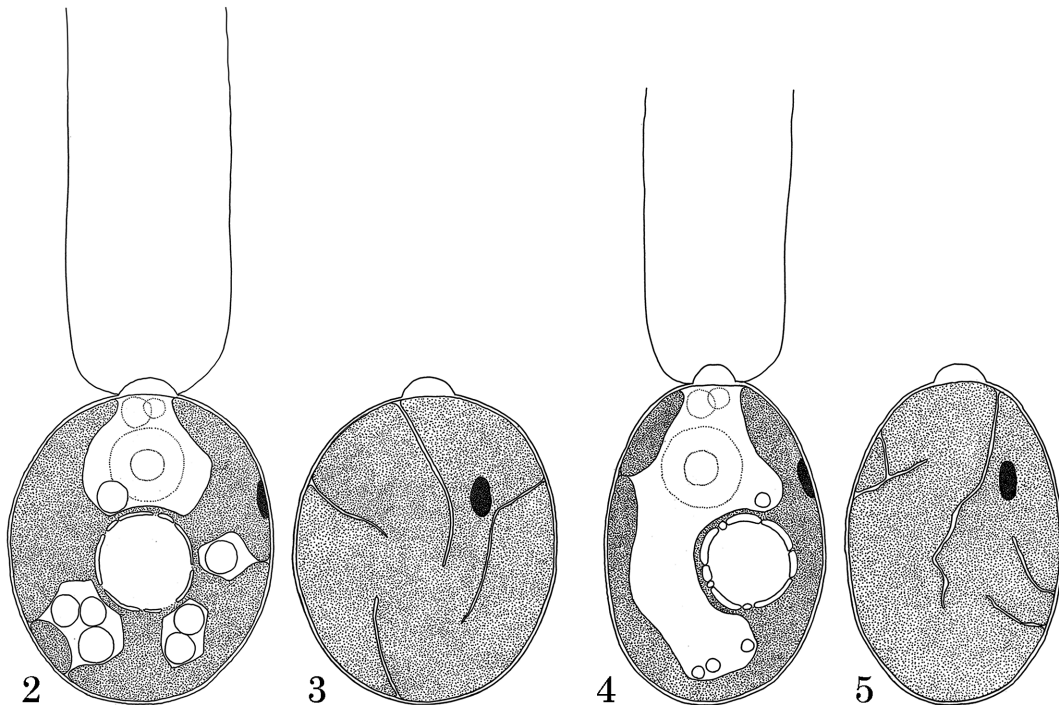
In the present phylogenetic tree, *Cr. typhlos* (including “*Cd. subangulosa*” strain NIES-2243) has pyrenoids and represents a weakly supported basal lineage within the *Reticulata* group, and other species within the group either contain (*Cr. chlorococcoides* and *Cr. difformis* [“*Cd. gerloffii*”]) or lack (*Cr. reticulata* and *Cr. rosae*) a pyrenoid. Thus, the most recent common ancestor of the *Reticulata* group

might have had a pyrenoid in the chloroplast. Subsequently, the pyrenoid may have independently been lost twice in ancestors of *Cr. rosae* and *Cr. reticulata*. The *Reticulata* group is a small clade within *Chloromonadinia* (Yumoto *et al.*, 2013), while pyrenoid loss may have occurred more than once during the evolution of this group. Thus, the *Reticulata* group represents a model for studying the pyrenoid evolution. More OTUs possibly belonging and closely related to the *Reticulata* group, such as *Cr. arctica* Barcytė & Hodač (Barcytė *et al.*, 2018a) and *Cr. svalbardensis* Barcytė & Hodač (Barcytė *et al.*, 2018b), are needed to understand the more detailed evolutionary events of pyrenoids within this group.

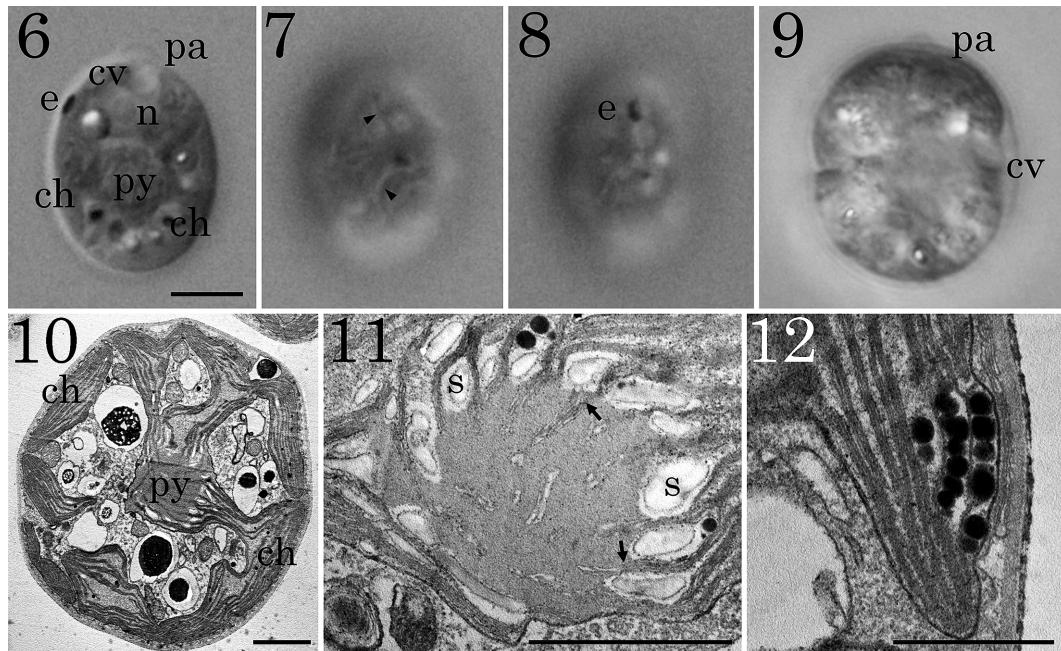
Morphology

Line drawings of vegetative cells of the two strains are shown in Figures 2–5.

“*Cd. gerloffii*” strain NIES-2215 (Figs. 6–12): Vegetative cells were unicellular, biflagellate, and



Figs. 2–5 Line drawings of light microscopic characteristics of vegetative cells in two strains of *Reticulata* group. **2, 3.** *Chloromonas difformis* Tomo. Makino, Matsuzaki & Nozaki *nom. nov.* strain NIES-2215 (formerly labeled “*Chlamydomonas gerloffii*”). **4, 5.** *Chloromonas typhlos* (Gerloff) Matsuzaki, Y. Hara & Nozaki strain NIES-2243 (formerly labeled “*Chlamydomonas subangulosa*”). **2, 4.** Optical sections of cells. **3, 5.** Surface views of cells.

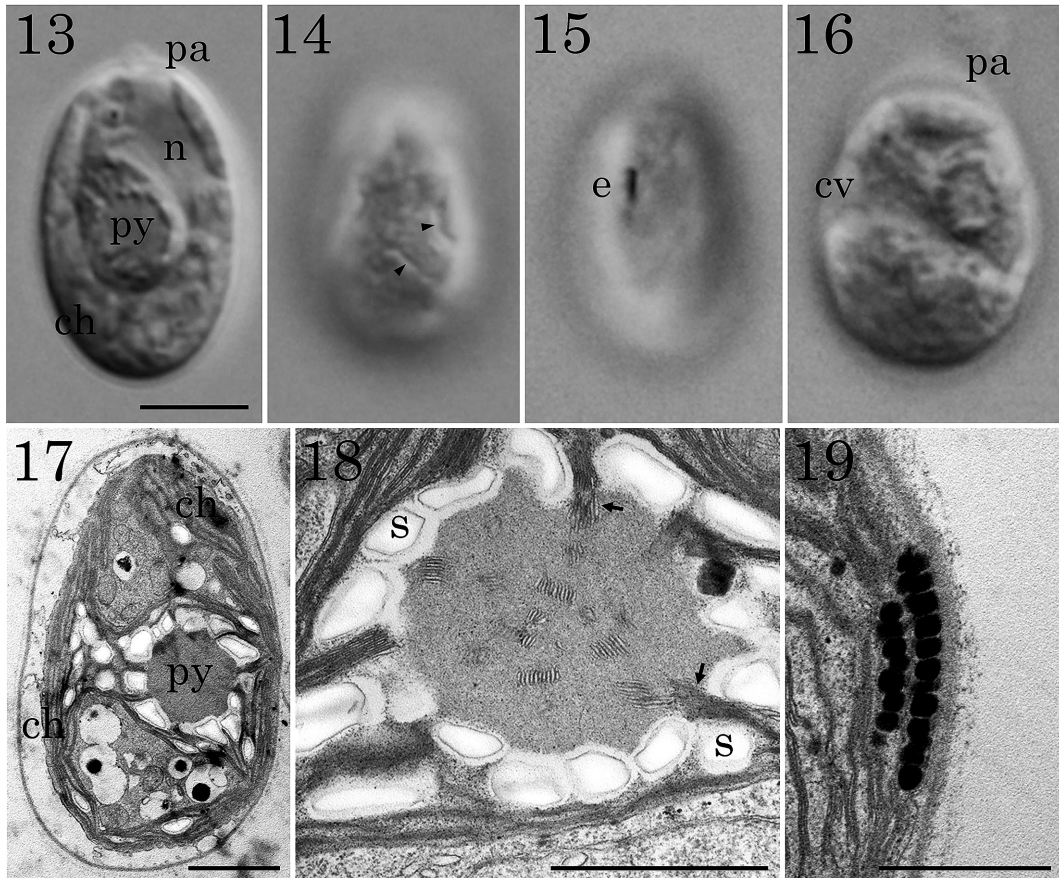


Figs. 6–12 *Chloromonas difformis* Tomo. Makino, Matsuzaki & Nozaki *nom. nov.* strain NIES-2215 (formerly labeled “*Chlamydomonas gerloffii*”). **6–9.** Nomarski interference micrographs of vegetative cells, shown at the same magnification. Scale bar=5 μ m. **6.** Optical section of a cell. **7.** Surface view of a cell. Arrowheads indicate irregular perforations and incisions on the chloroplast surface. **8.** Surface view of a cell showing a short-rod-shaped eyespot. **9.** Optical section of a dividing cell showing rotation of parental protoplast and apparently transverse first division. **10–12.** Transmission electron micrographs of vegetative cells. **10.** Longitudinal section of a cell. Scale bar=2 μ m. **11.** Pyrenoid showing thylakoid penetration into pyrenoid matrix (arrows). Scale bar=1 μ m. **12.** Eyespot composed of three layers of globules. Scale bar=1 μ m. **ch**, chloroplast; **cv**, contractile vacuole; **e**, eyespot; **n**, nucleus; **pa**, papilla; **py**, pyrenoid; **s**, starch grain.

broadly ellipsoidal in shape, measuring 10–18 μ m long and 8–15 μ m wide. The cell had a single nucleus, a prominent papilla at the base of the flagella, and a massive urn-shaped or stellate chloroplast with a single and somewhat indistinct pyrenoid in the middle of the cell (Fig. 6). The chloroplast had irregular perforations and incisions on the surface, but the lobes of the chloroplast were indistinct (Fig. 7). The eyespot was shaped as a short rod or ellipsoid and was positioned in the anterior 1/3 to 2/5 of the cell (Figs. 6, 8). The pyrenoid was spherical or ellipsoidal and positioned in the center of the chloroplast. The outline of the pyrenoid was obscure under light microscopy. The cytoplasm contained many globular vacuoles distributed in the incisions and perforations of the chloroplast. The papilla was hemispherical, often with a flat top face, and the nucleus was positioned in the cytoplasm anterior to the pyrenoid. The length of the flagella was nearly

1.5 times that of the cell. Two contractile vacuoles were located near the flagellar base. Asexual reproduction occurred via zoospore formation, producing four biflagellate zoospores within the parental cell wall. Before the first cell division, the protoplast rotated 90° (Fig. 9). Under TEM, the pyrenoid matrix was surrounded by starch granules, which were thin, meager and often separated from one another (Figs. 10, 11). The pyrenoid matrix was penetrated from various directions by thylakoid lamellae through the spaces between associated starch granules (Fig. 11). The eyespot was composed of three layers of globules (Fig. 12).

According to Ettl (1965, 1983), *Cd. gerloffii* is a species of the pyrenoid-containing genus “*Chlamydomonas*” and is characterized by a broadly ellipsoidal cell shape and indistinct structures of the chloroplast lobes and pyrenoid with peripheral starch granules. The present phylogenetic analyses



Figs. 13-19 *Chloromonas typhlos* strain NIES-2243 (formerly labeled "*Chlamydomonas subangulosa*"). **13-16.** Nomarski interference micrographs of vegetative cells, shown at the same magnification. Scale bar=5 μ m. **13.** Optical section of a cell. **14.** Surface view of a cell. Arrowheads indicate irregular perforations and incisions on the chloroplast surface. **15.** Surface view of a cell showing a rod-shaped eyespot. **16.** Optical section of a dividing cell showing rotation of parental protoplast and apparently transverse first division. **17-19.** Transmission electron micrographs of vegetative cells. **17.** Longitudinal section of a cell. Scale bar=2 μ m. **18.** Pyrenoid showing thylakoid lamellae penetrating into pyrenoid matrix (arrows). Scale bar=1 μ m. **19.** Eyespot composed of two layers of globules. Scale bar=1 μ m. **ch**, chloroplast; **cv**, contractile vacuole; **e**, eyespot; **n**, nucleus; **pa**, papilla; **py**, pyrenoid; **s**, starch grain.

demonstrated that *Cd. gerloffii* represents a distinct phylogenetic position separated from the other four species within the *Reticulata* group. Recently, Barcytė *et al.* (2018a) reported a new species, *Cr. arctica*, which is sister to *Cd. gerloffii* strain CCAP 11/72 (=NIES-2215) in the 18S *r*RNA gene tree. However, *Cr. arctica* lacks pyrenoids in the chloroplast and exhibits enough differences in its nuclear *r*DNA ITS2 sequence to be considered distinct from *Cd. gerloffii* at or above the species level (Barcytė *et al.*, 2018a). Although Barcytė *et al.* (2018a) showed that *Cr. paraserberinowii* (Skuja) Gerloff & H. Ettl

strain SAG 71.72 and *Cd. hydra* H. Ettl strain SAG 473 belong to the *Reticulata* group, the former species lack pyrenoids and the latter species is clearly different from *Cd. hydra* based on light microscopic characteristics (Ettl, 1983). Therefore, *Cd. gerloffii* should be recognized as a distinct species within the genus *Chloromonas*. However, the epithet *gerloffii* is unavailable in *Chloromonas* because *Cr. gerloffii* H. Ettl (1963) was described for different species. Thus, we propose the new name *Cr. difformis* to encompass *Cd. gerloffii* under the genus *Chloromonas*. Within the *Reticulata* group, *Cr. dif-*

formis can be distinguished from two other pyrenoid-containing species based on pyrenoid morphology. The pyrenoid matrix in vegetative cells of *Cr. chlorococcoides* and *Cr. typhlos* is entirely surrounded by thick starch plates with no space between starch granules except for the spaces through which thylakoid lamellae penetrate (Matsuzaki *et al.*, 2012). In contrast, the starch plates were thin and exhibited spaces between starch granules surrounding part of the pyrenoid matrix in *Cr. difformis* (Figs. 10, 11). This ultrastructural difference in the starch plates may reflect the difference in pyrenoids observed by light microscopy. Under light microscopy, the pyrenoids are prominent or distinct in the former two species (Matsuzaki *et al.*, 2012), whereas the *Cr. difformis* pyrenoids are somewhat indistinct (Ettl 1983; Fig. 6).

“*Cd. subangulosa*” strain NIES-2243 (Figs. 13–19): Vegetative cells were unicellular, biflagellate, and ellipsoidal in shape, measuring 13–18 μm long and 6–12 μm wide. The cells had one nucleus, a prominent papilla at the base of the flagella, and a parietal chloroplast with a single pyrenoid positioned in the middle of the cell (Fig. 13). The chloroplast was cup- or urn-shaped, with irregular perforations and incisions on the surface (Fig. 14). The eyespot was shaped as a short rod or ellipsoid and positioned in the anterior 1/3 of the cell (Fig. 15). The pyrenoid was prominent, spherical or ellipsoidal in shape, and lateral in position. The papilla was hemispherical, often with a flat top face, and the nucleus was positioned in the cytoplasm anterior to the pyrenoid. The flagella were the same length as the cell. Two contractile vacuoles were located near the base of the flagella. Asexual reproduction occurred via zoospore formation, producing four biflagellate zoospores within the parental cell wall. Before the first cell division, the protoplast rotated 90° (Fig. 16). TEM demonstrated that the pyrenoid matrix was surrounded entirely by starch plates, and thylakoid lamellae penetrated into the pyrenoid matrix from various directions (Figs. 17, 18). The eyespot was composed of two layers of globules (Fig. 19).

Ettl (1983) classified *Cd. subangulosa* into the “Euchlamydomonas” group because of its basal pyrenoid in the cup-shaped chloroplast. CCAP 11/28 was referred to be derived from one of the original author of *Cd. subangulosa* (“John”; George 1976). However, significant morphological differences were recognized between the original description

of *Cd. subangulosa* (Fritsch & John, 1942) and the present observations of NIES-2243. According to Fritsch & John (1942), this species has a basal pyrenoid, and the first division is longitudinal. However, the present cultured material has a lateral pyrenoid in the urn-shaped chloroplast, and the first division was apparently transverse after 90° rotation of the protoplast (Figs. 4, 5, 13–16). Moreover, the cell size according to the original description (Fritsch & John, 1942) conflicts with that of our observations. Fritsch & John (1942) reported that the cells are 16–20 μm long, whereas in our observation of NIES-2243, the cells were 13–18 μm long. These morphological features were essentially the same between strains NIES-2243 and CCAP 11/28, which were recently sent directly from CCAP, and the sequences of nuclear *rDNA* ITS2 in NIES-2243 and CCAP 11/28 were exactly the same (DDBJ/EMBL/GenBank accession numbers LC439250 and LC439251, respectively). Thus, CCAP 11/28 was misplaced with a different strain or misidentified as *Cd. subangulosa* (George 1976).

“*Cd. subangulosa*” strain NIES-2243 and *Cr. typhlos* strain SAG 26.86 formed a small robust clade within the *Reticulata* group. However, some morphological differences were recognized between these two strains. NIES-2243 has one eyespot per cell, whereas *Cr. typhlos* lacks eyespots (Matsuzaki *et al.*, 2012). Moreover, on average, the ratio of the cell width to length is lower in NIES-2243 than in *Cr. typhlos* strain SAG 26.86. The average ratio of the cell length to width (based on observations of 25 cells from each strain) is 1.39 in SAG 26.86 and 1.53 in NIES-2243. However, NIES-2243 is highly similar to *Cr. typhlos* strain SAG 26.86 in other morphological attributes (Matsuzaki *et al.*, 2012). Both strains have a hemispherical prominent papilla at the base of the flagella, a parietal chloroplast with irregular perforations and incisions on the surface and a single lateral pyrenoid positioned in the middle of the cell, and a nucleus positioned anterior to the pyrenoid (Figs. 4, 5, 13, 14) (Matsuzaki *et al.*, 2012). Because of these morphological characteristics, the stigma-containing species *Cd. media* G.A. Klebs is similar to NIES-2243 (Klebs, 1896; Ettl, 1983). However, *Cd. media* is different from *Cr. typhlos* and NIES-2243 due to its large cell length. Vegetative cells of *Cd. media* measure 18–20 μm long (Klebs, 1896; Ettl, 1983), whereas *Cr. typhlos* (including SAG 26.86) and NIES-2243 have vegeta-

tive cells measuring 6–18 μm long (Ettl, 1983; Matsuzaki *et al.*, 2012). In addition, the culture strain NIES-2743, recently determined to form the basis of the epitype of *Cd. media*, has a relatively smooth chloroplast surface, and its phylogenetic position is distantly separate from the genus *Chloromonas* (Nakada *et al.*, 2012). This species was recently transferred to the genus *Microglena* as *M. media* (G.A. Klebs) Nakada (Nakada & Tomita, 2014). Thus, NIES-2243 is assigned here to the monophyletic species *Cr. typhlos*. *Cr. typhlos* now exhibits morphological variability, particularly in terms of the presence or absence of an eyespot in the chloroplast. Such eyespot variability has been reported in another monophyletic species of the unicellular Volvocales, *Chlorogonium euchlorum* (Ehrenberg) Ehrenberg (Nozaki *et al.*, 1998) and among strains of the unicellular volvocalean species *Gloeomonas anomalipyrenoides* Nakada, Matsuzaki & Nozaki (Nakada *et al.*, 2015).

Recently, Boldina (2017) reported light and electron microscopy of a Russian strain of *Cr. typhlos*. Interestingly, this strain has a stigma as in NIES-2243. However, its phylogenetic position was not resolved. Further molecular phylogenetic studies using this Russian strain will resolve more accurate taxonomic status of *Cr. typhlos*.

TAXONOMIC TREATMENT

Chloromonas difformis Tomo. Makino, Matsuzaki & Nozaki *nom. nov.*

Replaced synonym: *Chlamydomonas gerloffii* H. Ettl 1965: 298, fig. 15 (holotype); pl. 30: figs. 1–3.

Priority for *Chloromonas gerloffii* H. Ettl 1963: 398.

Etymology: the name “*difformis*” meaning “with different forms from others” refers to the unique and distinct features of this species within the *Reticulata* group, such as a basal or central pyrenoid surrounded by meager starch plates, many globular vacuoles in the cytoplasm, and three layers of eyespot globules.

DISTRIBUTION: Czech (Ettl, 1965)

STRAIN EXAMINED: NIES-2215 (=CCAP 11/72, authentic strain of *Cd. gerloffii* strain; George, E.A., 1976).

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Chloromonas 属（ボルボックス目，緑藻綱）の *Reticulata* グループに所属する *Chlamydomonas* と表示されている NIES 保有 2 株の分類学的再同定

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Yumoto *et al.* (2013, *Microbiol. Cult. Coll.*) は 18S *rDNA* 系統から *Chlamydomonas* (*Cd.*) 属と表示されている NIES 保有の 2 株 (“*Cd. gerloffii*” NIES-2215 と “*Cd. subangulosa*” NIES-2243) が *Chloromonas* (*Cr.*) 属のタイプ種を含むクレード (*Reticulata* グループ) に所属することを明らかにしたが，詳細な研究は実施されなかった．本研究ではこれら 2 種の光学・透過型電子顕微鏡による観察と複数遺伝子による分子系統解析を実施した．その結果，NIES-2215 は *Reticulata* グループの他の 4 種と形態と系統で異なり，*Chloromonas* 属に所属すると判断したが，先行名として *Cr. gerloffii* H. Ettl 1963 が存在するため，新名 *Cr. difformis* Tomo. Makino, Matsuzaki & Nozaki を提唱した．NIES-2243 は眼点をもつという点以外は *Cr. typhlos* と形態的に類似し，*Cr. typhlos* SAG 26.86 と単系統群を構成したため，*Cr. typhlos* と再同定した．