

Taxonomic re-examination of *Chlamydomonas* strains maintained in the NIES-Collection

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INTRODUCTION

The genus *Chlamydomonas* (Volvocales, Chlorophyceae) comprises a large number of species (more than 600), and molecular phylogenetic analyses using 18S rRNA and chloroplast genes have shown it to be polyphyletic (Buchheim *et al.*, 1997; Hoham *et al.*, 2002; Nozaki *et al.*, 1998; Pröschold *et al.*, 2001). In previous studies, the principal clades within the genus were clearly, and for the most part consistently, distinguished, although the relationships among the clades have not always been well resolved (Buchheim *et al.*, 1997; Nakada *et al.*, 2008; Pröschold *et al.*, 2001). On the basis of these lineages, Pröschold *et al.* (2001) performed several taxonomic revisions and proposed two new genera, but the completion of the taxonomic revision of the genus is expected to take considerable time. Under such circumstances, Nakada *et al.* (2008) performed comprehensive molecular analyses of Volvocales, including *Chlamydomonas* species, based on the 18S rRNA gene sequence, and adopted PhyloCode (*International Code of Phylogenetic Nomenclature*, Cantino & de Queiroz, 2010) to explicitly define individual clades. In the PhyloCode system, names applied to clades are defined in terms of phylogenetic relationships (Cantino & de Queiroz, 2010). Accordingly, we consider the subgroups that were phylogenetically defined by Nakada *et al.* (2008) by following the PhyloCode to be the best way for indicating the appropriate taxonomic positions of individual *Chlamydomonas* strains maintained in the Microbial Culture Collection at the National Institute for Environmental Studies, Tsukuba, Japan (NIES-Collection).

Various *Chlamydomonas* species are familiar organisms that contribute to the algal biodiversity in soil and water (both freshwater and marine) environ-

ments, and they also occur in extreme environments such as acidic ponds and snow surfaces. Some *Chlamydomonas* strains are also used as models in basic research in many fields, such as genomics, genetics, physiology, and developmental biology (Harris, 2009). The NIES-Collection holds more than 80 *Chlamydomonas* strains, most of which were transferred from the University of Tokyo's IAM Collection in 2007, when the collection was closed, and some were deposited as *Chlamydomonas* species without species level identification. No molecular phylogenetic data are available for most of the *Chlamydomonas* strains in the NIES-Collection, although the 18S rDNA sequences of approximately 10 strains have been published (Nakada *et al.*, 2010; Nakada & Tomita, 2011). Therefore, to better characterize the *Chlamydomonas* strains in the NIES-Collection taxonomically, we sequenced their 18S rDNA, determined their phylogenetic positions, and assigned them to the subgroups defined by Nakada *et al.* (2008). Also of note, 18S rDNA sequences have been published for 30 strains that originated from the same strains as the NIES strains and have been maintained in other culture collections. Therefore, we investigated the strain histories and the published sequence data to confirm the taxonomic identity of these strains and to exclude the strains that may have been replaced by other strains during long-term subculturing.

MATERIALS AND METHODS

We determined the taxonomic positions of 79 *Chlamydomonas* strains maintained at the NIES-Collection by sequencing their 18S rDNA. Algal cultures were incubated in test tubes, each containing 10 ml of medium [AF-6, or C medium for freshwater strains, and f/2, ESM, or STP medium for marine strains (Kasai *et al.*, 2009)], at 20°C under an irradiance of 8–32 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ provided by day-light fluorescent lamps in a 12:12-h light:dark cycle.

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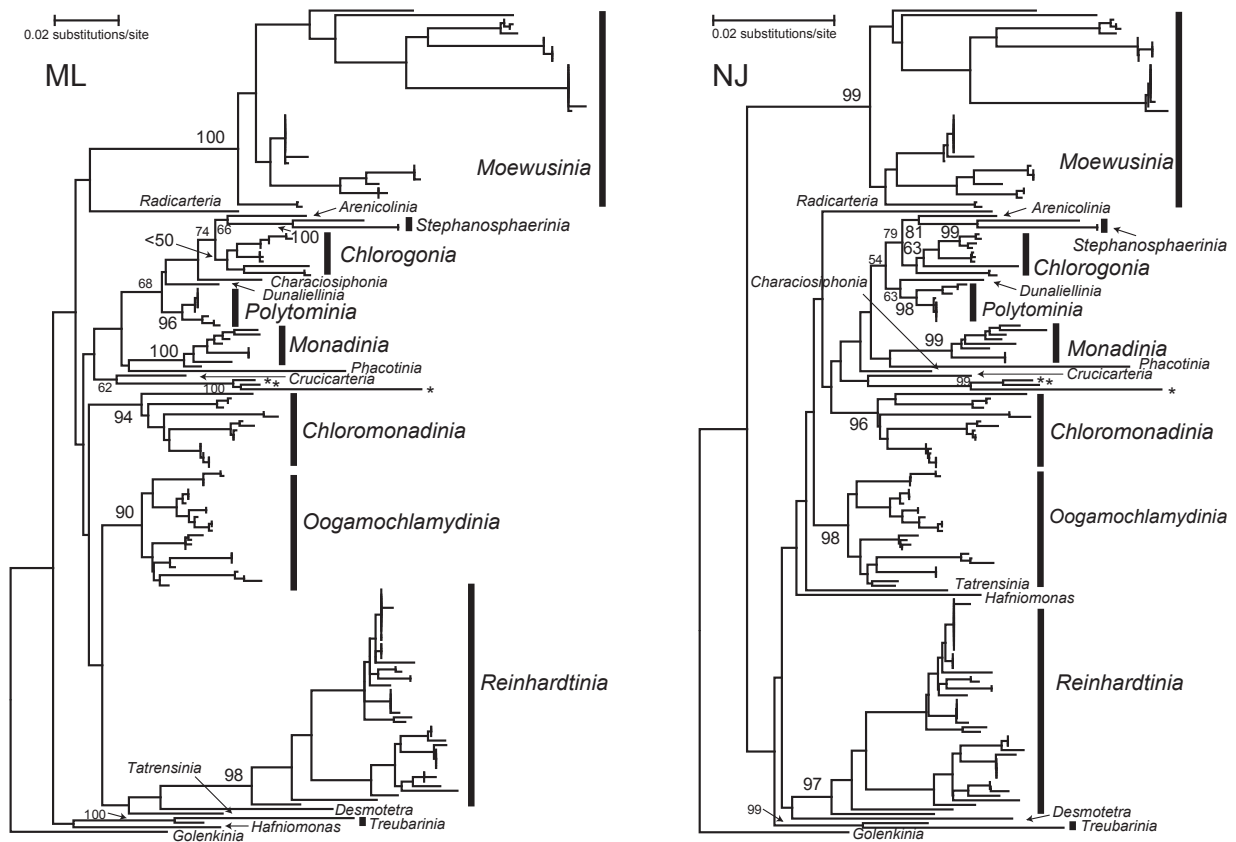


Fig. 1 Overview of the ML and NJ trees based on the aligned 1558 positions of 181 sequences of 18S rDNA. The tree is drawn to scale, with branch lengths measured for the number of substitutions per site. *Golenkinia* is arbitrarily placed at the base of the tree. Bootstrap values of more than 50% in the ML and NJ analyses are shown on each branch except for the inside of major clades, which are shown in Figs. 2 and 3.

Total DNA was extracted from the cells of 79 strains of *Chlamydomonas* by using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. The 18S rDNA was amplified by PCR using the originally designed primers: 63F (5'-ACGCTTGTCTCAAAGATTA-3') and 1818R (5'-ACGGAAACCTTGTTACGA-3'). PCR conditions were as follows: 94°C for 10 min; 30 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. All PCR amplifications were carried out in a PCR thermal cycler (TaKaRa PERSONAL, TaKaRa) in a total volume of 15 μ l, containing 0.2 mM of each dNTP, 0.5 μ M of each primer pair, 10 \times PCR buffer, 0.25 U *Ex Taq* DNA polymerase (TaKaRa), and 0.5–5 ng of template DNA. PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. The PCR products (1576–1680 base pairs of 18S rDNA) were then used to

sequence, using the primers reported by Nakayama *et al.* (1998), in a CEQ8000 DNA sequencer (Beckman Coulter). The 18S rDNA sequences of *Chlamydomonas* reported here have been submitted to the DDBJ with the accession numbers shown in Table 1.

Additional 102 sequences (Figs. 2 & 3) were selected using the NCBI BLAST-bl2seq program (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>) as well as from phylogenetic trees published by Nakada *et al.* (2008). In total, 181 sequences were aligned using ClustalX (Thompson *et al.*, 1997) obtained from the DDBJ, and manually refined on MEGA5 software (Tamura *et al.*, 2011), by referring to an alignment applied in Nakada *et al.* (2008). A total of 1558 positions were used for the analyses that followed.

We also used MEGA5 software to perform maximum likelihood (ML) and neighbor-joining (NJ) anal-

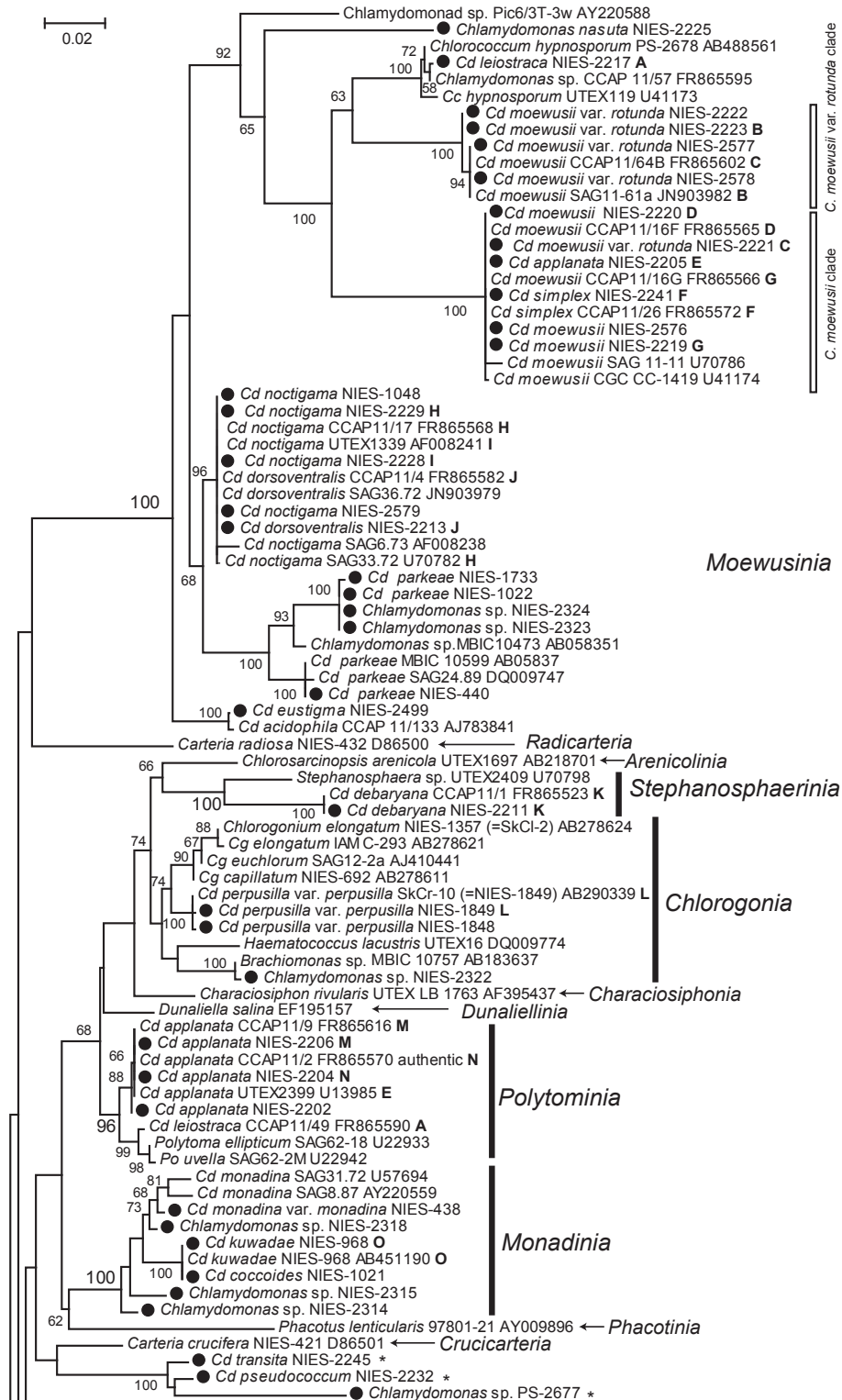


Fig. 2 Details of the ML tree obtained based on 18S rDNA sequences. *Moewusinia*, *Stephanosphaerinia*, *Chlorogonia*, *Polytomina*, and *Monadinia* are shown with representative strains of *Radicarteria*, *Arenicolinia*, *Characiosiphonia*, *Dunaliellinia*, *Phacotinia*, and *Crucicarteria*. Bootstrap values of more than 50% are shown on each branch. Sequences analyzed in the present study are indicated in filled circles. The same alphabetical characters (A–O) indicate strains of the same origin.

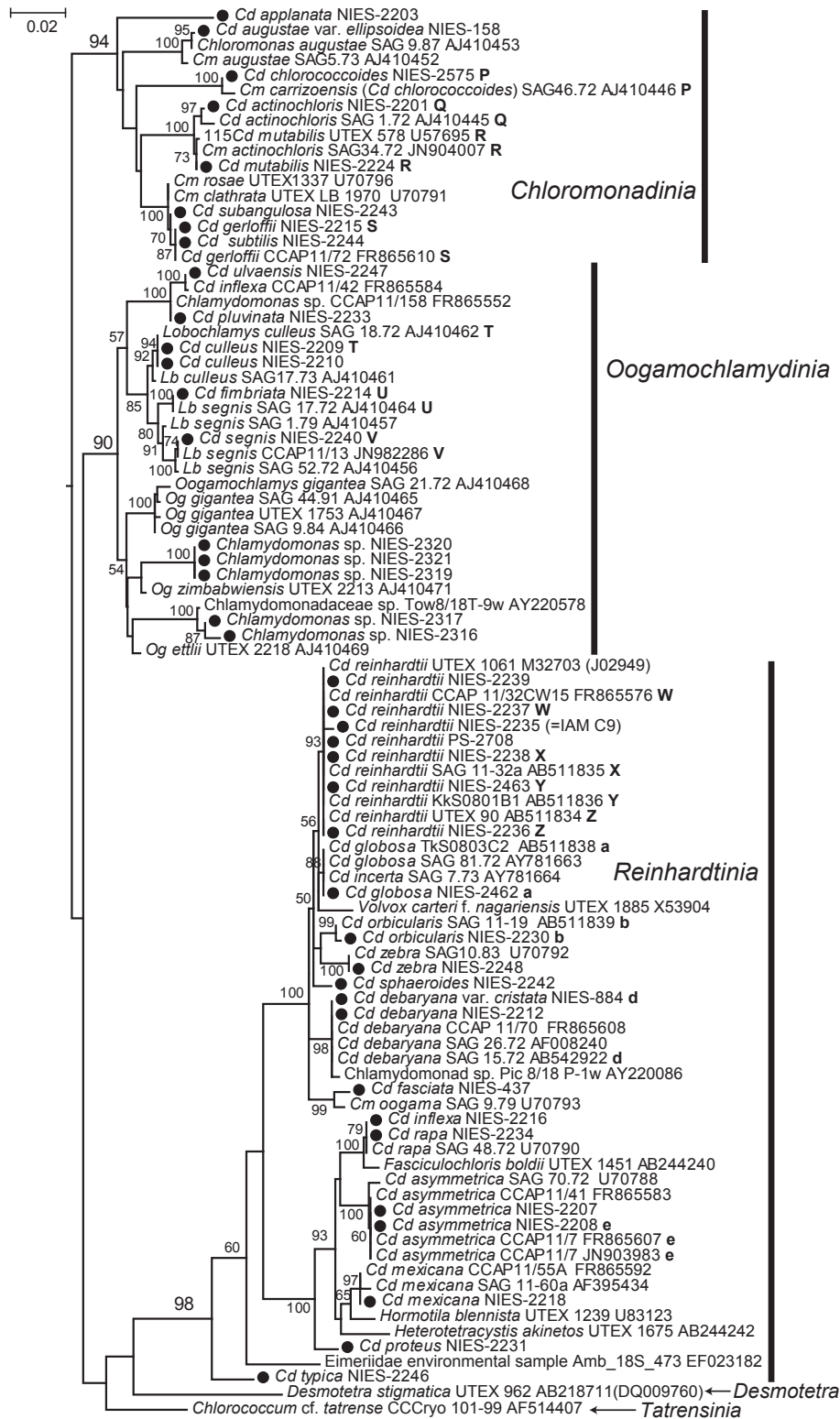


Fig. 3 Details of the ML tree obtained based on 18S rDNA sequences. *Chloromonadinia*, *Oogamochlamydia*, and *Reinhardtinia* are shown with representative strains of *Desmotetra*, and *Tatrensinia*. Bootstrap values of more than 50% are shown on each branch. Sequences analyzed in the present study are indicated in filled circles. The same alphabetical characters (P–e) indicate strains of the same origin.

yses. An ML tree was constructed by applying a 'General Time Reversible' model for nucleotide substitution with gamma-distributed among-site rate variation and invariant sites (GTR+G+I). An NJ tree was constructed by applying a 'Kimura-2-parameter' model. Bootstrap analyses were performed to assess the robustness of the trees (100 replicates for ML analysis, 1000 replicates for NJ analysis).

To measure sequence similarity among the NIES strains and strains of the same origin (but held in other culture collections), we used "Identities" obtained by aligning two (or more) sequences applying the NCBI BLAST-bl2seq program. These values show how many positions are identical among the query sequence and the portion of the subject sequence(s) when two (or more) sequences are aligned. Aligned positions were 1584–1666 that covered 99–100% of the query sequences (i.e., sequences of the NIES strains). "Identities" were also used to infer the taxonomic identity of three NIES strains that were not included in the subgroups defined by Nakada *et al.* (2008).

RESULTS AND DISCUSSION

Phylogenetic position and subgroups of *Chlamydomonas* strains

In both the ML and NJ analyses, each major clade, including representative species of the clade as defined by Nakada *et al.* (2008), was reproduced with high bootstrap support (Fig. 1, 90–100% in the ML tree; 96–99% in the NJ tree), except for the clade including *Chlorogonium euchlorum* (i.e., the subgroup *Chlorogonia*, <50% and 63% bootstrap values in the ML and NJ trees, respectively). We assigned the strains included in individual clades to the corresponding subgroups defined by Nakada *et al.* (2008) (Table 1), based on the results of our phylogenetic analyses (Figs. 2 & 3) as well as on their relationships to the species analyzed by Nakada *et al.* (2008). Although the ML and NJ topologies differed from one another (Fig. 1), major clades were similarly supported with high bootstrap values in the ML and NJ analyses. Therefore, we showed only the ML tree (Figs. 2 & 3), while the bootstrap values in the NJ analysis were shown only in the text, if necessary.

All NIES *Chlamydomonas* strains, except for three (see below), were assigned to eight subgroups: 23 strains belonged to *Moewusinia*, 1 to *Stephanosphaerina*, 3 to *Chlorogonia*, 3 to

Polytominia, 6 to *Monadinia*, 8 to *Chloromonadinia*, 11 to *Oogamochlamydia*, and 21 to *Reinhardtina* (Table 1).

We were unable to re-identify three strains, *Chlamydomonas transita* NIES-2245, *Cd. pseudococcum* NIES-2232, and *Chlamydomonas* sp. PS-2677 (indicated by asterisks in Figs. 1 & 2), based on our results, because they were not included in any of the subgroups defined by Nakada *et al.* (2008). However, we found several sequences that were most similar to (not the same as) the sequences of the above-mentioned three NIES strains by the Nucleotide BLAST search, and that these most similar sequences belonged to Sphaeropleales, not to Volvocales. For example, the sequence of *Cd. transita* NIES-2245 was most similar to six sequences including the sequence of *Asterarcys quadricellulare* strain KNUA020 (JQ043183) [Identity=99.2% (Identical position=1637/total position=1650)]. It was also similar to the sequence of *Scenedesmus vacuolatus* SAG 211-8b (X56104) [I=99.2% (1636/1650)]. The sequence of *Cd. pseudococcum* NIES-2232 was the most similar to four sequences including the sequence of Scenedesmaceae sp. Tow 9/21 P-1w (AY197641) [I=99.6% (1643/1650)]. It was also similar to the sequence of *Scenedesmus obliquus* SAG 276-3a (X56103) [I=99.5% (1641/1650)]. The sequence of *Chlamydomonas* sp. PS-2677 was most similar to that of *Scenedesmus armatus* var. *subalternans* CCAP 276/4A (FR865727) [I=99.7% (1572/1576)]. On the other hand, the sequence of *Cd. transita* NIES-2245, which positioned most basally within a clade the three species constituted, was less similar to the sequence of *Carteria crucifera* NIES-42 [I=95.0% (1572/1654)] compared to the sequences of Sphaeropleales. Therefore, we strongly suspect that these three NIES strains have been replaced or misidentified, and will not submit the DNA sequences of these strains until detailed morphological and comparative studies have been performed.

The sequences of 49 strains, among the 79 NIES strains analyzed in the present study, were newly determined (Table 1). These strains seemed to include interesting ones, which created new independent clades without any closely related strains that have been registered. We therefore speculate that these strains may belong to new taxa; i.e., four strains, NIES-1022, 1733, 2323 and 2324, which belonged to *Moewusinia* (among these four strains, two strains had been identified as *Cd. parkeae*, but

Table 1 List of 79 NIES strains, which were used to 18S rRNA gene sequencing

Subgroup names defined by Nakada <i>et al</i> (2008)	Strain no.	Species (revised species are underlined) < Original designation	History (depositor, deposit year) Strain number in other culture collections, boldface indicating strains DNA sequences of which have been published, with sequence identities ¹ , if appropriate, in parentheses	Accession no.
Moewusinia				
	NIES-2499	<i>Chlamydomonas eustigma</i> Ettl	<(Higuchi, 2009)	AB701493
	NIES-2217	<i>Chlamydomonas leiostraca</i> (Strehlow) Ettl	<IAM C-284 <BIU-466 (Starr, 1967) as <i>C. stercoraria</i> CCAP 11/49 [I=92.3% (1527/1655), A]; SAG 11-49	AB753034
	NIES-2219	<i>Chlamydomonas moewusii</i> Gerloff	<IAM C-258 <BIU-96 (Starr, 1967) CCAP 11/16G [I=99.9% (1644/1646), G]; UTEX 96; SAG 11-16g; ATCC 30418; CGC CC-56	AB701494
	NIES-2220	<i>Chlamydomonas moewusii</i> Gerloff	<IAM C-259 <BIU (Starr, 1967) CCAP 11/16F [I=99.8% (1663/1666), D]; SAG 11-16f; ATCC 30588	AB701495
	NIES-2241	<u><i>Chlamydomonas moewusii</i></u> Gerloff < <i>Chlamydomonas simplex</i> Pascher	<IAM C-281 <BIU-211 (Starr, 1967) CCAP 11/26 [I=99.9% (1646/1647), F]	AB701496
	NIES-2576	<i>Chlamydomonas moewusii</i> Gerloff	<IAM C- 5 <Tsubo L+ (Tsubo, 1967)	AB701497
	NIES-2222	<i>Chlamydomonas moewusii</i> var. <i>rotunda</i> Tsubo	<IAM C-19 <Tsubo 2475 (Tsubo)	AB701498
	NIES-2223	<i>Chlamydomonas moewusii</i> var. <i>rotunda</i> Tsubo	<IAM C-556 <UTEX 576 (1996); (=IAM C-16 <Tsubo 24(+)) (Tsubo) CCAP 11/64A ; SAG 11-61a [I=99.8% (1647/1651), B]	AB701499
	NIES-2577	<i>Chlamydomonas moewusii</i> var. <i>rotunda</i> Tsubo	<IAM C-18 <Tsubo 2470 (Tsubo)	AB701500
	NIES-2578	<i>Chlamydomonas moewusii</i> var. <i>rotunda</i> Tsubo	<IAM C- 26 <Tsubo 24122 (Tsubo)	AB701501
	NIES-2225	<i>Chlamydomonas nasuta</i> Korshikov	<IAM C-550 <UTEX 451(1996); (=IAM C-225 <BIU 451 (Starr, 1967))	AB701502
	NIES-1048	<i>Chlamydomonas noctigama</i> Korshikov	<2001-814-C10 (Nozai, 2003)	AB701503
	NIES-2213	<u><i>Chlamydomonas noctigama</i></u> Korshikov < <i>Chlamydomonas dorsoventralis</i> Pascher	<IAM C-228 <BIU-228 (Starr, 1967) CCAP 11/4 [I=99.9% (1623/1624), J]; UTEX 228; SAG23.87 ; ATCC 30594	AB753035
	NIES-2228	<i>Chlamydomonas noctigama</i> Korshikov	<IAM C-270 <BIU-1339 (Starr, 1967) as <i>C. pinicola</i> SAG 40.72; UTEX 1339 [I=99.9% (1640/1641), I]; Ettl 108	AB701504
	NIES-2229	<i>Chlamydomonas noctigama</i> Korshikov	<IAM C-557 <UTEX 220 (1996); (=IAM C-262 <BIU-220 (Starr, 1967) as <i>C. monoica</i>) ATCC 30629; CCAP 11/17 [I=99.9% (1640/1641), H]; SAG 33.72 [I=99.3% (1638/1650), H]	AB701505
	NIES-2579	<i>Chlamydomonas noctigama</i> Korshikov	<IAM C-264 <BIU-114 (Starr, 1967) SAG 35.72	AB701506
	NIES-440	<i>Chlamydomonas parkeae</i> Ettl	<I-29 (Suda, 1986)	AB701507
	NIES-1022	<u><i>Chlamydomonas</i> sp. (<i>Moewusinia</i>)</u> < <i>Chlamydomonas parkeae</i> Ettl	<#97 (Moriya, 2002)	AB701508
	NIES-1733	<u><i>Chlamydomonas</i> sp. (<i>Moewusinia</i>)</u> < <i>Chlamydomonas parkeae</i> Ettl	<M-77 (Sato, 2005)	AB701509
	NIES-2323	<i>Chlamydomonas</i> sp. (<i>Moewusinia</i>)	<Sa-38 (Suda, 1986)	AB701510
	NIES-2324	<i>Chlamydomonas</i> sp. (<i>Moewusinia</i>)	<IC-9 (Sawaguchi, 1987)	AB701511
	NIES-2205	Withdrawal < <i>Chlamydomonas applanata</i> Pringsheim	<IAM C-229 <BIU-342 (Starr, 1967) as <i>C. dysosmos</i> UTEX 2399 [I=92.0% (1521/1653), E]	–

Table 1 Continued

Subgroup names defined by Nakada <i>et al</i> (2008)	Strain no.	Species (revised species are underlined) < Original designation	History (depositor, deposit year) Strain number in other culture collections, boldface indicating strains DNA sequences of which have been published, with sequence identities ¹ , if appropriate, in parentheses	Accession no.
	NIES-2221	Withdrawal < <i>Chlamydomonas moewusii</i> var. <i>rotunda</i> Tsubo	<IAM C-15 <Tsubo 24(-) (Tsubo) CCAP 11/64B [I=94.5% (1565/1656), C]; UTEX 577; SAG 11-61b	–
<i>Polytomina</i>	NIES-2206	<i>Chlamydomonas applanata</i> Pringsheim	<IAM C-237<BIU-225 (Starr, 1967) as <i>C. humicola</i> ATCC 30455; CCAP 11/9 [I=99.9% (1628/1629), M]; SAG 11-9; UTEX 225	AB701512
	NIES-2204	<i>Chlamydomonas applanata</i> Pringsheim	<IAM C-218 <BIU-230 (Starr, 1967) CCAP 11/2 [I=99.9% (1642/1643), N]; SAG 6.72	AB701513
	NIES-2202	<i>Chlamydomonas applanata</i> Pringsheim	<IAM C-214 <BIU-969 (Starr, 1967) as <i>C. aggregata</i> Deason T-1-12; SAG 2.72; UTEX 969	AB701514
<i>Chlorogonia</i>	NIES-1848	<i>Chlamydomonas perpusilla</i> (Korshikov) Gerloff var. <i>perpusilla</i>	<SkCl-3 (Nakada, 2006)	AB753036
	NIES-1849	<i>Chlamydomonas perpusilla</i> (Korshikov) Gerloff var. <i>perpusilla</i>	< SkCr-10 (Nakada, 2006) [I=99.8% (1581/1584), L]	AB753037
	NIES-2322	<i>Chlamydomonas</i> sp. (<i>Chlorogonia</i>)	<w-36 (Suda, 1987)	AB701515
<i>Stephanosphaerina</i>	NIES-2211	<u><i>Chlamydomonas</i> sp. (<i>Stephanosphaerina</i>)</u> < <i>Chlamydomonas debaryana</i> Goroschankin	<IAM C-215 <BIU-231 (Starr, 1967) as <i>C. agloeformis</i> CCAP 11/1 [I=99.9% (1645/1647), K]; SAG 11-1; UTEX 231	AB701516
<i>Monadinia</i>	NIES-968	<i>Chlamydomonas kuwadae</i> Gerloff	<92-514-H-13 (Nozaki, 1996) NIES-968 (Nakada & Nozaki, 2009) [I=99.9% (1648/1649), O]	AB753038
	NIES-1021	<u><i>Chlamydomonas kuwadae</i> Gerloff</u> < <i>Chlamydomonas coccoides</i> Butcher	<#95 (Moriya, 2002)	AB701517
	NIES-438	<i>Chlamydomonas monadina</i> Stein var. <i>monadina</i>	<Kas-7 (Suda, 1986)	AB701518
	NIES-2314	<i>Chlamydomonas</i> sp. (<i>Monadinia</i>)	<SIS-ch (Suda, around 1986)	AB701519
	NIES-2315	<i>Chlamydomonas</i> sp. (<i>Monadinia</i>)	<H-3-1 (Suda, around 1986)	AB701520
	NIES-2318	<i>Chlamydomonas</i> sp. (<i>Monadinia</i>)	<KZ-5-17 (Suda, around 1986)	AB701521
<i>Oogamochlamydia</i>	NIES-2233	<i>Chlamydomonas pulvinata</i> Vischer	<IAM C-276 <BIU-212 (Starr, 1967) CCAP 11/25; SAG 45.72; UTEX 212	AB701522
	NIES-2247	<i>Chlamydomonas ulvaensis</i> Lewin	<IAM C-566 <UTEX (1996); (=IAM C-291 <BIU-724 (Satt, 1967)) CCAP 11/58; Lewin DD1/27; SAG 62.72	AB701523
	NIES-2240	<u><i>Lobochlamys segnis</i> (Ettl) Pröschold, Marin, Schlösser et Melkonian</u> < <i>Chlamydomonas segnis</i> Ettl	<IAM C-254 <BIU-222 (Starr, 1967) as <i>C. intermedia</i> ATCC 30631; CCAP 11/13 [I=99.9% (1648/1649), V]; SAG 11-13; UTEX 222	AB701524
	NIES-2214	<u><i>Lobochlamys segnis</i> (Ettl) Pröschold, Marin, Schlösser et Melkonian</u> < <i>Chlamydomonas fimbriata</i> Ettl	<IAM C-230 <BIU-1349 (Starr, 1967) CCAP 11/69; SAG 17.72 [I=99.9% (1645/1646), U]	AB701525

Table 1 Continued

Subgroup names defined by Nakada <i>et al</i> (2008)	Strain no.	Species (revised species are underlined) < Original designation	History (depositor, deposit year) Strain number in other culture collections, boldface indicating strains DNA sequences of which have been published, with sequence identities ¹ , if appropriate, in parentheses	Accession no.
	NIES-2210	<u><i>Lobochlamys culleus</i></u> (Ettl) Pröschold, Marin, Schlösser et Melkonian < <i>Chlamydomonas culleus</i> Ettl	<IAM C-553 <UTEX 1058 (1996); (=IAM C-232 <BIU-1058 (Starr, 1967) as <i>C. frankii</i> SAG 19.72; UTEX 1058	AB701526
	NIES-2209	<u><i>Lobochlamys culleus</i></u> (Ettl) Pröschold, Marin, Schlösser et Melkonian < <i>Chlamydomonas culleus</i> Ettl	<IAM C-552 <UTEX 1057 (1996); (=IAM C-231 <BIU-1057 (Starr, 1967) as <i>C. frankii</i>) SAG 18.72 [I=99.8% (1642/1645), T]; UTEX 1057	AB701527
	NIES-2316	<i>Chlamydomonas</i> sp. (<i>Oogamochlamydinia</i>)	<CChla (Suda, around 1986)	AB701528
	NIES-2317	<i>Chlamydomonas</i> sp. (<i>Oogamochlamydinia</i>)	<KZ-5-8 (Suda, around 1986)	AB701529
	NIES-2319	<i>Chlamydomonas</i> sp. (<i>Oogamochlamydinia</i>)	<H-3-6 (Suda, 1986)	AB701530
	NIES-2320	<i>Chlamydomonas</i> sp. (<i>Oogamochlamydinia</i>)	<w-14-5 (Suda, 1986)	AB701531
	NIES-2321	<i>Chlamydomonas</i> sp. (<i>Oogamochlamydinia</i>)	<w-30 (Suda, 1986)	AB701532
<i>Chloromonadinia</i>				
	NIES-158	<u><i>Chloromonas augustae</i></u> (Skuja) Pröschold, Marin, Schlösser et Melkonian < <i>Chlamydomonas augustae</i> Skuja var. <i>ellipsoidea</i> S. Watanabe	<ASE-242 (S. Watanabe, 1984)	AB701534
	NIES-2215	<i>Chlamydomonas gerloffii</i> Ettl	<IAM C-554 <UTEX (1996); (=IAM C-233 <BIU-1348 (Starr, 1967)) CCAP 11/72 [I=100% (1608/1608), S]; SAG 20.72; UTEX 1348, Ettl 136	AB701536
	NIES-2243	<i>Chlamydomonas subangulosa</i> Fritsch et John	<IAM C-563 <UTEX 209 (1996); (=IAM C-285 <BIU-209 (Starr, 1967)) CCAP 11/28; SAG 57.72; UTEX 209	AB701538
	NIES-2201	<u><i>Chloromonas actinochloris</i></u> (Deason et Bold) Pröschold, Marin, Schlösser et Melkonian < <i>Chlamydomonas actinochloris</i> Deason et Bold	<IAM C-213 <BIU-965 (Starr, 1967) SAG 1.72 [I=99.3% (1634/1645), Q]; UTEX 965, Deason C-2-14	AB701533
	NIES-2224	<u><i>Chloromonas actinochloris</i></u> (Deason et Bold) Pröschold, Marin, Schlösser et Melkonian < <i>Chlamydomonas mutabilis</i> Gerloff	<IAM C-558 <UTEX 578 (1996) [I=98.7% (1627/1649), R]; (=IAM C-7 <Tsubo T-A) SAG 34.72 [I=99.9% (1648/1649), R]	AB701537
	NIES-2575	<u><i>Chloromonas carrizoensis</i></u> (Ettl et Schwarz) Pröschold, Marin, Schlösser et Melkonian < <i>Chlamydomonas chlorococcoides</i> Ettl et Schwarz	<IAM C-277 <BIU-968 (Starr, 1967) SAG 46.72 [I=99.5% (1642/1650), P]; Deason C-2-4	AB701535
	NIES-2203	<u><i>Chloromonas</i></u> sp. (<i>Chloromonadinia</i>) < <i>Chlamydomonas applanata</i> Pringsheim	<IAM C-216 <BIU-967 (Starr, 1967) as <i>C. akinetos</i> SAG 3.72; UTEX 967, Deason C-1-11	AB701539
	NIES-2244	<i>Chlamydomonas subtilis</i> Pringsheim	<IAM C-564 <UTEX (1996); (=IAM C-287 <BIU-207 (Starr, 1967)) CCAP 11/30; UTEX 207	AB822371
<i>Reinhardinia</i>				
	NIES-2207	<i>Chlamydomonas asymmetrica</i> Korshikov	<IAM C-219 <BIU-450 (Starr, 1967) CCAP 11/41; SAG 11-41; UTEX 450	AB701540
	NIES-2208	<i>Chlamydomonas asymmetrica</i> Korshikov	<IAM C-234 <BIU-227 (Starr, 1967) as <i>C. gloeopara</i> ATCC 30586; CCAP 11/7 [I=99.9% (1608/1609), e]; SAG 11-7; UTEX 227; Rodhe 1635	AB701541

Table 1 Continued

Subgroup names defined by Nakada <i>et al</i> (2008)	Strain no.	Species (revised species are underlined) < Original designation	History (depositor, deposit year) Strain number in other culture collections, boldface indicating strains DNA sequences of which have been published, with sequence identities ¹ , if appropriate, in parentheses	Accession no.
	NIES-2212	<i>Chlamydomonas debaryana</i> Goroschankin	<IAM C-549 <UTEX (1996); (=C-1<Tsubo I16 as <i>C. angulosa</i>) UTEX 618; SAG 4.72	AB701542
	NIES-884	<i>Chlamydomonas debaryana</i> Goroschankin var. <i>cristata</i> Ettl	<Nozaki (2000) <UTEX UTEX 1344; SAG15.72 [I=99.9% (1640/1641), d]	AB701543
	NIES-437	<i>Chlamydomonas fasciata</i> Ettl	<H-3-4-2 (Suda, around 1986)	AB701544
	NIES-2462	<i>Chlamydomonas globosa</i> J. Snow	< TkS0803C2 (Nakada, 2009) [I=99.9% (1641/1642), a]	AB753039
	NIES-2216	<i>Chlamydomonas inflexa</i> Pringsheim	<IAM C-253 <BIU-727 (Starr, 1967) UTEX 727; SAG 24.72; R.A. Lewin DD1/72	AB701545
	NIES-2218	<i>Chlamydomonas mexicana</i> Lewin	<IAM C-555 <UTEX (1996); (=IAM C-257 <BIU-730 (Starr, 1967)) SAG 11-60b as <i>C. oblonga</i>	AB701546
	NIES-2230	<i>Chlamydomonas orbicularis</i> Pringsheim	<IAM C-559 <UTEX (1996); (=IAM C-266 <BIU-218 (Starr, 1967)) SAG 11-19 [I=99.9% (1606/1607), b]; UTEX 218	AB701547
	NIES-2231	<i>Chlamydomonas proteus</i> Pringsheim	<IAM C-560 <UTEX 216 (1996); (=IAM C-271 <BIU-216 (Starr, 1967)) ATCC 30452; CCAP 11/21; SAG 41.72	AB701548
	NIES-2234	<i>Chlamydomonas rapa</i> Ettl	<IAM C-279	AB701549
	NIES-2235	<i>Chlamydomonas reinhardtii</i> Dangeard	<IAM C-9 <Tsubo R (-)	AB701550
	NIES-2236	<i>Chlamydomonas reinhardtii</i> Dangeard	<IAM C-239 <BIU-90 (Starr, 1967) CCAP 11/32A; SAG 11-32b; UTEX 90 [I=100% (1631/1631), Z]; NIBB 4014; CGC CC-1010; CGC CC-409; 137c mt+	AB701551
	NIES-2237	<i>Chlamydomonas reinhardtii</i> Dangeard	<IAM C-540 <Mihara (1991) CCAP 11/32CW15 [I=99.9% (1643/1644), W]; cw-15 strain	AB701552
	NIES-2238	<i>Chlamydomonas reinhardtii</i> Dangeard	<IAM C-541<Kuchitsu (1991); (=IAM C-238 <BIU-89 (Starr 1967)) CCAP 11/32B; SAG 11-32a [I=99.9% (1644/1645), X]; UTEX 89; NIBB 4013; CGC CC-1009; 137c mt -	AB701553
	PS-2708	<i>Chlamydomonas reinhardtii</i> Dangeard	<IAM C-238 <BIU-89 (Starr 1967); (=IAM C-541)	AB753040
	NIES-2239	<i>Chlamydomonas reinhardtii</i> Dangeard	<IAM C-562 <Tsubo (1996); (=IAM C-8 <Tsubo) Tsubo R (+)	AB701554
	NIES-2463	<i>Chlamydomonas reinhardtii</i> Dangeard	< KkS0801B1 (Nakada, 2006) [I=99.9% (1640/1641), Y]	AB701555
	NIES-2242	<i>Chlamydomonas sphaeroides</i> Gerloff	<IAM C-255 <BIU-221 (Starr, 1967) as <i>C. iyengari</i> UTEX 221; SAG 25.72	AB701556
	NIES-2246	<i>Chlamydomonas typica</i> Deason et Bold	<IAM C-565 <UTEX 971 (1996); (=IAM C-290 <BIU-971 (Starr, 1967)) SAG 61.72; UTEX 971; Deason T-2-11	AB701557

Table 1 Continued

Subgroup names defined by Nakada <i>et al</i> (2008)	Strain no.	Species (revised species are underlined) < Original designation	History (depositor, deposit year) Strain number in other culture collections, boldface indicating strains DNA sequences of which have been published, with sequence identities ¹ , if appropriate, in parentheses	Accession no.
	NIES-2248	<i>Chlamydomonas zebra</i> Korshikov ex Pascher	<IAM C-221 <BIU-229 (Starr, 1967) as <i>C. brannonii</i> SAG 8.72; UTEX 229; CCAP 11/3; Brannon5	AB701558
Uncertain phylogenetic position				
	NIES-2232	<i>Chlamydomonas pseudococcum</i> Lucksch	<IAM C-273 CCAP 11/23 as <i>Fusola</i> sp.; UTEX 214	–
	NIES-2245	<i>Chlamydomonas transita</i> Ettl	<IAM C-289 <BIU-1345 (Starr, 1967) SAG 60.72 as <i>C. boldii</i> ; Ettl No. 130	–
	PS-2677	<i>Chlamydomonas</i> sp.	<IAM C-622 (=C-130)	–

¹ I=percentage identities (identical positions/total positions compared), alphabetical characters indicating strains of the same origin in Figs. 2 & 3. See text.

reidentified to *Chlamydomonas* sp. in the present study as mentioned below in the ‘Revisions’ section), and three strains of *Chlamydomonas* sp., NIES-2319, 2320 and 2321, which belonged to *Oogamochlamys*. The identification of these strains may contribute to further understand *Chlamydomonas* classification.

Comparison of DNA sequences of the NIES strains and strains of the same origin

18S rDNA sequences have been previously published for 30 strains that originated from the same strains as the NIES strains, which were analyzed in the present study and are maintained in a depositor or other culture collections (Table 1, strains shown in boldface). Using “Identities,” we compared similarities between these published sequences and sequences of the corresponding NIES strains to confirm the taxonomic identity. Among 33 sequences registered for the 30 strains, two sequences were 100% identical to the corresponding sequences of the NIES strains, 24 sequences differed in 1–4 positions (Identities=99.9–99.8%), and 4 sequences differed in 8–22 positions (Identities=99.5–99.3%). On the other hand, three sequences differed in more than 90 positions (Identities=94.5–92.0%).

We assume that the differences were produced as a consequence of different sequencers used and/or a degree of accuracy in analyses when differences were small such as in less than 4 positions. In the case with differences in 8–22 positions, in addition to the aforementioned reasons, sequence incongruity

may occur through independent DNA substitutions in different subcultures, which were held in different culture collections for a long time. Two examples of sequence comparisons among the three strains of the same origin may support this assumption. The sequence of *Chlamydomonas noctigama* NIES-2229 differed in one and a total of 12 distinct positions from CCAP 11/17 and SAG 33.72, respectively, both of which originated from the same strain as NIES-2229 (Fig. 2, H). *Chlamydomonas mutabilis* NIES-2224 differed in one and a total of 22 distinct positions from SAG 34.72 (as *Cd. actinochloris*) and UTEX 578, respectively, both of which originated from the same strain as NIES-2224 (Fig. 3, R). Although these different positions were not always included in the phylogenetic analyses because they were sometimes outside of the region used for the analyses, these strains were included in their respective, strongly supported clades.

On the other hand, a comparison of sequences from NIES-2221 and CCAP 11/64B showed differences in 91 positions and both strains originated from the same strain Tsubo 24 (-). In addition, NIES-2221 and CCAP 11/64B belonged to markedly different clades within *Moewusinia* (Fig. 2, C). NIES-2221 did not belong to the originally identified *Cd. moewusii* var. *rotunda* clade, but to the *Cd. moewusii* clade. In addition, sexually complementary strains of NIES-2221 and CCAP 11/64B, which are NIES-2223 and SAG 11-61a respectively, and both originated from the same strain Tsubo 24 (+), belong to the *Cd. moewusii* var. *rotunda* clade (Fig. 2, B).

Therefore, we concluded that NIES-2221 had been replaced by another strain after it was transferred to Japan. The data also indicate that organisms identified as *Cd. moewusii* var. *rotunda* are clearly distinguished from those identified as *Cd. moewusii* (Fig. 2) by phylogenetic analysis of 18S rDNA, although the species name *Cd. moewusii* has sometimes been used for both organisms.

Larger sequence differences were observed in the following cases: 132 positions between *Chlamydomonas applanata* NIES-2205 and UTEX 2399 (Fig. 2, E), and 128 positions between *Chlamydomonas leiostraca* NIES-2217 and CCAP 11/49 (Fig. 2, A). Each strain belonged to a different subgroup defined by Nakada *et al.* (2008): *Cd. applanata* NIES-2205 in *Moewusinia* whereas UTEX 2399 in *Polytominia* (Fig. 2, E); *Cd. leiostraca* NIES-2217 in *Moewusinia* whereas CCAP 11/49 in *Polytominia* (Fig. 2, A). Since *Cd. applanata* was not identified to belong to the subgroup *Polytominia*, as its authentic strain CCAP 11/2, which belongs to *Polytominia*, we concluded that NIES-2205 had been replaced by another strain after it was transferred to Japan.

As a result, we withdrew two strains, *Cd. applanata* NIES-2205 and *Cd. moewusii* var. *rotunda* NIES-2221 from our collection list. *Cd. leiostraca* NIES-2217 could not be reidentified due to the unavailability of the authentic strain of the species in culture collections. Detailed phylogenetic, morphological, and comparative studies are required.

Revisions

Among the strains that belong to the eight subgroups, 15 strains were revised (depicted as underlined words in Table 1) as follows:

***Moewusinia*:** Two strains, *Chlamydomonas simplex* NIES-2241 and *Cd. dorsoventalis* NIES-2213 were revised to *Cd. moewusii* and *Cd. noctigama*, respectively (Table 1), based on the phylogenetic analysis results that classified them in the subclades *Cd. moewusii* and *Cd. noctigama*, respectively, with a high bootstrap support (100% and 96%, respectively, in the ML tree, Fig. 2; 99% for both in the NJ tree). In addition, published sequences of CCAP 11/26 and CCAP 11/4 that originated from the same strains as NIES-2241 and 2213, respectively, also showed that these strains belonged to *Cd. moewusii* and *Cd. noctigama*, respectively (Fig. 2, F & J). These findings indicate the possibility of errors, such as mislabeling, during the time since their orig-

inal isolation can probably be excluded. Therefore, we retained these two strains in our collection list with a revision of the species names. Two strains of *Cd. parkeae*, NIES-1022 and 1733, were downgraded to *Chlamydomonas* sp. (Table 1), because the phylogenetic analysis results showed that, along with two other strains, NIES-2323 and 2324, they constituted a distinctly different subclade from *Cd. parkeae* (100% bootstrap value in the ML tree, Fig. 2, and 99% in the NJ tree).

***Stephanosphaerinia*:** *Chlamydomonas debaryana* NIES-2211 was revised to *Chlamydomonas* sp. The species *Cd. debaryana* are included in the subgroup *Reinhardtinia* (Nakada *et al.*, 2008; cf. 'Reinhardtii-clade' in Pröschold *et al.*, 2001); however, this strain was not classified as such. In addition, the published sequence of CCAP 11/1, which originated from the same strain as NIES-2211, is highly similar to the NIES-2211 sequence (Fig. 2, K, Table 1). Therefore, we concluded the possibility of errors, such as mislabeling, since its original isolation could probably be excluded. The strain was added to our collection list with revision, although a specific name for the strain could not be determined.

***Monadinia*:** *Chlamydomonas coccooides* NIES-1021 was revised to *Cd. kuwadae* because this strain was shown to be included in a subclade that consisted of *Cd. kuwadae* with high bootstrap support (100% in the ML tree, Fig. 2, and 99% in the NJ tree) based on the results of phylogenetic analyses.

***Oogamochlamydia*:** Recently, Pröschold *et al.* (2001) established two new genera, *Oogamochlamys* and *Lobochlamys*, in this subgroup and redefined the species included in these genera. In this revision, two strains, *Lobochlamys segnis* SAG 17.72 and *Lb. culleus* SAG 18.72, that originated from the same strains as NIES-2214 and 2209, respectively, were included. According to this revision (Pröschold *et al.* 2001) and based on the phylogenetic analyses performed in the present study, the four NIES strains were revised, as follows (Table 1): Both *Cd. segnis* NIES-2240 (Fig. 3, V) and *Cd. fimbriata* NIES-2214 (Fig. 3, U) were revised to *Lb. segnis*, and two strains of *Cd. culleus*, NIES-2209 (Fig. 3, T) and 2210, were revised to *Lb. culleus*.

***Chloromonadinia*:** Pröschold *et al.* (2001) emended the genus *Chloromonas* and revised several species (including subspecies) within the genus. In this revision, *Chlamydomonas augustae*, and two varieties of the species, *Cd. augustae* var. *eupapillata* (the

authentic strain, SAG 5.73) and *Cd. augustae* var. *ellipsoidea* (the authentic strain, SAG 9.87) were revised together to *Chloromonas augustae*; both *Cd. actinochloris* (represented by SAG 1.72) and *Cd. mutabilis* (represented by SAG 34.72) were emended to *Chloromonas actinochloris*; *Cd. chlorococcoides* (represented by SAG 46.72) was revised to *Chloromonas carrizoensis*. According to these revisions, and based on our phylogenetic analysis, four NIES strains included in this subgroup were revised (Table 1): *Cd. augustae* var. *ellipsoidea* NIES-158 was revised to *Cm. augustae*. *Chlamydomonas actinochloris* NIES-2201 (Fig. 3, Q) and *Cd. mutabilis* NIES-2224 (Fig. 3, R) were revised to *Cm. actinochloris*. *Chlamydomonas chlorococcoides* NIES-2575 (Fig. 3, P) was revised to *Cm. carrizoensis* (Fig. 3, P). In addition, *Chlamydomonas applanata* NIES-2203 (= IAM C-216) was revised and downgraded to *Chloromonas* sp. (Table 1), because authentic strain of *C. applanata* (CCAP 11/2) is included in *Polytomia* (Fig. 2). It is not unlikely that NIES-2203 has been replaced by another strain. However, we included this strain in our collection list as *Chloromonas* sp., because the sequence is unique at present and no relevant sequences have been published.

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