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Two Species of *Chlamydomonas* (Volvocales, Chlorophyceae) New to Japan

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Chlamydomonas is a unicellular volvocalean genus with two equal flagella and single or multiple pyrenoids in the chloroplast. In this study, culture strains originating from two localities in Japan were identified as *Chlamydomonas* (*Cd*.) *perpusilla* (Korshikov) Gerloff var. *perpusilla* and *Cd*. *pumilio* H. Ettl var. *pumilio* based on light microscopy. Neither species has been previously recorded in Japan. Molecular phylogenetic analyses based on 18S ribosomal RNA genes showed that *Cd*. *perpusilla* is closely related to *Chlorogonium*, and *Cd*. *pumilio* formed a clade with some *Chlamydomonas* species and *Polytoma*.

Key words: *Chlamydomonas perpusilla*, *Chlamydomonas pumilio*, culture strain, taxonomy.

Chlamydomonas Ehrenb. is a unicellular volvocalean genus that is traditionally characterized by having two equal flagella and single or multiple pyrenoids in the chloroplast and lacking specialized features in protoplasts and cell walls (Ettl 1976, 1983, Melkonian and Preisig 2000). Pröschold et al. (2001) amended the *Chlamydomonas* genus and restricted it to few species closely related to their proposed "conserved type species", *Cd*. *reinhardtii* P. A. Dang. However, around 400 other species have not yet been reclassified at the genus level. To advance the natural classification of "*Chlamydomonas*", more phylogenetic studies of morphologically characterized strains are needed.

Although more than 400 species of *Chlamydomonas* were recorded from various freshwater habitats worldwide (Ettl 1976, 1983), just less than 30 Japanese species have been identified to the species level (Akiyama et al. 1977, Ichimura 1997, Nozaki 2000, Kasai et al. 2004, Pocock et al. 2004). Most of these Japanese species have been recorded without culture strains, and their taxonomic re-examination and determination of phylogenetic position within Volvocales are thus almost impossible.

Recently, we isolated strains of two species of *Chlamydomonas* and identified them as *Cd*. *perpusilla* (Korshikov) Gerloff var. *perpusilla* and *Cd*. *pumilio* H. Ettl var. *pumilio*, both of which are new to Japan. Morphology, taxonomy and 18S ribosomal RNA (*r*RNA) gene phylogeny of these two Japanese algae are described in this report.

Materials and Methods

The soil samples from which *Cd*. *perpusilla* var. *perpusilla* SkCr-10 and SkCl-3 were isolated originated from the bottom of Sakataga-ike Pond (N35°49′6″E140°16′26″, pH 7.1, 22.1°C for SkCr-10 and N35°49′

Table 1. List of 18S *r*RNA gene sequences of *Chlamydomonas* taxa obtained in this study

Taxa	Strain designation	Accession number
Cd. perpusilla var. *perpusilla*	SkCr-10 (= NIES-1849)	AB290339
Cd. pumilio var. *pumilio*	ArC-7 (= NIES-1850)	AB290340
Cd. sordida	SAG 18.73*	AB290341

*Schlösser (1994).

10″E140°16′28″, pH 6.2, 17.5°C for SkCl-3), Ootake, Narita-shi, Chiba, on 23 July, 2003. A small amount of the dried soil samples were re-wetted with ion-exchanged water in a Petri dishes. *Cd. pumilio* var. *pumilio* ArCp-7 was isolated from a water sample collected from a pond (approximately N35°38′55″E139°43′40″, pH 7.2, 18.0°C) in Arisugawanomiya Memorial Park, Minato-ku, Tokyo, on 28 April, 2003. Clonal cultures were established from the water sample or re-wetted soil samples in Petri dishes using the pipette-washing method (Pringsheim 1946). For comparison, a strain (SAG 18.73) labeled "*Cd. pumilio*" was obtained from Sammlung von Algenkulturen der Universtät Göttingen (SAG; Schlösser 1994). The cultures were grown in screw-cap tubes (18 × 150 mm) containing 9–10 mL of MG medium (Kasai et al. 2004) and maintained at about 20°C, with an alternating 14 h light / 10 h dark cycle, at a light intensity of about 130–200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool white fluorescent lamps. SkCr-10, SkCl-3 and ArCp-7 were deposited in the Microbial Culture Collection at the National Institute for Environmental Studies (NIES) as NIES-1849, -1848 and -1850, respectively.

Because *Cd. perpusilla* was shown to be closely related to some species of *Chlorogonium* (Fig. 20) for which pyrenoid stability is a key distinguishing character at the species level (Nozaki et al. 1998), we determined whether the pyrenoids were stable or unstable (Nozaki et al. 1994, 1995) in *Cd. perpusilla* SkCr-10. The cells were grown photoheterotrophically in AF-6 medium (Kato 1982, Kasai et al. 2004) supplemented with major organic compounds (modified acetate medium: 400 mg/L each of sodium acetate·$3H_2O$, glucose, bacto yeast extract, and bacto tryptone) as described by Nozaki et al. (1995). Light microscopy was carried out using an Olympus BX60 microscope equipped with Nomarski interference optics.

For phylogenetic analyses, partial 18S *r*RNA genes from SkCr-10, ArCp-7 and SAG 18.73 (Table 1) were sequenced as described previously (Nozaki et al. 1997, Fawley and Fawley 2004, Nakada and Nozaki 2007). 18S *r*RNA gene sequences were aligned by Clustal X (Thompson et al. 1997), and manually refined. The rRNA secondary structure of *Volvox carteri* F. Stein f. *nagariensis* M. O. P. Iyengar (Rausch et al. 1989) was used as a reference for the alignment. The region used for phylogenetic analyses corresponded to positions 57-1732 of the *V. carteri* f. *nagariensis* (Rausch et al. 1989). Two alignments were constructed. One ("volvocalean alignment") included 38 volvocalean OTUs (Fig. 19) and the other ("*Dunaliella* alignment") included 33 OTUs (Fig. 20) selected from *Dunaliella* and *Lobocharacium* lineages *sensu* Buchheim et al. (2002). *Carteria crucifera* Korshikov and *Lobocharacium* lineage were designated as outgroups for the volvocalean and *Dunaliella* alignments, respectively, in accordance with previous phylogenetic studies (Pröschold et al. 2001, Buchheim et al. 2002, Nozaki et al. 2003).

These two alignments were subjected to maximum likelihood (ML), most parsimonious (MP) and neighbor-joining (NJ) analyses that were performed using PAUP 4.0b10 (Swofford 2002). For ML analyses, we applied a TrN + I + G model for volvocalean alignment and a TrNef + I + G model for *Dunaliella* alignment selected by hLRT using Modeltest 3.7 (Posada and Crandall 1998). The phylogenetic analyses were performed as described previously (Nakada and Nozaki 2007), except that bootstrap probabilities (BP) of ML analyses were performed using a subtree pruning-regrafting branch-swapping algorithm.

For Bayesian analyses, we applied the GTR + I + G model for volvocalean alignment and the SYM + I + G model for *Dunaliella* alignment selected by hLRT using MrModeltest 2.2 (Nylander 2004). Posterior probabilities (PP) were calculated based on the Bayesian analyses with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), as described previously (Nakada and Nozaki 2007), except for the generation of Markov chain Monte Carlo (MCMC) iterations (1,500,000 generations for volvocalean alignment and 1,000,000 generations for *Dunaliella* alignment). The average standard deviation of split frequencies of the two MCMC iteration runs was below 0.01 for each analysis, indicating convergence.

Results and Discussion

Taxonomic accounts

Chlamydomonas perpusilla (Korshikov) Gerloff var. **perpusilla**: Gerloff (1940), p. 471. [Figs. 1–8, 21]

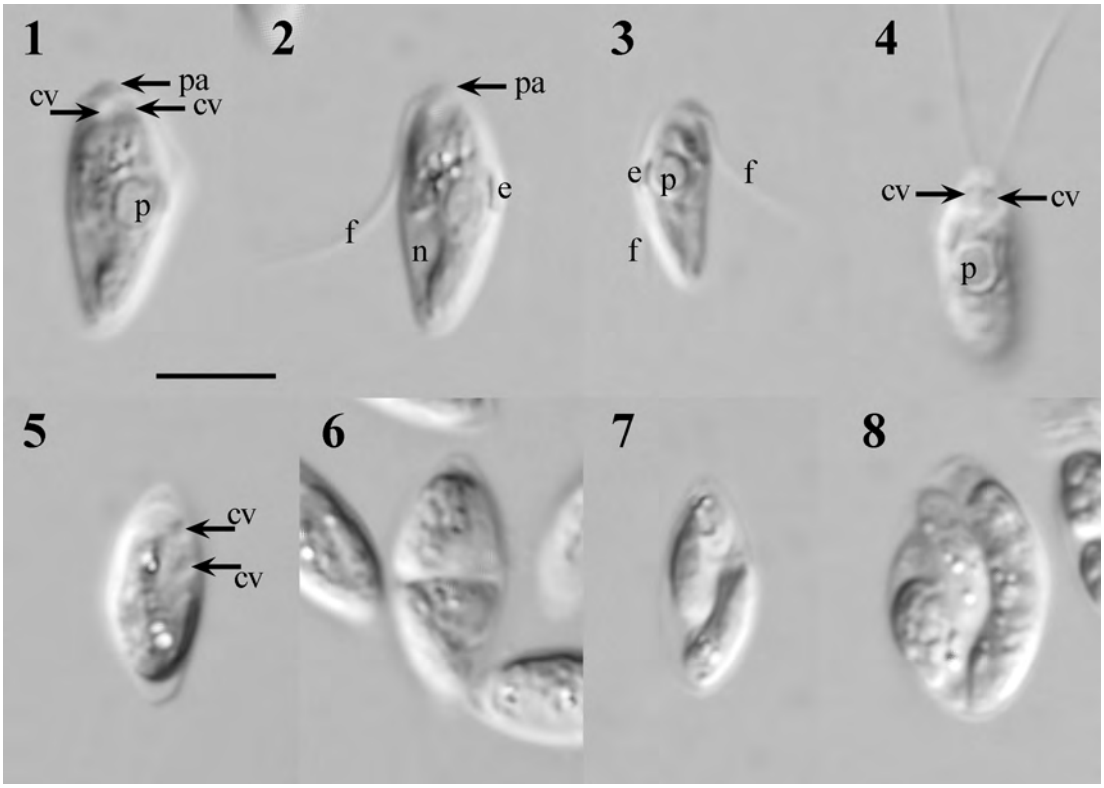
Chlamydomonas minima Korshikov in Pascher non J. Schiller (1925). Korshikov in Pascher (1927), p. 280, fig. 241.

Cells biflagellate, with a thin cell wall, fusiform with blunt anterior and posterior ends (Figs. 1–3). Anterior papilla semicircular and inconspicuous (Figs. 1–3). Cells asymmetric, with a nearly straight side ("ventral" side) and more convex side ("dorsal" side) (Figs. 1–3). Two contractile vacuoles located in the anterior end of the protoplast (Fig. 1). Chloroplast parietal, filling the dorsal side of the protoplast, reaching to the anterior and posterior ends of the protoplast, with a single large pyrenoid positioned centrally (Figs. 1–3). Pyrenoid nearly spherical, stable (Figs. 1, 3, 4). Nucleus located posterior to the pyrenoid on the ventral side of the cell (Fig. 2). Stigma single, elliptical to pear-shaped, positioned in the anterior 1/4–1/2 of the cell (Figs. 2, 3). Flagella as long as or slightly longer than the cell length (Figs. 2, 3). Cells 6–11 μm in length, 2–4 μm in width. Asexual reproduction accomplished by formation of two or four zoospores (Figs. 7, 8). The first cell division transverse, following loss of flagella and movement of two contractile vacuoles and the nucleus toward the division plane (Figs. 5, 6).

Strains examined: SkCr-10 and SkCl-3.

Distribution: British Isles (Pentecost 2003), Czech Republich (Ettl 1958), Romania (Péterfi and Péterfi 1966), Russia (Dedusenko-Ŝegoleva et al. 1959), Tajikistan (Vaulina et al. 1959), Ukraine (Pascher 1927), USA (Alaska; Hortobágyi and Hilliard 1965) and Japan.

Remarks: The Japanese isolate was almost identical to Korshikov's original description (Pascher 1927) in that it possesses fusiform and asymmetric vegetative cells, two anterior contractile vacuoles, parietal chloroplast with a single central pyrenoid and a stigma, and basal nucleus (Figs. 1–3, 21). Both Japanese material and Ukrainian material by Korshikov (Pascher 1927) show the first transverse cell division during asexual reproduction (Fig. 6). Two other varieties have been described in *Cd. perpusilla* (Ettl 1976), *Cd. perpusilla* var. *limnicola* (Kol) Nakada & Nozaki, comb. nov. (see nomenclature) and *Cd. perpusilla* var. *monovacuolata* Fott & H. Ettl. *Cd. perpusilla* var.



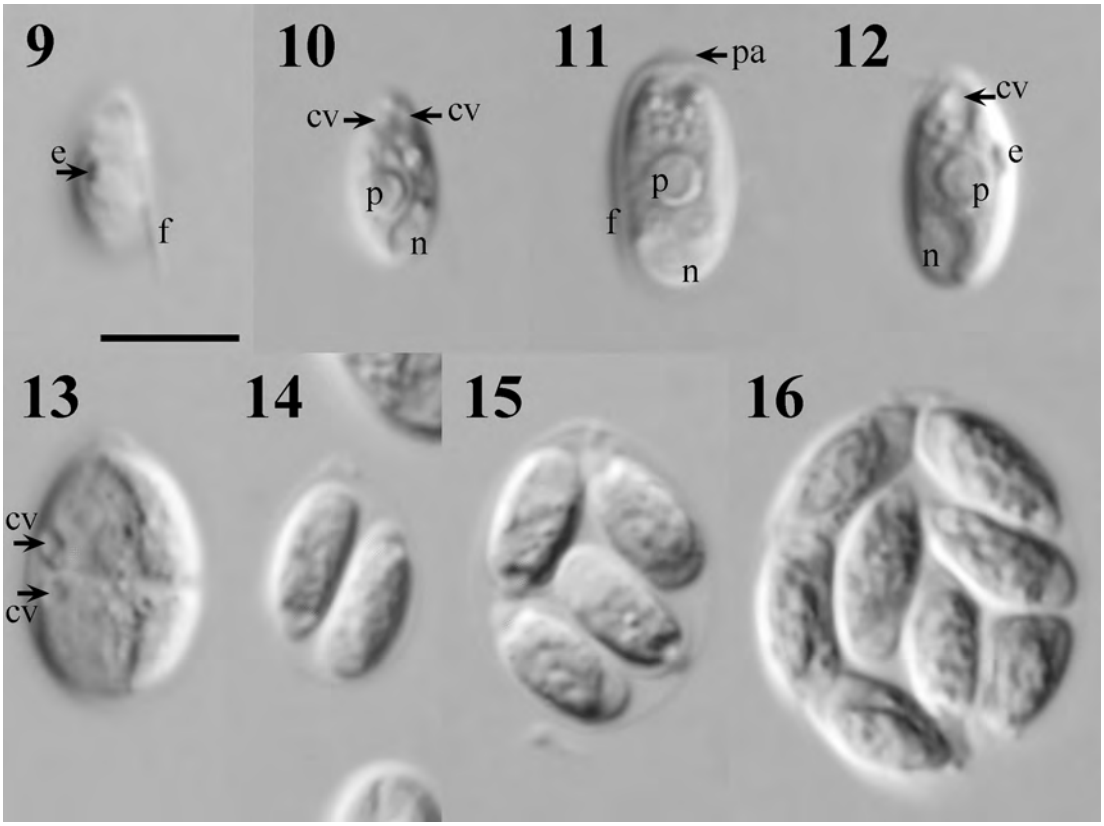
Figs. 1–8. *Chlamydomonas perpusilla* (Korshikov) Gerloff var. *perpusilla* (SkCr-10). Nomarski interference microscopy shown at the same magnification. Figs. 1–4. Vegetative cells. Figs. 1–3. Cells grown photoautotrophically (3 days old in MG medium). Fig. 4. Cell grown photoheterotrophically (24-h culture in the modified acetate medium), showing stable pyrenoids. Figs. 5–8. Asexual reproduction. Fig. 5. Cell at the beginning of asexual reproduction. Note two contractile vacuoles on the way to the division plane. Fig. 6. Transverse first cell division. Fig. 7. Two daughter cells in the parental cell wall. Fig. 8. Four daughter cells in the parental cell wall. Abbreviations: pa, papilla; cv, contractile vacuole; p, pyrenoid; f, flagellum; n, nucleus; e, stigma. Scale = 5 μm.

limnicola is different from the type variety including the present Japanese strain in its tapered anterior end of the protoplast and reduced chloroplast, which is distant from both anterior and posterior end of the protoplast (Kol 1938). *Cd. perpusilla* var. *monovacuolata* has only a single contractile vacuole (Fott and Ettl 1959), while the type-variety has two contractile vacuoles (Fig. 1; Pascher 1927). *Chlamydomonas fusus* H. Ettl is also similar to *Cd. perpusilla* in possessing fusiform vegetative cells, two anterior contractile vacuoles, parietal chloroplast with a single central pyrenoid and a stigma, and a basal nucleus (Ettl 1965). However, *Cd. fusus* has symmetric vegetative cells (Ettl 1965), while *Cd. perpusilla* has asymmetric vegetative cells (Figs. 1–3; Pascher 1927).

Chlamydomonas pumilio H. Ettl var. **pumilio**: Ettl (1965), p. 382, fig. 76.

[Figs. 9–16, 22]

Cells biflagellate, with a thin cell wall, oblong, ellipsoidal to ovoid (Figs. 9–12). Anterior papilla keel-like. Two contractile



Figs. 9–16. *Chlamydomonas pumilio* H. Ettl var. *pumilio* (ArCp-7). Nomarski interference microscopy shown at the same magnification. Cells grown photoautotrophically (3 days old in MG medium). Figs. 9–12. Vegetative cells showing variations of cell shape. Figs. 13–16. Asexual reproduction. Fig. 13. Transverse first cell division. Note two contractile vacuoles positioned across the division plane. Fig. 14. Two daughter cells in the parental cell wall. Fig. 15. Four daughter cells in the parental cell wall. Fig. 16. Eight daughter cells in the parental cell wall. For abbreviations, see Figs. 1–8. Scale = 5 μm.

vacuoles located near the anterior end of the protoplast (Fig. 10). Chloroplast parietal, reaching to the both anterior and posterior ends of the protoplast, with a single large pyrenoid positioned centrally (Figs. 10–12). Pyrenoid nearly spherical (Figs. 10–12). Nucleus located in the basal region of the protoplast (Figs. 10–12). Stigma single, circular to elliptical, positioned in the anterior 1/3–1/2 of the cell (Figs. 9, 12). Flagella as long as or slightly longer than the cell length (Figs. 9, 11). Cells 6–10 μm in length, 2–5 μm in width. Asexual reproduction accomplished by formation of two, four or eight zoospores (Figs. 14–16). The first cell division transverse, following loss of flagella and movement of two contractile vacuoles and the nucleus toward the division plane (Fig. 13).

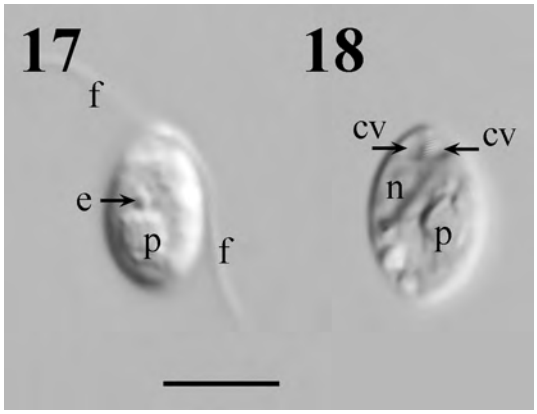
Strain examined: ArCp-7.

Distribution: Austria (Ettl 1968), Czech Republich (Ettl 1965), Spain (Cambra and Hindák 1998), Brazil (Bicudo 2004) and Japan.

Remarks: The Japanese isolate was almost identical to the original description (Ettl 1965) in that it possesses more or less ellipsoidal vegetative cells with keel-like papilla,

two anterior contractile vacuoles, parietal chloroplast with a single central pyrenoid and a stigma, and basal nucleus (Figs. 9–12). Ettl (1968) described a single variety, *Cd. pumilio* var. *ovoidea* H. Ettl. *Chlamydomonas pumilio* var. *ovoidea* has regularly ovoid or ovoid-ellipsoidal small vegetative cells measuring 3.5–5 μm in length (Ettl 1968), and is distinguished from the type variety which has oblong, ellipsoidal to ovoid vegetative cells measuring 4.5–10 μm in length (Figs. 9–12; Ettl 1965). *Cd. asymmetrica* Korshikov var. *minima* Bourr., *Cd. aggregata* Deason & H. C. Bold and *Cd. kakosmos* F. Moewus are similar to *Cd. pumilio* in that they possess small (5–11 μm in length) ellipsoidal vegetative cells, two anterior contractile vacuoles, parietal chloroplast with a single pyrenoid, and basal nucleus (Ettl 1976, 1983). However, *Cd. asymmetrica* var. *minima* has asymmetrical cells, and neither *Cd. aggregata* nor *Cd. kakosmos* has distinct papilla on the anterior end of the cell (Ettl 1976, 1983).

Because SAG 18.73 was previously identified as "*Cd. pumilio*" (Schlösser 1994), we also observed SAG 18.73 for comparison. However, SAG 18.73 was clearly different from *Cd. pumilio* as SAG 18.73 has cells with rounded papilla and anterior nucleus (Fig. 18) while *Cd. pumilio* has a keel-like papilla and a nucleus that is always positioned in the basal region of the protoplast (Figs. 10–12; Ettl 1965). Therefore, we identified strain SAG 18.73 as *Cd. sordida* H. Ettl (Figs. 17, 18). *Cd. sordida* SAG 18.73 was phylogenetically distant from *Cd. pumilio* (Fig. 19).



Figs. 17–18. *Chlamydomonas sordida* H. Ettl (SAG 18.73 "*Cd. pumilio*"). Nomarski interference microscopy of the vegetative cells, shown at the same magnification. Cells grown photoautotrophically (3 days old in MG medium). For abbreviations, see Figs. 1–8. Scale = 5 μm.

Phylogenetic Analyses

Based on the volvocalean alignment, several major lineages of the Volvocales were resolved (Fig. 19). For example, a *Dunaliella* lineage (Buchheim et al. 2002) was resolved with moderate statistical support (with BP of 71%, 76% and 76% in the ML, MP and NJ analyses, respectively, and 1.00 PP). The *Lobocharacium* lineage (Buchheim et al. 2002) was sister to the *Dunaliella* lineage in ML, NJ and Bayesian trees, but this relationship was supported only by 57% BP of NJ analysis. *Chlamydomonas perpusilla* and *Cd. pumilio* were included within the *Dunaliella* lineage. Although *Cd. sordida* SAG 18.73 was previously identified as "*Cd. pumilio*" (Schlösser 1994), it was not related to *Cd. pumilio* but included within the *Tetracystis* lineage (100% BP in ML, MP and NJ analyses, and 1.00 PP).

In the phylogenetic analyses based on the *Dunaliella* alignment (Fig. 20), *Cd. perpusilla* was included within the '*Chlorogonium*'-Clade (Pröschold et al. 2001) with moderate statistical support (BP of 86%, 75% and 59% in the ML, MP and NJ analyses, respectively, and 1.00 PP). In the '*Chlorogonium*'-Clade, *Cd. perpusilla*, three *Chlorogonium* species, chlamydomonad sp. NDem 9/21 T-14d and two 18S *r*RNA gene sequences derived from unidentified organisms formed a clade with moderate support (BP of 66%, 58% and 74% in the ML, MP

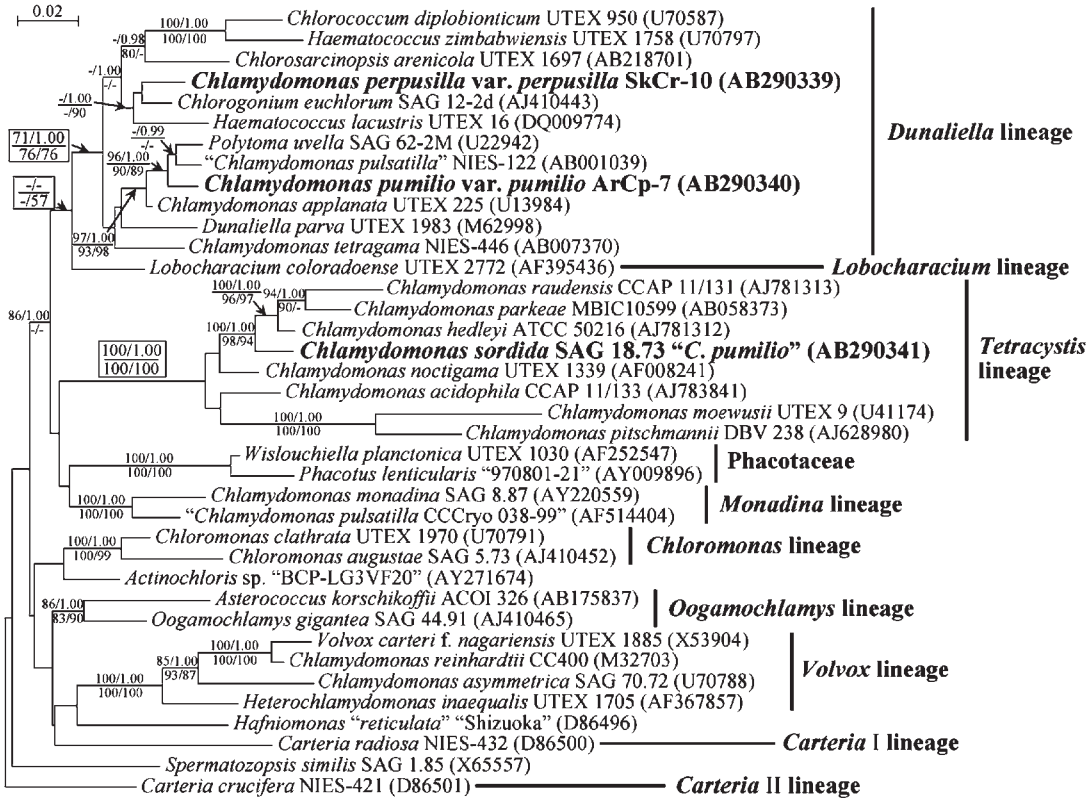


Fig. 19. Maximum likelihood (ML) tree based on the aligned 18S *r*RNA genes from volvocalean OTUs (volvocalean alignment). Numbers indicate bootstrap values from ML (top left), most parsimonious (bottom left), neighbor-joining (bottom right) analyses, and Bayesian posterior probabilities (top right). Bootstrap values ≥80% and Bayesian PPs ≥0.95 are shown except for some nodes discussed in the text (boxed). Branch lengths represent nucleotide substitutions per site. Accession numbers are shown right to each OTUs.

and NJ analyses, respectively, and 0.93 PP). *Chlamydomonas perpusilla* was closely related to chlamydomonad sp. NDem 9/21 T-14d and uncultured eukaryotic picoplankton clone VN9 (>85% BP in the ML, MP and NJ analyses, and 1.00 PP).

The 18S *r*RNA gene sequences of chlamydomonad sp. NDem 9/21 T-14d and uncultured eukaryote VN9 are very similar to that of *Cd. perpusilla* (99.6% over 1688 bp and 98.9% over 887 bp, respectively). The similarity between *Cd. perpusilla* SkCr-10 and chlamydomonad sp. NDem 9/21 T-14d is comparable with intraspecific variation of volvocalean algae. For example, the similarity between two strains of *Chlorogonium elongatum* (P. A. Dang.) Francé, UTEX 2561 and IAM C-293, is 99.3% over 1687 bp. Therefore, *Cd. perpusilla* SkCr-10 and chlamydomonad sp. NDem 9/21 T-14d are probably conspecific or closely related, and detailed morphological observation of strain NDem 9/21 T-14d would be helpful in resolving its taxonomic relationship to *Cd. perpusilla*.

Chlamydomonas pumilio was included within the '*Polytoma*'-Clade (Pröschold et al. 2001) with strong statistical support (BP of 95%, 92% and 100% in the ML, MP and NJ analyses, and 1.00 PP). *Chlamydo-*

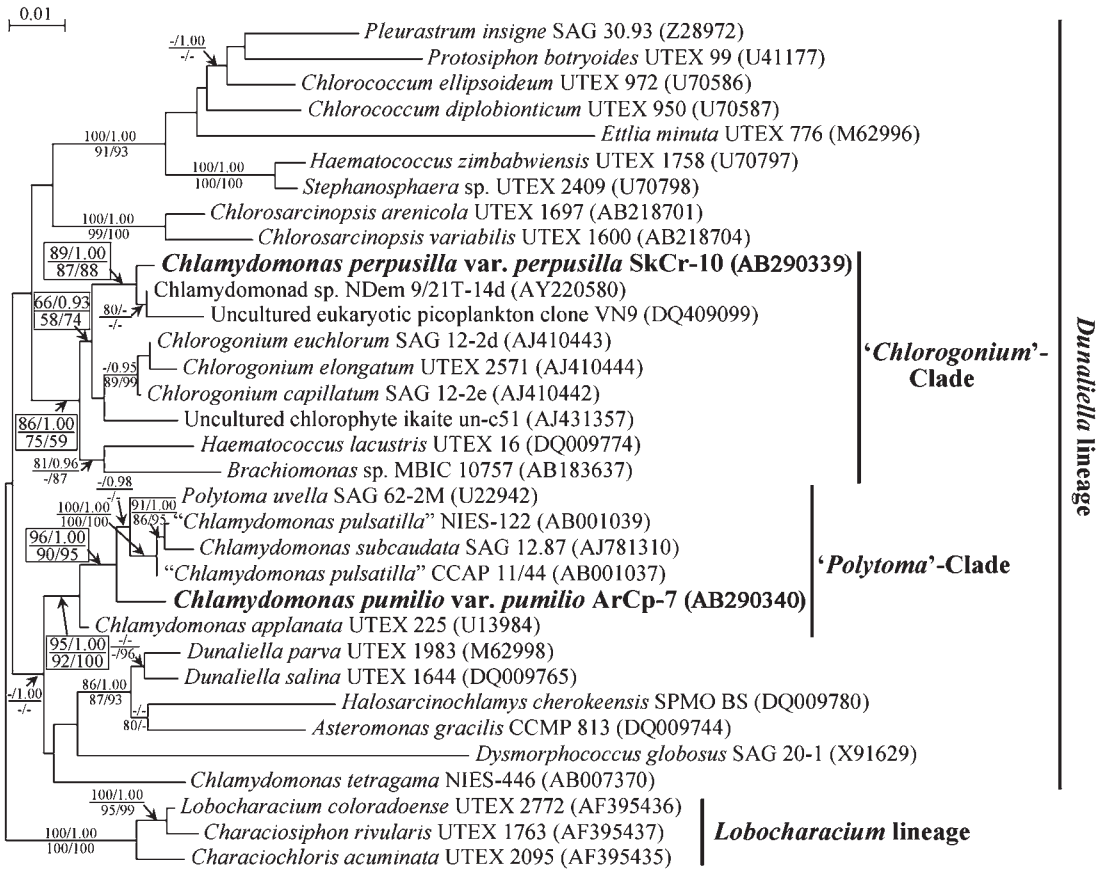


Fig. 20. Maximum likelihood (ML) tree based on the aligned 18S *r*RNA genes of OTUs of *Lobocharacium* and *Dunaliella* lineages (*Dunaliella* alignment). For details, see Fig. 19.

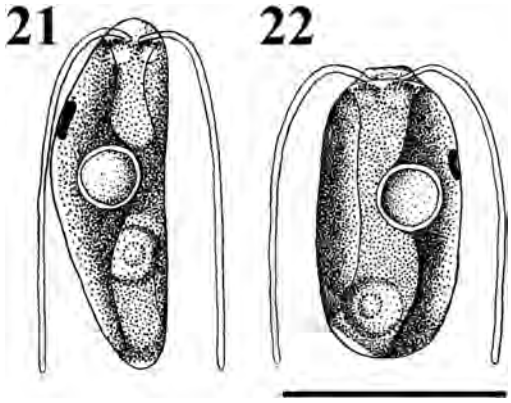
monas pumilio formed a clade with three strains of *Chlamydomonas* (NIES-122, CCAP 11/44 and SAG 12.87) and with *Polytoma uvella* (≥90% BP in the ML, MP and NJ analyses, and 1.00 PP). Though strains NIES-122 and CCAP 11/44 were identified as "*Cd. pulsatilla*" (Ichimura 1997, Thompson et al. 1988), they were not monophyletic (Fig. 20). Another 18S *r*RNA gene sequence of "*Cd. pulsatilla* CCCryo 038-99" was distantly related to the *Dunaliella* lineage and closely related to *Cd. monadina* F. Stein to form the *Monadina* lineage (Fig. 19). Therefore, the strains labeled "*Cd. pulsatilla*" are to be re-examined.

Conclusions

The first phylogenetic analyses of 18S *r*RNA gene sequences of *Cd. perpusilla* and *Cd. pumilio* showed that they represent new lineages of *Chlamydomonas*. More than 100 other organisms related to Volvocales represented by unique 18S *r*RNA sequences have been already reported (e.g., Fawley et al. 2004), and morphological studies on such organisms are necessary for the comprehensive revision of the genus *Chlamydomonas*.

Nomenclature

Chlamydomonas perpusilla (Korshikov) Gerloff var. **limnicola** (Kol) Nakada & Nozaki, comb. nov.



Figs. 21–22. Line drawings of vegetative cells of two species of *Chlamydomonas*, shown at the same magnification. Fig. 21. *Cd. perpusilla* (Korshikov) Gerloff var. *perpusilla*. Fig. 22. *Cd. pumilio* H. Ettl var. *pumilio*. Scale = 5 μm.

Basionym: *Chlamydomonas minima* Korshikov var. *limnicola* Kol in Arb. Ung. Biol. Forsch.-Inst. **10**: 168 (1938).

Chlamydomonas perpusilla (Korshikov) Gerloff var. "*limicola*" Hub.-Pest., nom. nud.

Huber-Pestalozzi (1961) considered the original spelling of the variety "*limnicola*" (dweller in water) as an error for "*limicola*" (dweller on mud). However, there is no clear evidence to indicate the original spelling was in fact an error (Kol 1938, Huber-Pestalozzi 1961). Huber-Pestalozzi failed to indicate the full and direct reference to the basionym, and thus did not validate the intended new combination (Huber-Pestalozzi 1961). Therefore, the combination, *Chlamydomonas perpusilla* var. *limnicola*, is here validated.

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仲田崇志，野崎久義：**コナミドリムシ属（*Chlamydomonas*；緑藻綱，オオヒゲマワリ目）の日本新産 2 種について**

コナミドリムシ属（*Chlamydomonas*）は単細胞二鞭毛性でピレノイドを有するオオヒゲマワリ目（Volvocales）藻類である．本邦千葉県成田市，東京都渋谷区の池より分離された株は，光学顕微鏡観察に基づき日本未記録種の *Chlamydomonas perpusilla* (Korshikov) Gerloff var. *perpusilla* (新称：チョビコナミドリ)と *Cd. pumilio* H. Ettl var. *pumilio* (新称：アリスガワコナミドリ)に同定された．18S リボソーマル RNA 遺伝子を用いた系統解析の結果，チョビコナミドリはヤリミドリ属（*Chlorogonium*）と，アリスガワコナミドリは幾つかのコナミドリムシ属やイロナシコナヒゲムシ属（*Polytoma*）と単系統群をなすことが示された．

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