

Article

Description of *Limnomonas* gen. nov., *L. gaiensis* sp. nov. and *L. spitsbergensis* sp. nov. (Chlamydomonadales, Chlorophyta)

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Abstract: Two novel *Chlamydomonas*-like species, belonging to the *Moewusii* clade, have been described. The first species inhabits eutrophic and neutral to basic pH waters in Sweden and England. It is easily recognizable under a light microscope due to its morphology (a small green prolate spheroidal shape with a large and truncated papilla at its anterior end, two equal flagella, a single lateral eyespot, a basal nucleus, and a well-defined pyrenoid) and to its peculiar whole-body pendulum movement while resting on surfaces or attached to floating particles. The species occurs as free-living individuals and is able to gather temporarily into groups of individual cells. No particular binding structures or palmelloid cells were observed in cultures. The second species, previously assigned to *Chlamydomonas* cf. *proboscigera*, was collected from persistent snow in Svalbard, Norway. Its morphology is revised herein. Using SSU rDNA sequence analyses, these two species formed a well-supported clade. Moreover, ITS-2 secondary structure analyses confirmed sexual incompatibility between these biological species. Considering these results, a new genus *Limnomonas* and its type species *L. gaiensis* and *L. spitsbergensis* are proposed.



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Keywords: genetic fingerprint; lake-dwelling microalgae; morpho-species; organism ultrastructure; swimming behavior; *Chlamydomonas*

1. Introduction

Species of the genus *Chlamydomonas* Ehrenberg 1833 are an important component of the phytoplanktonic diversity in forest lakes in Sweden [1]. They usually bloom in April–May [2] and are observed in lower abundance during cooler and darker periods of the year [1,2]. Long-term monitoring provides a rich database of the variation of environmental parameters and organismic diversity and abundances (e.g., environmental databases from Länsstyrelsen and from Swedish Universities). It is of the utmost importance to investigate the biodiversity, organism dispersal, and the invasion of new species.

However, certain analyses remain quite constrained both spatially and taxonomically. On one hand, intensive monitoring provides meticulous descriptions of new taxa [3], phytoplanktonic diversity, and trends [4,5] in few targeted lakes, often taking a life-time to achieve [6]. On the other hand, large-scale monitoring using intensive sampling often lacks a description at the species level due to technical limitations (e.g., time allocated, expertise of the investigator for each taxon, and standardization of the monitoring method using preservative agents that impede the observation of organismal movement). For instance, a closer look at the Chlorophyceae does not grasp all the taxonomic levels of *Chlamydomonas*-like species often reported as Chlamydomonadales, *Chlamydomonas* sp., or green algae/dots (e.g., <http://miljodata.slu.se/mvm/> (accessed on 4 May 2022); [7,8]). Deep organismic taxonomy requires diverse microscopic observations and a comparison with taxonomic keys, which is a time-consuming process that often requires experts. Alternatively, genetic

barcoding is employed to assess the diversity present in environmental samples or to rapidly identify organisms of interest. However, the technique remains limited by the number, diversity, and quality of available sequences in genetic databases [9]. A solution is the combination of morphological and phylogenetic analyses [10]. In addition, for most of the algal lineages, no universal barcode marker has been proposed.

Chlamydomonas is known to be a large polyphyletic genus comprising hundreds of species collected from different environments [11]. Most of these have been described morphologically [11,12] and some have been characterized genetically (e.g., [13–15] and see references therein). The genus was revised several times with the recognition of at least eight major monophyletic lineages [13]. More recently, a study of the *Moewusii* clade (*Moewusinia sensu* Nakada et al. [14]) showed substructures composed of five distinct lineages sustained by phenotypic traits and molecular phylogenetic analyses [16]. It also reported a few unresolved species originally assigned as *Chlamydomonas*, of which strains either occurred singly or regrouped within defined lineages [16].

The particularities of the novel species investigated here are its rather small body size, its peculiar whole-body pendulum motion, and its capacity to co-occur and interact with the potentially harmful *Microcystis* species. Due to their distinctive morphological traits, the species can be spotted quickly in live samples. Strains were collected in eutrophic lakes in which *Chlamydomonas* sp. were previously reported (miljodata.slu.se; 2018) but without being described. Moreover, its occurrence is of economic relevance as it is able to live in neutral-basic pH waters, commonly used for recreational purposes. In the present study, the morphology, cell ultrastructure, pendulum motion, and taxonomy of this taxon were investigated using diverse microscopic observations and phylogenetic analyses. In light of the results, a new name is proposed to characterize a novel genus within *Moewusinia* clade: *Limnomonas* gen. nov. with its type species *L. gaiensis* and *L. spitsbergensis*.

2. Materials and Methods

Thirty single cells of a small and ellipsoidal shaped *Chlamydomonas* were isolated to established cultures from three eutrophic lakes in Sweden (Table 1), i.e., 18 strains from Lake Ryssbysjön, 7 strains from Lake Skedviken, and 5 strains from Lake Västra Ringsjön. Their morphology was compared with the commonly studied round and larger *Chlamydomonas* species known in Swedish lakes [3]. Moreover, the peculiar whole-body pendulum motion caught our attention. Single-cell isolation was performed under an Olympus light microscope (CKX31SF, Olympus) using a micro-capillary glass pipette [17]. Each individual was washed several times in clean droplets of MWC medium [18], transferred into a volume of 200 µL of MWC medium, and grown at 20 °C under a light regime 12:12 light:dark with 30 µmol photons m⁻² s⁻¹. Environmental parameters from their natural habitat, such as average water temperature and pH, were retrieved from the Swedish University of Agricultural Science's miljödatabas för mark-vatten-miljö (<http://miljodata.slu.se/mvm/> (accessed on 4 May 2022)) and Bergman [19].

Additionally, *Chlamydomonas* strains were acquired from culture collections for morphological comparison. The freshwater strain CCAP 11/119 isolated from Blelham Tarn, Cumbria, England was ordered from the Culture Collection of Algae and Protozoa. The strain CCCryo 217-05 isolated from a persistent snow field in Doktorbreen, Nathorst Land, Spitsbergen, Svalbard, Norway was ordered from the Culture Collection of Cryophilic Algae.

Table 1. Sampling information and water chemistry of the Swedish eutrophic lakes. Geographical coordinates; Environmental data collected from Miljödata-MVM [20] timeseries: range of water temperature (Twater), turbidity and pH, average and standard deviation of alkalinity, conductivity, total nitrogen (TotN), total phosphorous (TotP) and sulfate (SO4), and information on the reference station, timeseries span and period, and number of data points (Ndata); and Ecological status: humic status and water color with lake and station references [21–23]. Water temperature at the time of the sampling is indicated between brackets.

Parameters	Lake Skedviken	Lake Ryssbysjön	Lake Västra Ringsjön
Coordinates	59°47.30' N; 18°17.44' E	57°41.75' N; 14°37.68' E	55°54.78' N; 13°27.17' E
Twater (°C)	5.8–24.3 (20)	1.5–23.4 (23)	1.3–23.6 (25)
Turbidity (FNU)	6–30	3.6–86	3.0–26.0
pH	7.9–9.1	6.8–9.1	7.6–9.1
Alkalinity (mekv/L)	1.9 ± 0.1	0.4 ± 0.1	1.9 ± 0.2
Conductivity (mS/m)	23.1 ± 1.5	15.4 ± 2.4	26.9 ± 2.2
Salinity (psu)	<0.5, freshwater	<0.5, freshwater	<0.5, freshwater
TotN (µg/L)	1206 ± 140	1376 ± 566	961.2 ± 256.3
TotP (µg/L)	80 ± 22	107 ± 58	98.3 ± 33.0
SO4 (mg/L)	5.9 ± 4.0	5.7 ± 2.2	6.9 ± 4.8
Ndata	13 to 17	20 to 57	12 to 43
Station data [20]	2588	6171	1314
Timeseries data	2006–2020	2004–2020	1998–2021
Period data	August–November	August–November	August–December
Humic status	oligo to meso-humic	meso- to poly-humic	oligo to meso-humic
Water color	slightly to moderately colored water	significant to strongly colored water	slightly to moderately colored water
Lake (WA) and monitoring station (SE)	WA80739913, SE663072-164112 [21]	WA77935742, SE639905-143013 [22]	WA55412723, SE620062-135224 [23]

The external morphology and the cell ultrastructure were investigated in four of the 30 established strains (VR66-07, VR66-10, R86-45, and R86-47). These strains were deposited at the Culture Collection of Algae (SAG), University of Göttingen, Germany, under the numbers SAG 2637, SAG 2636, SAG 2638, and SAG 2639, respectively.

The morphology and the phenotypic plasticity were documented in photographs and compared with the identification keys from Ettl [11,12], Ettl and Gärtner [24], and the meticulous work of Skuja on *Chlamydomonas* species in different habitats including Swedish lakes [3–6]. The individual pendulum motion and their distribution pattern were assessed under a light microscope (Eclipse Ts2, Nikon) using NIS-Elements BR (version 4.50, Nikon) software for image recording and ImageJ, version 1.51a [25], for image analyses. The oscillation frequency (in Hertz) of the cell motion was inversely proportional to the number of frames necessary for a complete oscillation movement divided by the number frames per second of the registration (see the results section for details on the movement performed).

Cell ultrastructure was investigated using a transmission electron microscope (TEM) at the Microscopy Facility, Department of Biology, Lund University. A loose pellet of individual cells was fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylic buffer (pH 7.4) for three hours at 4 °C and post-fixed in 1% osmium tetroxide in distilled water for an hour at 4 °C. The pellet was dehydrated in a graded ethanol series and embedded in epoxy resin (Agar 100, Resin kit, R1031) via acetone and polymerized for 48 h at 60 °C. Ultra-thin sections (50 nm) were cut using an ultramicrotome (EM UC7, Leica, Wetzlar, Germany) using a diamond knife. The sections were mounted on a pioloform-coated copper grid and stained with uranyl acetate (2%, 30 min) and lead citrate (4 min). The ultra-thin sections were examined at 100 kV using a transmission electron microscope (JEM-1400 PLUS, JEOL Ltd., Tokyo, Japan). Micrographs were recorded with a JEOL Matataki CMOS camera using TEM Centre for JEM1400 Plus software.

Complementary morphological information on chloroplast position and shape was inferred using an automated microscope with phase gradient contrast (Zeiss Cell discoverer 7) at the Lund university Bioimaging Center, Lund University. Cells were loaded in a µ-Plate 24-well black ibi-Treat (ibidi GmbH, Gräfelfing, Germany) and an equivalent volume of

carbonated mineral water was used to sedate the organisms. Chlorophyll autofluorescence signal was used to detect the chloroplast.

Genomic DNA was extracted following the CTAB extraction protocol described in Tesson et al. [26]. The SSU rDNA of strains VR66-07, VR66-10, R86-45, and R86-47 were amplified by PCR reactions using the protocol described in Genitsaris et al. [27] with 35 cycles and the primers EukA (5'-AAC CTG GTT GAT CCT GCC AGT-3') and Euk1633rE (5'-GGG CGG TGT GTA CAA RGRG-3'). The ITS rDNA was amplified using the protocol of Goff and Moon [28] with 30 cycles and the primers ITS-A (5'-GGG ATC CGT TTC CGT AGG TGA ACC TGC-3') and ITS-B (5'-GGG ATC CAT ATG CTT AAG TTC AGC GGG-3') [29]. All PCR products were purified and sequenced as described by Lebret et al. [30]. To identify which taxa are closely related to the isolated strains, we used the SSU rDNA sequences (ON124833-ON124836) and the ITS rDNA sequences (ON124901-ON124904) for BLAST N search [31]. The results showed that these strains belong to the *Moewusii* clade and the closest relative is an unidentified *Chlamydomonas* species (uncultured clones AY2009D11 (HQ219443) and AY2009D32 (HQ219442)). In addition, the sequence of the strain CCAP 11/119 (FR865531) was absolutely identical in SSU (ON124833-ON124836) and ITS (ON124901-ON124904) to SAG 2636-2639.

The SSU rDNA sequences were aligned according to the secondary structures and included in a dataset of a total of 36 sequences (1782 bp) of representatives belonging to the *Moewusii* clade of the Chlamydomophyceae. Among these were the sequences of strains CCCryo 216-05 (GU117580) and CCCryo 217-05 (GU117581). To test which evolutionary model fitted best for the dataset, we calculated the log-likelihood values of 56 models using the automated model selection tool implemented in PAUP, version 4.0b169 [32], and the best model according to the Akaike criterion by PAUP was chosen for the analyses. The settings of the best model are given in the figure legend. The following methods were used for the phylogenetic analyses: distance, maximum parsimony, maximum likelihood, and Bayesian inference. Programs used included PAUP version 4.0b169 [32] and the PHASE package 2.0 [33–37]. For the Bayesian calculations, the secondary structure models of SSU and ITS (RNA7D in PHASE) were also taken into account.

The secondary structures of ITS-2 sequences were folded using the computer programs *mfold* [38] and analyzed with the ITS-2/Compensatory Base Changes (CBC) approach [39] described as follows: Those sequences were folded using the three programs mentioned above with the three constraints described as follows: (i) the last 25 bases of the 5.8S rRNA and the first 25 of the LSU rRNA must bind and form the 5.8S/LSU stem; (ii) the pyrimidine/pyrimidine mismatch (the first RNA processing site) in Helix II after the 5–7th base pair has to be present in the structure; and (iii) the second RNA processing site, the GGU motif characteristic for green algae, has to be at the 5' site in Helix III (for details about the processing sites and constraints, see Coleman [40] and Cote et al. [41]). For the usage of ITS-2 as DNA Barcode, it is necessary to obtain ITS sequences free of ambiguities with the flanking regions of 5.8S and LSU rDNA to avoid analyzing pseudogenes. Only then is it possible to fold a unique secondary structure model of ITS-2 with the constraints mentioned above. The structures were visualized with the program PseudoViewer 3 [42].

Considering this approach, we extracted the conserved region of ITS-2 from each strain as follows: (i) 16 base pairs of the 5.8S/LSU stem, (ii) five basal base pairs of Helix I, (iii) eleven base pairs of Helix II including the pyrimidine-pyrimidine mismatch, and (iv) all base pairs of Helix III. The resulting dataset was then aligned manually. These alignments were translated into base pair alignment using a number code for each base pair (1 = A-U; 2 = U-A; 3 = G-C; 4 = C-G; 5 = G•U; 6 = U•G). The resulting number barcodes are given in the diagnoses below. These barcodes of each species were compared to detect Compensatory Base Changes (CBCs), hemi-CBCs (HCBCs) and/or insertion/deletion, single, or unpaired bases.

3. Results

The four isolated strains from Swedish lakes belonged to the well-supported *Moewusii* clade of the Chlamydomphyceae Ettl (=clockwise-group of the Chlorophyta *sensu* Mattox & Stewart). Phylogenetic analyses of the SSU rDNA revealed that these isolates formed together with the strain CCAP 11/119, the uncultured clones AY2009D32 and AY2009D11, and the strains CCCryo 216-05 and CCCryo 217-05, a monophyletic lineage within this clade, which is highly supported in all Bayesian and bootstrap analyses (Figure 1). This monophyletic lineage is described below as the new genus *Limnomonas*.

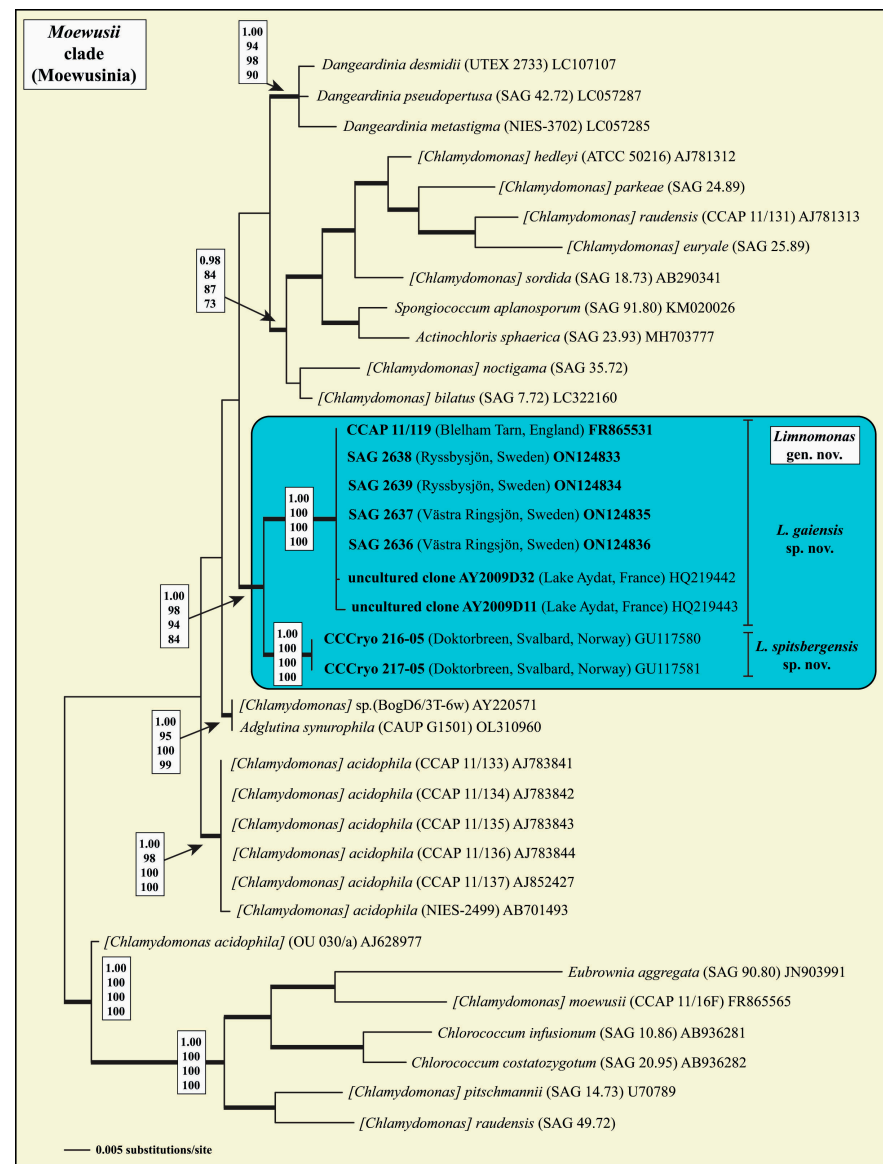


Figure 1. Molecular phylogeny of the *Moewusii* clade (Chlamydomphyceae) based on SSU rDNA sequence comparisons. The phylogenetic tree shown was inferred using the maximum likelihood method based on a dataset of 1782 aligned positions of 36 taxa using PAUP 4.0a build169. For the analysis, the GTR+I+G (base frequencies: A 0.2466, C 0.2164, G 0.2740, U 0.2630; rate matrix A-C 0.7875, A-G 2.5023, A-U 1.4014, C-G 0.6678, C-U 5.5103, G-U 1.0000) with the proportion of invariable sites (I = 0.7014) and gamma shape parameter (G = 0.6614) was chosen, which was calculated as the best model by the automated model selection tool implemented in PAUP. The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE; bootstrap values > 70% calculated with PAUP using maximum likelihood, neighbor-joining, and maximum parsimony). The newly sequenced strains are highlighted in bold.

To determine if the two lineages within *Limnomonas* represent different species, we analyzed the ITS-2 rDNA sequences and their secondary structures. As presented in Figure 2, the two lineages differed in their ITS-2 secondary structures. The applied ITS-2/CBC approach revealed five compensatory base changes and four hemi-CBCs in the conserved regions of ITS-2 among the lineages (Figure 3). This indicates that these lineages represent species. As a consequence of the phylogenetic results, we propose the new genus *Limnomonas* with two species as follows.

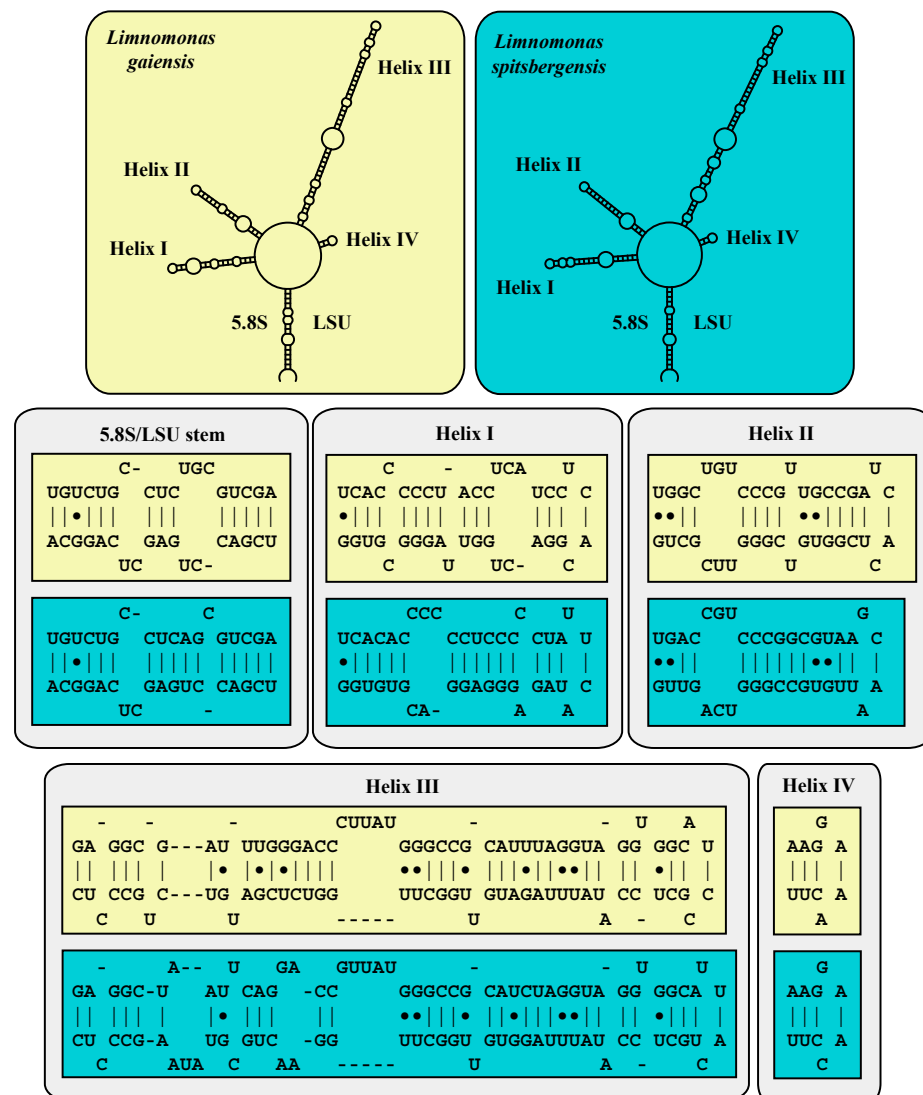


Figure 2. ITS-2 rRNA secondary structure models of *Limnomonas gaiensis* and *L. spitsbergensis*.

Limnomonas S.V.M. Tesson & T. Pröschold gen. nov. (Figure 4).

Description: Cells are unicellular, with motile and solitary vegetative stages, with two flagella, 1 to 2 times longer than the cell body. Chloroplast cup-shaped containing a single pyrenoid of type 1 *sensu* Watanabe & Lewis [16], often ellipsoidal to spherical shape, surrounded by starch grains and penetrated by thylakoids. Two apical contractile vacuoles. The eyespot, often elliptic, part of the chloroplast and located in the anterior half of the cell. With or without frustoconical papilla.

Diagnosis: Differ from other biflagellated unicellular genera by SSU and ITS rDNA sequences.

Etymology: The generic name *Limnomonas* is a combination of two words—i.e., ‘Limnology’, which refers to the versatile inland waters from which available species were collected (e.g., lakes, mine drainage, snow) and ‘monas’ which refers to ‘unit’ in ancient Greek.

Type species (designated here): *Limnomonas gaiensis* S.V.M. Tesson & T. Pröschold sp. nov.

Limnomonas gaiensis S.V.M. Tesson & T. Pröschold sp. nov. (Figure 4A–F).

Description: Individuals were distributed as single cells or in groups of cells. Each individual possessed a brown-red eyespot visible under light microscope. Their bodies were characterized by delicate walls, uniformly thick with no noticeable local thickening. The shape of the body was ellipsoidal, rounded in the base and truncated towards the front (head) by the presence of a frustoconical papilla, and no further visible extensions. The papilla base ranged from 0.7 to 1.1 μm ($0.9 \pm 0.1 \mu\text{m}$) with a narrower length on one side of the organism. The body length was between 5.5 and 9.2 μm with an average of $6.8 \pm 0.8 \mu\text{m}$. The body width spanned from 2.1 and 4.5 μm with an average of $2.7 \pm 0.5 \mu\text{m}$. The organism exhibited two smooth flagella, close to each other. Each flagellum passed through one of the two apertures disposed oppositely on each side of the papilla. The flagella were of nearly equal size, of 1 to 1.5 times the organismal body length, spanning from 6.5 to 11 μm with a mean of $8.9 \pm 1.3 \mu\text{m}$. One basal nucleus was located in the first-half of the organism.

Diagnosis: Differs from other species of biflagellated chlorophytes by SSU-ITS (ON124833 and ON124901).

Etymology: Individuals were isolated from lakes, i.e., aquatic reservoirs surrounded by lands. The name of the species '*gaiensis*' is a two-word combination of the root of the ancient Greek '*gaia*' (signification: land/Earth) and the suffix '*ensis*' indicating the origin.

Holotype (designated here): The strain SAG 2638 is permanently cryopreserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the SAG, University of Göttingen, Germany.

Type locality: Lake Ryssbysjön (WA77935742, [22]), Sweden (57°41'44.9" N 14°37'40.5" E). Forest lake with eutrophic, basic to neutral pH waters.

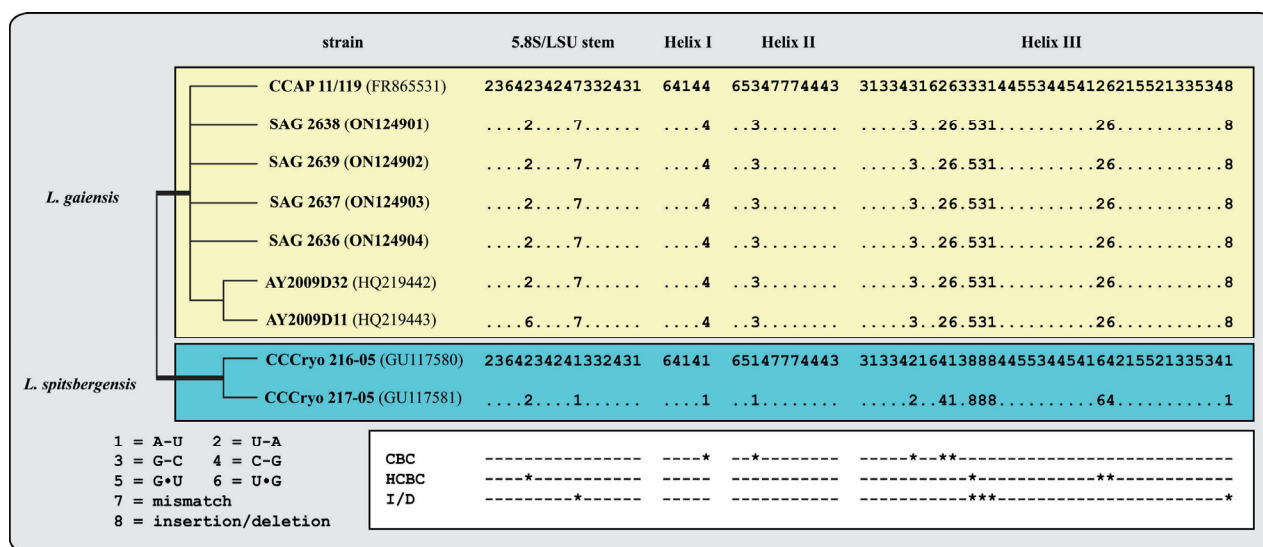


Figure 3. ITS-2 barcodes of *Limnomonas gaiensis* and *L. spitsbergensis* determined by the ITS-2/Compensatory Base Change approach. The Compensatory Base Changes (CBCs), hemi-Compensatory Base Changes (HCBCs), and non-homoplasious synapomorphies (NHSs) characterized by insertions (I) or deletions (D) are marked with an asterisk.

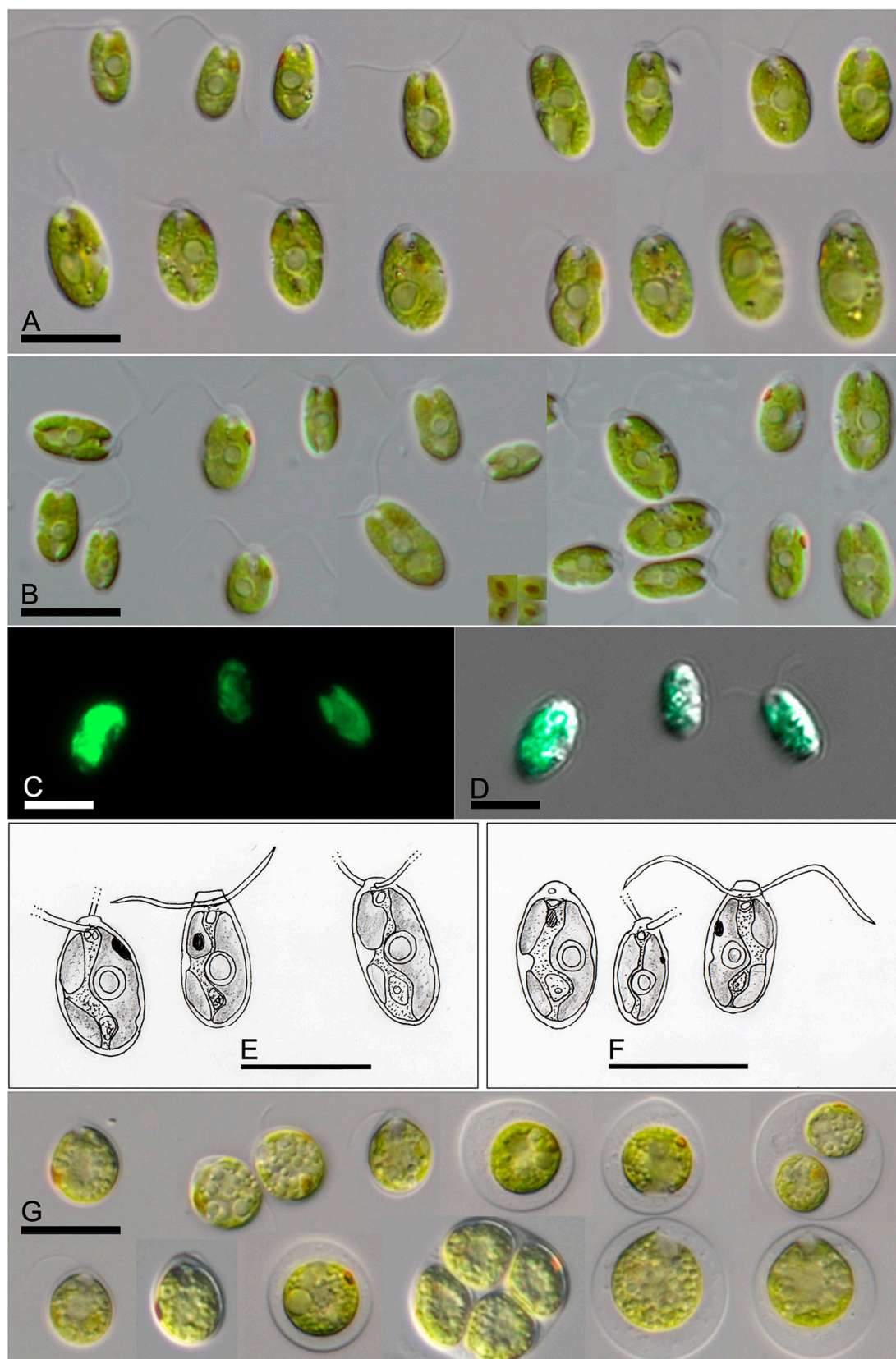


Figure 4. Morphology and phenotypic plasticity of the *Limnomonas* species. (A,B) *L. gaiensis*, strains SAG 2638 (A) and CCAP 11/119 (B); (C,D) *L. gaiensis* chlorophyll autofluorescence; (E,F) Line drawing of vegetative cells of *L. gaiensis* SAG 2638 (E) and CCAP 11/139 (F); and (G) *L. spitsbergensis*, strain CCCryo 217-05; scale bar = 10 µm for all pictures (A–G).

Limnomonas spitsbergensis S.V.M. Tesson & T. Pröschold sp. nov. (Figure 4G)

Description: Biflagellated cells 9.8×11.8 to 13.5×15.5 μm in size, broadly ellipsoidal, broadly ovoid, sometimes slightly dorsalventral in shape, with two flagella of cell body length. Cell wall $0.5\text{--}0.8$ μm thick with small cone-like papilla (~ 1.2 μm). Chloroplast cup-shaped similar to *Chlamydomella* type, with a single spherical pyrenoid surrounded by barely visible starch grains located towards the middle of the cell. Nucleus central. Elliptical eyespot rod-like positioned laterally, in the middle of the cell. After reaching a cell size of $15\text{--}16$ μm , the cell wall started to become distant from the protoplast. Mature cells with distant cell walls 15.3×16.7 μm up to 20.1×21.0 μm in size. Asexual reproduction by 2–4 zoospores. Zoosporangia 21.9×22.4 to 23.5×25.3 μm in size. Sexual reproduction not observed.

Diagnosis: Deposited in GenBank and in the Culture Collection of Cryophilic Algae as *Chlamydomonas* cf. *proboscigera* Korshikov but differs from the original species description by chloroplast morphology (massive cup-shaped of the *Euchlamydomonas* type), cell shape (broadly ovoid), and ecology (plankton and soil of temperate zones around the world). From other species of biflagellated chlorophytes by SSU-ITS (GU117581).

Etymology: The name of the species originated from its origin.

Holotype (designated here): The strain CCCryo 217-05, collected from snow, is permanently cryopreserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the CCCryo, Fraunhofer Institute Potsdam-Golm, Germany.

Type locality: Doktorbreen, Nathorst Land, Spitsbergen, Svalbard, Norway ($77^{\circ}34'00.0''$ N $16^{\circ}54'00.0''$ E).

The morphology of both species *L. gaiensis* and *L. spitsbergensis* showed a high phenotypic plasticity (Figure 4). The cell shape and size, in addition to the position of the pyrenoid, varied. The ellipsoidal cell shape of vegetative cells was prolate spheroidal in *L. gaiensis* strains (Figure 4A–F) and round to oblate spheroidal in *L. spitsbergensis* (Figure 4G). Sporangia were observed in *L. spitsbergensis*. In both *Limnomonas* species, the ellipsoidal to spherical-shaped pyrenoid was located along the apical-basal axis in the mid region of the organisms.

All 30 strains of *L. gaiensis* performed a pendulum movement characterized by a continuous and full-body length oscillation around the front-apical cellular axis, with an oscillation frequency of 3.1 ± 2.0 Hz, and an amplitude spanning from acute to straight angles along the basal-apical axis (Video S1, Supplementary Materials). The ultrastructure of *Limnomonas gaiensis* (Figure 5) showed a single and large pyrenoid of ellipsoidal to round shape, surrounded by two starch plates looking like hourglass-shaped bowls (Figure 5B,H), disposed laterally, halfway along the front-basal organism axis (Figure 5B,D). A network of thylakoids passed through the structure bisecting the pyrenoid (Figure 5H). The parietal bowl-shaped chloroplast was appended to one side of the organismal cell wall and smooth (Figure 5E).

The basal body apparatus at apical position showed the typical $9 \times 2 + 2$ structure of flagellar axonemes in cross-section (Figure 5A,B). The flagellar membrane and axoneme in longitudinal section are demonstrated in Figure 5C. The two flagella were close to each other, going out on each side of the papilla (Figure 5G). A bed of small vesicles and irregularly distributed larger vacuoles (Figure 5H,J) and their fusion into two distinct contractile vacuoles (Figure 5E,J) were visible near the flagella system. The cell wall was relatively thick and complex with several visible layers and a tubular-like structure beneath the outer membrane (Figure 5I).

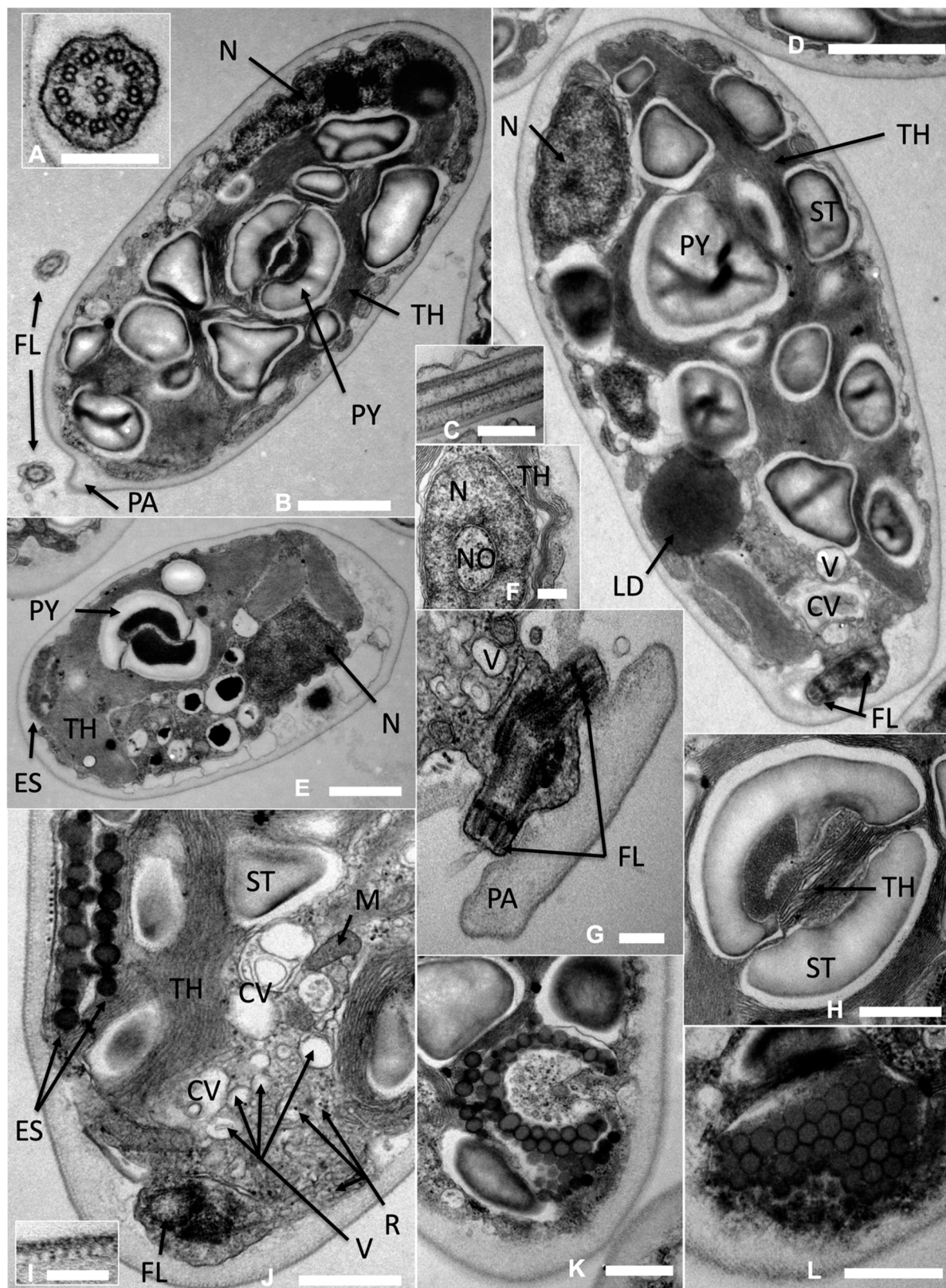


Figure 5. Ultrastructure of *Limnomonas gaiensis*. Scale bars: 100 nm (I), 200 nm (A,C,F,G), 500 nm (H,J,K,L), and 1 µm (B,D,E). Visible cell components are an eyespot (ES), a flagella system (FL) and papilla (PA), contractile vesicles (CV) and larger vesicles (V), a pyrenoid (PY), thylakoids (TH), a nucleus (N) and nucleolus (NO), mitochondria (M), ribosomes (R), starch (ST) and lipid (LD) bodies.

The nucleus in *L. gaiensis* was located in the first-half of the organism towards the basal/posterior end (Figure 5B,D), sometimes spanning along the apical-basal axis up to the pyrenoid location. The nucleus section showed nuclear pores, a central nucleolus, and patches of heterochromatin around the nuclear periphery and the nucleolus (Figure 5F).

An ellipsoidal and curved eyespot was located in the anterior part of the cell (Figure 5J,K). The tangential section of the eyespot showed a crystalline-like plate characterized by close-packing and regularly spaced hexagonally shaped granules (Figure 5L). The cross-sections of the eyespot showed two layers of dense granules (Figure 5J). The length of the outer-most layer of the eyespot was between 1.05 and 1.47 μm and contained an irregular size and arrangement of granules. The inner layer length spanned from 0.7 to 1.29 μm . The two layers formed a complex of 0.30–0.36 μm sandwiched between the organismal cell wall and a network of thylakoids (e.g., Figure 5J), which occupied one-sixth to one-ninth of the organismal cell width. The granules were of irregular size spanning from 70 to 190 nm in diameter.

Further ultrastructure features were visible, such as the presence of ribosomes and mitochondria (e.g., Figure 5J), and numerous inclusions of starch (e.g., Figure 5B,D). *Limnomonas gaiensis* did not show dorso-ventral axis development, nor caudal extensions, but a regular ellipsoid shape without local thickening of the cell wall.

In environmental samples and mixed cultures, the co-occurrence of *Limnomonas gaiensis* and *Microcystis* sp. were monitored. It was possible to observe *L. gaiensis* organisms contacting both precipitates and colonies of *Microcystis* sp.

4. Discussion

As demonstrated in Figure 1, the genus *Limnomonas*, composed of two species, formed a highly supported lineage within the *Moewusii* clade of the Chlamydomphyceae. The two species of *Limnomonas* vary in morphology and molecular phylogeny, and they also differ in their ecology. The first species, *L. gaiensis*, seems to be widely distributed in plankton of cold-temperate lakes in Sweden and the UK. The second species, *L. spitsbergensis*, represented by strains CCCryo 216-05 and CCCryo 217-05, is known, to date, only from snow fields (Spitsbergen, Svalbard, Norway). The two CCCryo strains were originally assigned to the taxon *Chlamydomonas* cf. *proboscigera* (Figure 1). Unfortunately, no authentic strain of *C. proboscigera* is available in public culture collections. However, comparing the habitats of all records (different water bodies and soils of temperate climate zones around the world) published for *C. proboscigera*, it is clear that the two psychrophilic strains do not represent *C. proboscigera* (see details in Ettl [11]). In addition, the morphology of both strains also differs from the original description of Korshikov in Pascher [43]. *Limnomonas spitsbergensis* has an ovoid, slightly elongated cell shape, a *Chlamydeella*-type cup-shaped chloroplast (not *Euchlamydomonas* type), a single and round pyrenoid located in the middle of the cell (not basal), and an elliptical (not spotted) eyespot located laterally in the middle of the cells. As a consequence, we described both strains CCCryo 216-05 and CCCryo 217-05 as a new species, *Limnomonas spitsbergensis* (see above).

The morphology of *L. gaiensis* showed similarities to the descriptions of *Chlamydomonas pumilio* and *C. sordida* [11,12,44,45]. It has a regular ellipsoid shape, without local thickening of the cell wall. It also possesses a broader and straight-truncated papilla, a rather large eyespot, and no caudal extensions. As in *C. pumilio*, *L. gaiensis* showed delicate cell walls, flagella about the same length of the body, two apical contractile vacuoles, a lateral chloroplast, an ellipsoidal to spherical pyrenoid located half-height of the body and a nucleus located in the third of the cell towards the basal apex. Its elliptic eyespot is often in the front third of the cell. However, its body length was slightly bigger than expected in *C. pumilio* (4.5–8 μm long, 1.5–3 μm wide). The differences with *C. pumilio* var. *pumilio* [12,24,45] reside in both the presence of numerous starch reserves and two hourglass-shaped bowls of starch around the pyrenoid instead of the multiple starch plates drawn in the taxonomic key. However, this feature may be a minor issue, as several studies showed that pH and CO₂ conditions may affect the ultrastructural organization of both

starch reserves and the pyrenoid starch sheath in *Chlamydomonas* species [46,47]. Despite similar morphology, Nakada and Nozaki [48] showed that *C. pumilio* is a sister taxon to *C. pulsatilla* and *C. subcaudata*, belonging to the ‘*Polytoma*’-clade *sensu* Pröschold et al. [13]. Considering our results, we therefore presented in this study *L. gaiensis* as a new taxon of the ‘*Moewusii*’-clade. Similarly, to Nakada and Nozaki [48], the strain SAG 18.73 assigned as *C. sordida* represented with high statistical support an own lineage within the ‘*Moewusii*’-clade, and does not belong to *Limnomonas* (Figure 1).

The morphology varies between species of the *Limnomonas* genus and with the two clades sharing a last common ancestor with *Limnomonas*, i.e., *Adglutina* and *Chlamydomonas acidophila* (strains CCAP 11/133-137, NIES-2499). *Limnomonas gaiensis* and *C. acidophila* possess comparable inner and outer morphology, characterized by a monad ellipsoidal body shape, a basal nucleus, two apical contractile vacuoles, a cup-shaped chloroplast, and an ellipsoidal stigma (eyespot) located towards the anterior half of the cell [12,16,49]. However, *L. gaiensis* misses the basal roundness and dorsoventral axis orientation described in both *C. acidophila* and *C. proboscigera* (Ettl [12]) and in *L. spitsbergensis* (this study). Contrary to the above-mentioned sister-clades, *Adglutina* does not possess a pyrenoid and the position of several morphological features (eyespot and nucleus) are variable [50]. The papilla in *Adglutina* (low keel shape [50]) and in *L. spitsbergensis* (small cone-shape [this study]) are low, in the continuity of the cell, whereas *L. gaiensis* presents a pronounced frustoconical papilla (this study). Moreover, the size of the eyespot is similar among species of *Limnomonas* but differs in shape from that of *Adglutina* (ellipsoidal somewhat triangular [50]). Morphological similarities between *C. acidophila* and *Limnomonas*, and dissimilarities with *Adglutina*, raise the question of the reciprocal impact of organism behavior and habitat on morphology.

Chlamydomonas-like taxa can exhibit cell adhesion to substrates. A study from Kreis et al. [51] reveals that cell adhesion, investigated in the model species *Chlamydomonas reinhardtii*, is independent from the nature of the substrate. Cell adhesion was reported in the above-mentioned sister-clades in *Adglutina synurophila* to the eukaryotic microalga *Synura* [50], in *L. gaiensis* to precipitates, abiotic surfaces, and the cyanobacteria *Microcystis* (this study), and possibly in *C. acidophila* NIES-2499 to microfilm [49,52]. The pronounced attachment of these taxa to substrate may fulfil similar ecological requirements (e.g., nutrition), but rise from different genetic and morphologic backgrounds. Further investigations are necessary to decipher the factors triggering cell adhesion in these species.

The natural environment in which these species live may be the cause of morphological variations reported above. Investigated strains were collected from a wide range of water acidity levels, e.g., *C. acidophila* from acidic (ca pH 3) river microfilm (NIES-2499 [50,53]) and water column (CCAP 11/133-137 [53–55]), *A. synurophila* from slightly acidic to neutral freshwater bodies (pH 6–7.9 [50]), *L. spitsbergensis* from acidic snow (pH 5.4, ‘Red-snow’, ‘*Moewusii*’-clade/B in [56]), and *L. gaiensis* from slightly acidic to neutral freshwater lakes (pH 6.7–9.1, this study and [57]). Water acidity can affect the ultrastructure and behavior in *Chlamydomonas* species [58]. For instance, it can increase vacuolization and auto-sporulation [58] or the pyrenoid volume, cell wall thickness and the formation of palmelloid colonies in other species, possibly reducing the cell volume, the chloroplast volume, the starch reserves, and the cell motility [49]. Acidification can also lead to flagella amputation [59]. Intermediately, certain strains of *Chlamydomonas acidophila* are not acidophilic *sensu stricto* and are able to tolerate a wide range of pH values (1.5–8.4) [55,58]. Yet, the impact of water acidity on the size of the papilla remains to be demonstrated, which is one of the main discordant features between *C. acidophila* and *L. gaiensis*. Moreover, the impact of water acidity does not seem to explain the cell roundness observed in the sister-clades (*C. acidophila* and *A. synurophila*) and in the sister-species *L. spitzbergensis*, which is absent in *L. gaiensis* strains, nor the absence of pyrenoid in *A. synurophila* only. Future genomic analyses of *L. gaiensis* and its relatives would uncover valuable missing information in understanding the evolution of *Chlamydomonas*-like organisms.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d14060481/s1>, Video S1: Pendulum movement and cell oscillation of *Limnomonas gaiensis*.

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References

1. Ramberg, L. Relations between phytoplankton and light climate in two Swedish forest lakes. *Internat. Rev. Ges. Hydrobiol.* **1979**, *64*, 749–782. [\[CrossRef\]](#)
2. Nygaard, G. *New or Interesting Plankton Algae—With a Contribution on Their Ecology*; Munksgaard, Ltd.: Copenhagen, Denmark, 1977.
3. Skuja, H. Taxonomische und biologische Studien über das Phytoplankton schwedischer Binnengewässer. *Nova Acta Regiae Soc. Sci. Ups. Ser. IV* **1956**, *16*, 404.
4. Skuja, H. Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. *Symb. Bot. Ups.* **1948**, *9*, 399.
5. Skuja, H. Grundzüge der Algenflora und Algenvegetation der Fjeld-Gegenden um Abisko in Schwedisch-Lappland. *Nova Acta Regiae Soc. Sci. Ups. Ser. IV* **1964**, *18*, 465.
6. Willén, T. Notes on Swedish plankton algae. *Nova Hedwig.* **1963**, *6*, 9–13.
7. Cronberg, G. *Växtplankton i Vombsjön 1989–1995*; Limnologi, Ekologiska Institutionen: Lund, Sweden, 1996; 20p.
8. Gustafsson, S.; Cronberg, G. *Sammanställning Och Utvärdering Av Planktonsamhället i Nio Skånska Sjöar*; Länsstyrelsen i Skåne län: Malmö, Sweden, 2012; Volume 15, p. 86.
9. Spouge, J.L. Measurement of a barcode’s accuracy in identifying species. In *DNA Barcoding in Marine Perspectives Assessment and Conservation of Biodiversity*; Trivedi, S.P., Ansari, A.A., Ghosh, S.K., Rehman, H., Eds.; Springer International Publishing: Cham, Switzerland, 2016; pp. 29–41.
10. Manoylov, K.M. Taxonomic identification of algae (morphological and molecular): Species concepts, methodologies, and their implications for ecological bioassessment. *J. Phycol.* **2014**, *50*, 409–424. [\[CrossRef\]](#)
11. Ettl, H. Die Gattung *Chlamydomonas* Ehrenberg (*Chlamydomonas* und die nächstverwandten Gattungen II). *Beihefte zur Nova Hedwigia* **1976**, *49*, 1122.
12. Ettl, H. Chlorophyta I-Phytomonadina. In *Süßwasserflora von Mitteleuropa, Bd. 9*; Ettl, H., Gerloff, J., Heynig, H., Mollenhauer, D., Eds.; Spektrum Akademischer Verlag: Heidelberg, Germany, 1983; 808p.
13. Pröschold, T.; Marin, B.; Schlösser, U.G.; Melkonian, M. Molecular phylogeny and taxonomic revision of *Chlamydomonas* (Chlorophyta). I. Emendation of *Chlamydomonas* Ehrenberg and *Chloromonas* Gobi, and description *Oogamochlamys* gen. nov. and *Lobochlamys* gen. nov. *Protist* **2001**, *152*, 265–300. [\[CrossRef\]](#)
14. Nakada, T.; Misawa, K.; Nozaki, H. Molecular systematics of Volvocales (Chlorophyceae, Chlorophyta) based on exhaustive 18S rRNA phylogenetic analyses. *Mol. Phylog. Evol.* **2008**, *48*, 281–291. [\[CrossRef\]](#)

15. Demchenko, E.; Mikhailyuk, T.; Coleman, A.W.; Pröschold, T. Generic and species concepts in *Microglena* (previously the *Chlamydomonas monadina* group) revised using an integrative approach. *Eur. J. Phycol.* **2012**, *47*, 264–290. [CrossRef]
16. Watanabe, S.; Lewis, L.A. Phylogenetic interpretation of light and electron microscopic features of selected members of the phylogroup *Moewusinia* (Chlorophyceae), with new generic taxonomy. *Phycologia* **2017**, *56*, 329–353. [CrossRef]
17. Pringsheim, E.G. *Pure Cultures of Algae, Their Preparation and Maintenance*; Cambridge University Press: Cambridge, UK, 1946.
18. Guillard, R.R.L.; Lorenzen, C.J. Yellow-green algae with chlorophyllide C¹². *J. Phycol.* **1972**, *8*, 10–14.
19. Bergman, E. Changes in nutrient load and lake water chemistry in Lake Ringsjön, southern Sweden, from 1966 to 1996. *Hydrobiologia* **1999**, *404*, 9–18. [CrossRef]
20. Miljödata-MVM. Swedish University of Agricultural Sciences (SLU). National Data Host Lakes and Watercourses, and National Data Host Agricultural Land. 2022. Available online: <https://miljodata.slu.se/mvm/> (accessed on 27 April 2022).
21. Ecological Status for the Lake Skedviken (MS_CD: WA80739913 VISS EU_CD: SE663072-164112). Available online: [https://Skedviken-Sjö-VISS-VattenInformationsSystemförSverige\(lansstyrelsen.se\)](https://Skedviken-Sjö-VISS-VattenInformationsSystemförSverige(lansstyrelsen.se)) (accessed on 4 May 2022).
22. Ecological Status for the Lake Ryssbysjön (MS_CD: WA77935742/VISS EU_CD: SE639905-143013). Available online: [https://Skedviken-Sjö-VISS-VattenInformationsSystemförSverige\(lansstyrelsen.se\)](https://Skedviken-Sjö-VISS-VattenInformationsSystemförSverige(lansstyrelsen.se)) (accessed on 4 May 2022).
23. Ecological Status for the Lake Västra Ringsjön (MS_CD: WA55412723/VISS EU_CD: SE620062-135224). Available online: [https://Skedviken-Sjö-VISS-VattenInformationsSystemförSverige\(lansstyrelsen.se\)](https://Skedviken-Sjö-VISS-VattenInformationsSystemförSverige(lansstyrelsen.se)) (accessed on 4 May 2022).
24. Ettl, H.; Gärtner, G. *Syllabus der Boden-, Luft- und Flechtenalgen*; Springer Spektrum: Heidelberg, Germany, 2014; p. 773.
25. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671. [CrossRef] [PubMed]
26. Tesson, S.V.M.; Borra, M.; Kooistra, W.H.; Procaccini, G. Microsatellite primers in the planktonic diatom *Pseudo-nitzschia multistriata* (Bacillariophyceae). *Amer. J. Bot.* **2011**, *98*, e33–e35. [CrossRef]
27. Genitsaris, S.; Kormas, K.A.; Moustaka-Gouni, M. Microscopic eukaryotes living in a dying lake (Lake Koronia, Greece). *FEMS Microbiol. Ecol.* **2009**, *69*, 75–83. [CrossRef]
28. Goff, L.J.; Moon, D.A. PCR amplification of nuclear and plastid genes from algal herbarium specimens and algal spores. *J. Phycol.* **1993**, *29*, 381–384. [CrossRef]
29. Coleman, A.W.; Suarez, A.; Goff, L.J. Molecular delineation of species and syngenes in volvoclean green algae (Chlorophyta). *J. Phycol.* **1994**, *30*, 80–90. [CrossRef]
30. Lebet, K.; Tesson, S.V.M.; Kritzberg, E.S.; Tomas, C.; Rengefors, K. Phylogeography of the freshwater raphidophyte *Gonyostomum semen* confirms a recent expansion in northern Europe by a single haplotype. *J. Phycol.* **2015**, *51*, 768–781. [CrossRef]
31. Altschul, S.F.; Gish, W.; Myers, E.W.; Miller, W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
32. Swofford, D.L. *PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10*; Sinauer Associates: Sunderland, MA, USA, 2002.
33. Jow, H.; Hudelot, C.; Rattray, M.; Higgs, P. Bayesian phylogenetics using an RNA substitution model applied to early mammalian evolution. *Mol. Biol. Evol.* **2002**, *19*, 1591–1601. [CrossRef] [PubMed]
34. Higgs, P.; Jameson, D.; Jow, H.; Rattray, M. The evolution of tRNA-Leu genes in animal mitochondrial genomes. *J. Mol. Evol.* **2003**, *57*, 435–445. [CrossRef] [PubMed]
35. Hudelot, C.; Gowri-Shankar, V.; Jow, H.; Rattray, M.; Higgs, P. RNA-based phylogenetic methods: Application to mammalian mitochondrial RNA sequences. *Mol. Phylog. Evol.* **2003**, *28*, 241–252. [CrossRef]
36. Gibson, A.; Gowri-Shankar, V.; Higgs, P.; Rattray, M. A comprehensive analysis of mammalian mitochondrial genome base composition and improved phylogenetic methods. *Mol. Biol. Evol.* **2005**, *22*, 251–264. [CrossRef] [PubMed]
37. Telford, M.J.; Wise, M.J.; Gowri-Shankar, V. Consideration of RNA secondary structure significantly improves likelihood-based estimates of phylogeny: Examples from the bilateria. *Mol. Biol. Evol.* **2005**, *22*, 1129–1136. [CrossRef]
38. Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acid Res.* **2003**, *31*, 3406–3615. [CrossRef]
39. Darienko, T.; Gustavs, L.; Eggert, A.; Wolf, W.; Pröschold, T. Evaluating the species boundaries of green microalgae (*Coccomyxa*, Trebouxiophyceae, Chlorophyta) using integrative taxonomy and DNA barcoding with further implications for the species identification in environmental samples. *PLoS ONE* **2015**, *10*, e0127838. [CrossRef]
40. Coleman, A.W. ITS2 is a double-edged tool for eukaryote evolutionary comparisons. *Trends Genet.* **2003**, *19*, 370–375. [CrossRef]
41. Cote, C.A.; Greer, C.L.; Peculis, B.A. Dynamic conformational model for the role of ITS2 in pre-rRNA processing in yeast. *RNA* **2002**, *8*, 786–797.
42. Byun, Y.; Han, K. PseudoViewer3: Generating planar drawings of large-scale RNA structures with pseudoknots. *Bioinformatics* **2009**, *25*, 1435–1437. [CrossRef]
43. Pascher, A. Volvocales. In *Süßwasser-Flora Deutschlands, Österreichs und der Schweiz. Heft 4*; Pascher, A., Ed.; Gustav Fischer: Jena, Germany, 1927; pp. 1–506.
44. Ettl, H. Beitrag zur Kenntnis der Morphologie der Gattung *Chlamydomonas* Ehrenberg. *Arch. Protistenkd.* **1965**, *107*, 271–430.
45. Ettl, H. Ein Beitrag zur Kenntnis der Algenflora Tirols. *Ber. Nat.-Med. Ver. Innsbr.* **1968**, *56*, 177–354.
46. Ramazanov, Z.; Rawat, M.; Mason, C.E.; Moroney, J.V. Ultrastructural and biochemical adaptation of algal cells to limiting CO₂ concentrations. *Sci. Mar.* **1996**, *60*, 141–148.

47. Visviki, I.; Santikul, D. The pH tolerance of *Chlamydomonas applanata* (Volvocales, Chlorophyta). *Arch. Environ. Contam. Tox.* **2000**, *38*, 147–151. [\[CrossRef\]](#)
48. Nakada, T.; Nozaki, H. Two species of *Chlamydomonas* (Volvocales, Chlorophyceae) new to Japan. *J. Jpn. Bot.* **2007**, *82*, 179–189.
49. Hirooka, S.; Hirose, Y.; Kanesaki, Y.; Higuchi, S.; Fujiwara, T.; Onuma, R.; Era, A.; Ohbayashi, R.; Uzuka, A.; Nozaki, H.; et al. Acidophilic green algal genome provides insights into adaptation to an acidic environment. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E8304–E8313. [\[CrossRef\]](#)
50. Barcytė, D.; Pusztai, M.; Škaloud, P.; Eliáš, M. When you Like Other Algae: *Adglutina synurophila* gen. et sp. nov. (Moewusinia, Chlorophyceae), a Clingy Green Microalga Associated with *Synura* Colonies. *Protist* **2022**, *173*, 125858. [\[CrossRef\]](#)
51. Kreis, C.T.; Grangier, A.; Bäumchen, O. In vivo adhesion force measurements of *Chlamydomonas* on model substrates. *Soft Matter* **2019**, *15*, 3027–3035. [\[CrossRef\]](#)
52. Yumoto, K.; Kasai, F.; Kawachi, M. Taxonomic re-examination of *Chlamydomonas* strains maintained in the NIES-Collection. *Microbiol. Cult. Coll.* **2013**, *29*, 1–12.
53. Bissinger, V.; Jander, J.; Tittel, J. A new medium free of organic carbon to cultivate organisms from extremely acidic mining lakes (pH 2.7). *Acta Hydrochim. Hydrobiol.* **2000**, *28*, 310–312. [\[CrossRef\]](#)
54. Pedrozo, F.; Kelly, L.; Diaz, M.; Temporetti, P.; Baffico, G.; Kringel, R.; Friese, K.; Mages, M.; Geller, W.; Woelfl, S. First results on the water chemistry, algae and trophic status of an Andean acidic lake system of volcanic origin in Patagonia (Lake Caviahue). *Hydrobiologia* **2001**, *452*, 129–137. [\[CrossRef\]](#)
55. Gerloff-Elias, A.; Spijkerman, E.; Pröschold, T. Effect of external pH on the growth, photosynthesis and photosynthetic electron transport of *Chlamydomonas acidophila* Negoro, isolated from an extremely acidic lake (pH 2.6). *Plant Cell Environ.* **2005**, *28*, 1218–1229. [\[CrossRef\]](#)
56. Remias, D.; Karsten, U.; Lütz, C.; Leya, T. Physiological and morphological processes in the Alpine snow alga *Chloromonas nivalis* (Chlorophyceae) during cyst formation. *Protoplasma* **2010**, *243*, 73–86. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Monchy, S.; Sancier, G.; Jobard, M.; Rasconi, S.; Gerphagnon, M.; Chabé, M.; Cian, A.; Meloni, D.; Niquil, N.; Christaki, U.; et al. Exploring and quantifying fungal diversity in freshwater lake ecosystems using rDNA cloning/sequencing and SSU tag pyrosequencing. *Environ. Microbiol.* **2011**, *13*, 1433–1453. [\[CrossRef\]](#)
58. Visviki, I.; Palladino, J. Growth and cytology of *Chlamydomonas acidophila* under acidic stress. *Bull. Environ. Contam. Tox.* **2001**, *66*, 623–630. [\[CrossRef\]](#)
59. Lefebvre, P.A. Flagellar Amputation and Regeneration in *Chlamydomonas*. *Methods Cell Biol.* **1995**, *47*, 5.