

ORIGINAL PAPER

When you Like Other Algae: *Adglutina synurophila* gen. et sp. nov. (*Moewusinia*, Chlorophyceae), a Clingy Green Microalga Associated with *Synura* Colonies



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Unicellular green biflagellates of the order Volvocales (Chlorophyceae, Chlorophyta) are common inhabitants of various types of habitats, and can also form peculiar interspecific relationships. Most of their morphological diversity has historically been assigned to the two prominent genera *Chlamydomonas* and *Chloromonas*. Ongoing reclassification of these algae, aided by molecular phylogenetics, has resulted in numerous newly proposed genera, but there are certainly brand-new taxa awaiting recognition. In this study, based on morphological and ultrastructural observations together with sequence data of the nuclear 18S and ITS2 rDNA and the plastid *rbcl* gene, we describe *Adglutina synurophila* gen. et sp. nov., a volvocalean biflagellate isolated from colonies of the golden-brown alga *Synura petersenii* (Chrysophyceae). Phylogenetic analyses placed *Adglutina* in the phylogroup *Moewusinia* as a sister lineage to the acidophilic “*Chlamydomonas*” species. It is characterised by having oval to broadly ellipsoidal cells with a low keel-shaped papilla and a cup-shaped chloroplast lacking a pyrenoid, but possessing a lateral eyespot of a variable position. The unique set of features, together with its *Synura*-loving nature, anchor *Adglutina* as a well distinguishable phylogenetic lineage within the *Moewusinia*. The novel alga has a widespread distribution; it has been found in three European countries to date.

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Introduction

Green algae (Chlorophyta and Streptophyta) are a diverse and widespread group of primarily photosynthetic eukaryotes constituting important primary producers in both aquatic and terrestrial ecosystems. They exhibit great diversity not only in morphological and ultrastructural features, but also in their habitation of a wide range of ecological niches and adoption of different lifestyles, including the formation of peculiar interspecific relationships (Leliaert 2019). The most extensively studied green algae come from the three largest core Chlorophyta classes (Trebouxiophyceae, Ulvophyceae, and Chlorophyceae), and have repeatedly been observed in symbiotic relationships or other types of intimate interactions. Highly illustrative examples include lichens where multiple genera of coccoid microalgae from the class Trebouxiophyceae (e.g. *Trebouxia*, *Asterochloris*) play key roles as photobionts (Muggia et al. 2020; Škaloud et al. 2015). Additional examples of relationship-forming trebouxiophytes include, *Chlorella*, *Micractinium*, and *Carolibrandtia* living inside the cells of various ciliates (Hoshina et al. 2018, 2021; Pröschold et al. 2020; Summerer et al. 2007), or *Coccomyxa* cells thriving in higher plants (Sciuto et al. 2019; Trémouillaux-Guiller et al. 2002), as well as viciously infecting different molluscan species (Sokolnikova et al. 2016). Additionally, the parasitic trebouxiophyte genus *Phyllosiphon* can form specific endophytic siphonous stages within members of the monocotyledonous flowering plants of the family Araceae (Aboal and Werner 2011). Notably, the colourless genera *Prototheca* and *Helicosporidium* parasitise both vertebrates and invertebrates, including humans (Kano 2020; Tartar et al. 2002). The class Ulvophyceae also harbours parasitic taxa, such as *Cephaleuros* and *Stomatochroon* (members of the exclusively terrestrial order Trentepohliales) that infect various land plants (Brooks 2004), while *Scotinosphaera* (Scotinosphaerales) occurs as an endophyte of aquatic plants (Wujek and Thompson 2005). Members of the class Chlorophyceae include, for example, the disk-shaped *Pseudovellula*, encompassing epiphytic or epizoic microalgae living in freshwater and marine habitats (Sanchez-Puerta et al. 2006), and *Koshicola*, described as an epiphyte on the aquatic angiosperm *Spirodela polyrhiza* (Watanabe et al. 2016); both of these algae belong to the order Chaetopeltidales. The largest order Volvocales (syn. Chlamydomonadales) notably encompasses the aquatic endo-

phytic and parasitic genus *Chlorochytrium* (Wujek and Thompson 2005) and *Oophila* microalgae growing within amphibian egg capsules and invading their embryonic tissues and cells (Kim et al. 2014). In many cases, the ecological role of the epibiotic and endobiotic growth is not known, nor are the association specificity or threshold for the shift from mutualistic or commensal behaviour to a parasitic relationship.

Within the Volvocales, most of the phylogenetic diversity is represented by chlamydomonad-type biflagellate members. They are scattered across the whole order and fall within different phylogroups, as defined by Nakada et al. (2008). However, the majority of the sequenced phylogenetic lineages are not yet robustly resolved or defined taxonomically, as they lack proper identification and continue to bear the name of the polyphyletic genera *Chlamydomonas* or *Chloromonas* (Pröschold et al. 2001). Moreover, a comprehensive understanding of the ecology, behaviour, and distribution is lacking for many such lineages. Investigations of additional strains of the poorly known or understudied lineages fill such gaps, thereby improving the organisation of the known taxonomic diversity and characterisation of hitherto undescribed organisms (Barcytė et al. 2020; Nakada et al. 2016). Since the morphological diversity provides little phylogenetic resolution among chlamydomonad-morphotype algae, ecological, behavioural (including host specificity), in addition to molecular data, could be crucial for assessing the phylogenetic novelty and final taxonomic conclusions.

Here we report the isolation and characterisation of three green biflagellate strains that have close interactions with the colonial golden-brown alga *Synura petersenii* (Synurales, Chrysophyceae). Light and transmission electron microscopy were used to inspect and describe the cells, and molecular phylogenetic analyses were used to elucidate their phylogenetic position within the Volvocales. The data obtained call for a proposal of a new genus and species to accommodate a lineage of widespread freshwater microalgae associated with *Synura* colonies.

Results

Morphological and Ultrastructural Observations

We isolated and cultivated three strains (S163, S165, S182) of green biflagellate algae found living attached to and within *S. petersenii* colonies

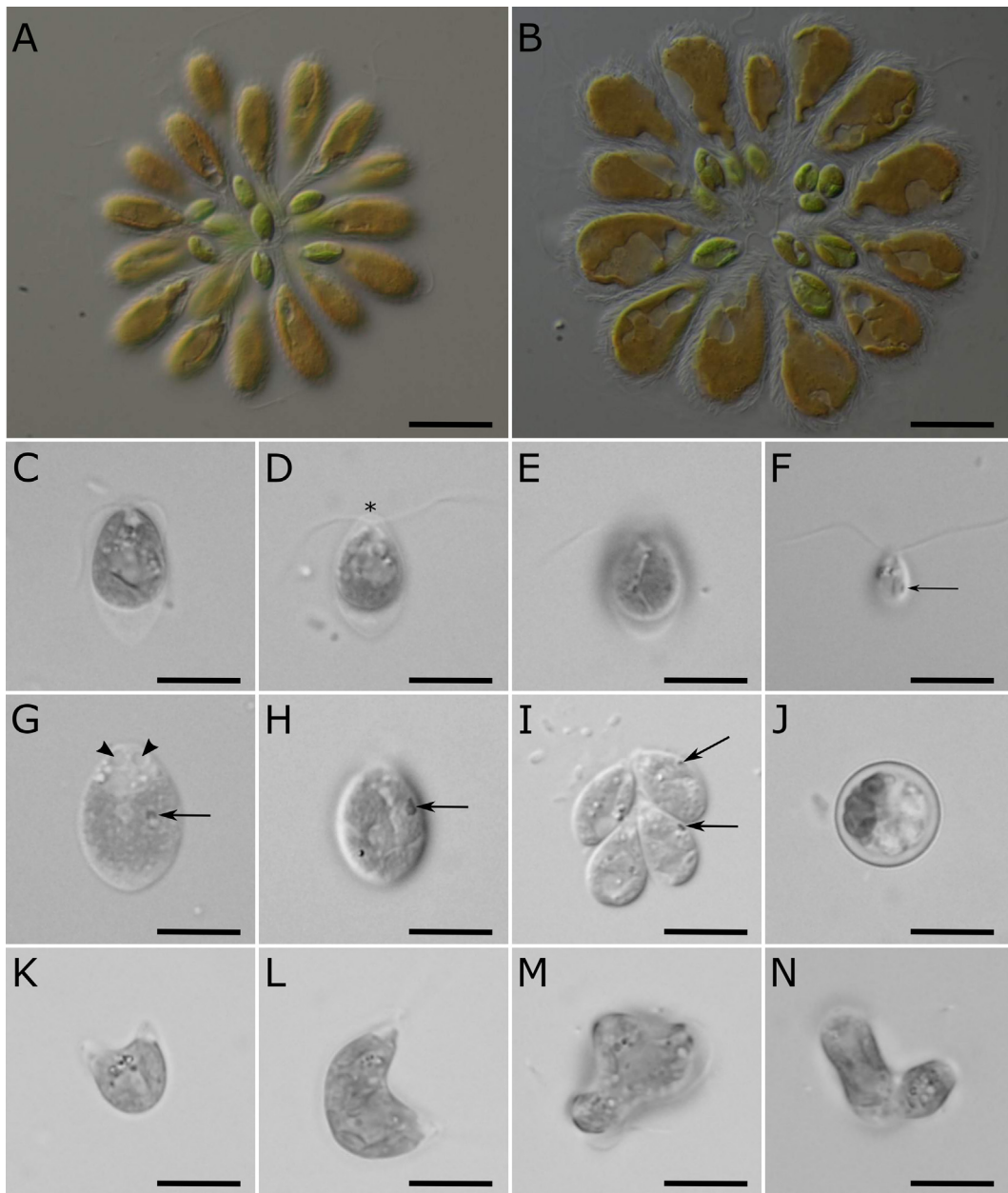


Figure 1. **A, B.** A green biflagellate alga, *Adglutina synurophila* gen. et sp. nov., living inside colonies of *Synura petersenii* (Chrysophyceae). **C.** A mature vegetative cell showing a cup-shaped lobed chloroplast, the posterior cell end noticeably detached from the protoplast, and flagella approximately the size of the cell. **D.** A mature cell with a low keel-shaped papilla (asterisk). **E.** A deep wedge-like incision. **F.** A young vegetative cell with flagella longer than the cell and a posterior eyespot (arrow). **G.** Two apical contractile vacuoles (arrowheads) and a middle eyespot (arrow). **H.** An old cell with additional chloroplast holes and a lateral middle eyespot (arrow). **I.** Four cells formed in a single zoosporangium with ellipsoidal eyespots (arrows). **J.** A thick-walled globular cell. **K.** The beginning of asexual reproduction through budding. **L.** The half-way separation of the sister buds. **M.** The formation of additional two buds. **N.** The soon-ready to separate three sister cells. Scale bars A, B = 10 μm C–N = 5 μm .

(Fig. 1A, B), sampled in different countries in Europe. The new strains, described here as *Adglutina synurophila* gen. et sp. nov., were morphologically highly alike and existed as biflagellate oval or

broadly ellipsoidal single cells (Fig. 1C–I). Their size varied from 4.0–11.0 μm in length and 2.0–8.0 μm in width, with the average cell being approximately 7.0 μm long and 4.0 μm wide. Two equal flagella

were of variable length: shorter than, equal to, or longer than the cell. Generally, mature cells had shorter flagella than young ones (Fig. 1C–F). The cell wall was thin, with an anterior low keel-shaped papilla (Fig. 1D, asterisk). At the posterior cell end, the cell wall was distant from the protoplast and formed a slightly narrower rear (Fig. 1C–E). Two apical contractile vacuoles were present (Fig. 1G, arrowheads). The chloroplast was cup-shaped and occupying most of the cell volume, often divided into three or four unequal lobes (Fig. 1C). A deep and branched incision reaching almost to the posterior was occasionally observed, splitting the chloroplast into two main parts (Fig. 1E, H). Additional lateral short slits were present giving the chloroplast a chopped-look (Fig. 1H). Old cells additionally developed few open spaces within the plastid (Fig. 1G, H). The eyespot, when seen, was prominent, ellipsoidal to somewhat triangular, and of variable position, but usually laying in the lateral middle of the chloroplast or slightly anteriorly in mature cells, and posteriorly in young smaller cells (Fig. 1F–I, arrows). Asexual reproduction occurred via formation of two to four zoospores (Fig. 1I). Cell division, including the formation of one or two additional buds was also observed (Fig. 1K–N). The conjoint sister cells that already contained flagella moved chaotically with the ongoing cytoplasmic division and secretion of separate cell walls. Sexual mating was not detected even when mixing strains together to account for possible heterothallism. Thick-walled globular cells were present in older cultures (Fig. 1J). The single nucleus was of variable position: central, anterior, or slightly posterior, often pushed to the side. Its position was confirmed by DAPI staining (Fig. 2A, arrow). The same staining also visualised multiple chloroplast nucleoids, which were scattered throughout the organelle (Fig. 2A, smaller bright blue spots). Their number was variable, likely depending on the number of the chloroplast lobes or the cell age. The DAPI dye did not stain thick-walled globular cells (Fig. 2B).

The main ultrastructural features of *Adglutina* detected through transmission electron microscopy (TEM) matched the general characteristics of the volvocalean green flagellates and are presented in Supplementary Material Figure S1. Notably, the cell wall of flagellate cells was thin, without additional thickenings or protrusions (Supplementary Material Fig. S1A). The presence of the keel-shaped papilla and two apical contractile vacuoles was confirmed (Supplementary Material Fig. S1B). Similarly to light

microscopy observations, the chloroplast was cup-shaped and divided into several main lobes; no pyrenoids were evident (Supplementary Material Fig. S1A). At times, the posterior cell end was densely filled by small cytoplasmic vacuoles of up to 0.22 μm in diameter, which contained electron-dense grain-like particles (Supplementary Material Fig. S1C). The detected mitochondrial profiles appeared to contain tubulo-vesicular cristae (Supplementary Material Fig. S1D).

Phylogenetic Analyses

The three newly isolated and sequenced *Adglutina* strains were identical in their 18S rDNA and *rbcL* sequences. They nested within the *Moewusinia* phylogroup of Volvocales as a separate phylogenetic lineage not attributable to any so far known or taxonomically resolved genus-level clade. The closest relative of the algae that has been studied is a strain designated as *Chlamydomonas* sp. BogD6/3T-6w (GenBank: AY220571), isolated from a tycho plankton sample of the mesotrophic Lake Itasca located in the Itasca State Park in Minnesota, USA (Fawley et al. 2004). With only a single nucleotide substitution it likely constitutes the same species as the three newly isolated strains presented in this study. In the phylogenetic trees obtained from the 18S rDNA with the maximum likelihood (ML) and Bayesian inference (BI), *Adglutina* (including the BogD6/3T-6w strain) formed a sister lineage to a clade, designated here as ‘clade *acidophila*’, consisting of “*Chlamydomonas*” (*Cd.*) *acidophila* Rsa6 (GenBank: KM016996) and CCAP 11/136 (GenBank: AJ783844), isolated from Bor Copper mine in Serbia and Františkovy Lázně in Czech Republic, respectively, and *Cd. eustigma* NIES-2499 (GenBank: AB701493), isolated from mosses growing in the acid mining drainage of an abandoned sulfur mine in Nagano Prefecture, Japan. Several sequences of uncultured eukaryotes reported from extremely acidic Tinto River (GenBank: AY082979) and pit lakes (GenBank: KC619619 and KC619620) from the Iberian Pyrite Belt in Spain were part of ‘clade *acidophila*’ as well. However, the sister relationship between *Adglutina* and ‘clade *acidophila*’ received only moderate statistical support in the ML analysis and no support in BI. The other close relatives of *Adglutina* uncovered by the 18S rDNA analyses included *Cd. proboscigera* CCCryo 216-05 (GenBank: GU117580) isolated from snow collected on the north-eastern part of Doktorbreen

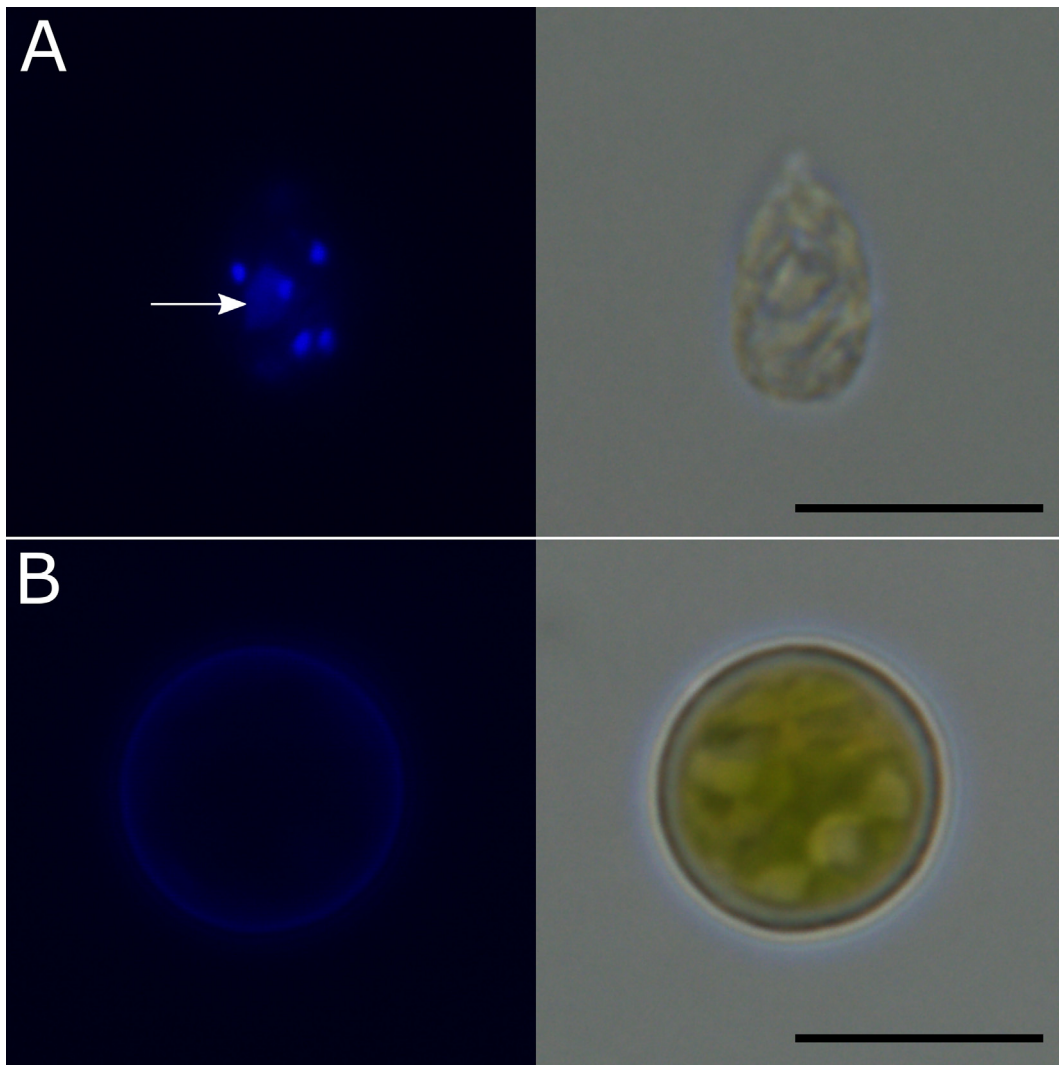


Figure 2. Fluorescence micrographs of fixed DAPI-stained cells of *Adglutina synurophila* S163. **A.** The nucleus (arrow) and chloroplast nucleoids (smaller bright blue spots) were visualised. **B.** Globular thick-walled cells were not stained. Scale bars = 5 μm .

glacier in Svalbard, Norway, and Chlorophyta sp. I-155 (GenBank: EF432529) of an unknown origin. They altogether constituted a group of significant statistical support, occupying a rather isolated position within the *Moewusinia* phylogroup (Fig. 3). In the *rbcL* analyses *Adglutina* also clustered as a sister lineage to representatives of ‘clade *acidophila*’, with ultrafast bootstrap support of 82% and a maximum posterior probability of 1.00 (Fig. 4). Additional close relatives of *Adglutina* remained unclear in the *rbcL* analysis, since only low support values were obtained for a potential sisterhood with a branch represented by *Cd. bilatus* SAG 7.72 (GenBank: AF517082) isolated from a pool in the High Tatra Mountains, Slovakia, and for the existence of an even broader clade additionally comprising two taxa

with unclear taxonomic affiliations, “*Chloromonas*” sp. ANT3 (GenBank: U80809) collected on Petrel Island, Antarctica, and Chlorophyta sp. WC6-3 (GenBank: MF278343), isolated from coal mine drainage in Butler County, Pennsylvania, USA.

ITS2 rDNA and its Secondary Structure

Adglutina strains S163 and S165 had identical ITS2 rDNA sequences, consisting of 224 nucleotides. Strain S182 differed from them by two nucleotide substitutions and contained an additional single-nucleotide insertion, making it 225 bp long. Based on a BLAST search, the closest relative represented in GenBank was the uncultured organism termed “Chlamydomonadales isolate soil 2907” (GenBank:

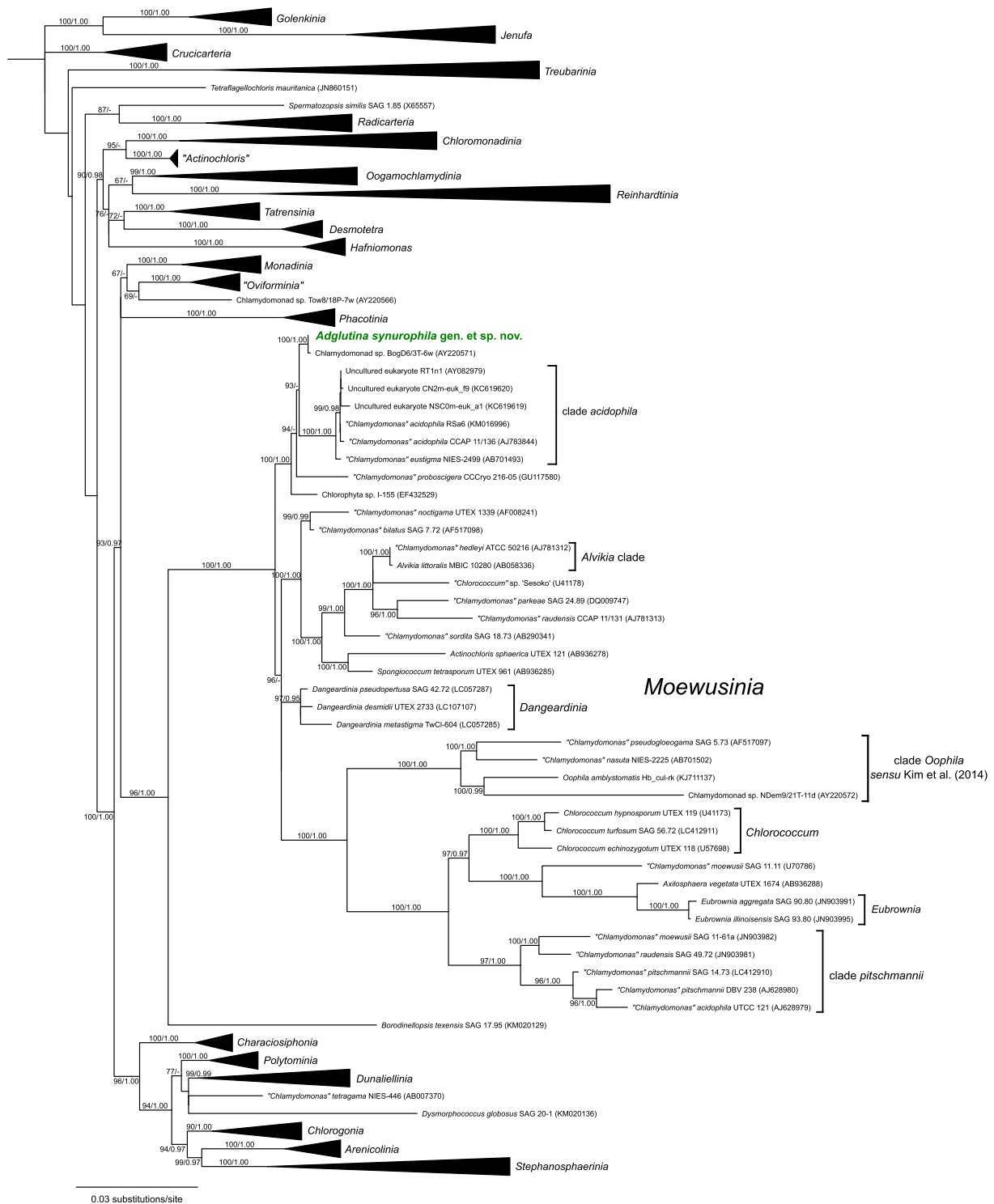


Figure 3. Maximum likelihood phylogenetic tree of the order Volvocales (Chlorophyceae) based on 18S rDNA sequences. The new genus and species described in this study is highlighted in green. Support values are shown near the nodes as follows: ML bootstrap (>50)/Bayesian posterior probability (≥ 0.95). The phylogroup “*Actinochloris*” as defined by Nakada et al. (2008) requires taxonomic re-assessment and should not be confused with the genuine *Actinochloris* (represented by the type species *Actinochloris sphaerica*) belonging to the *Moewusinia* phylogroup. The sequence from *Tetraselmis striata* SAG 41.85 (Chlorodendrophyceae) is included as an outgroup.

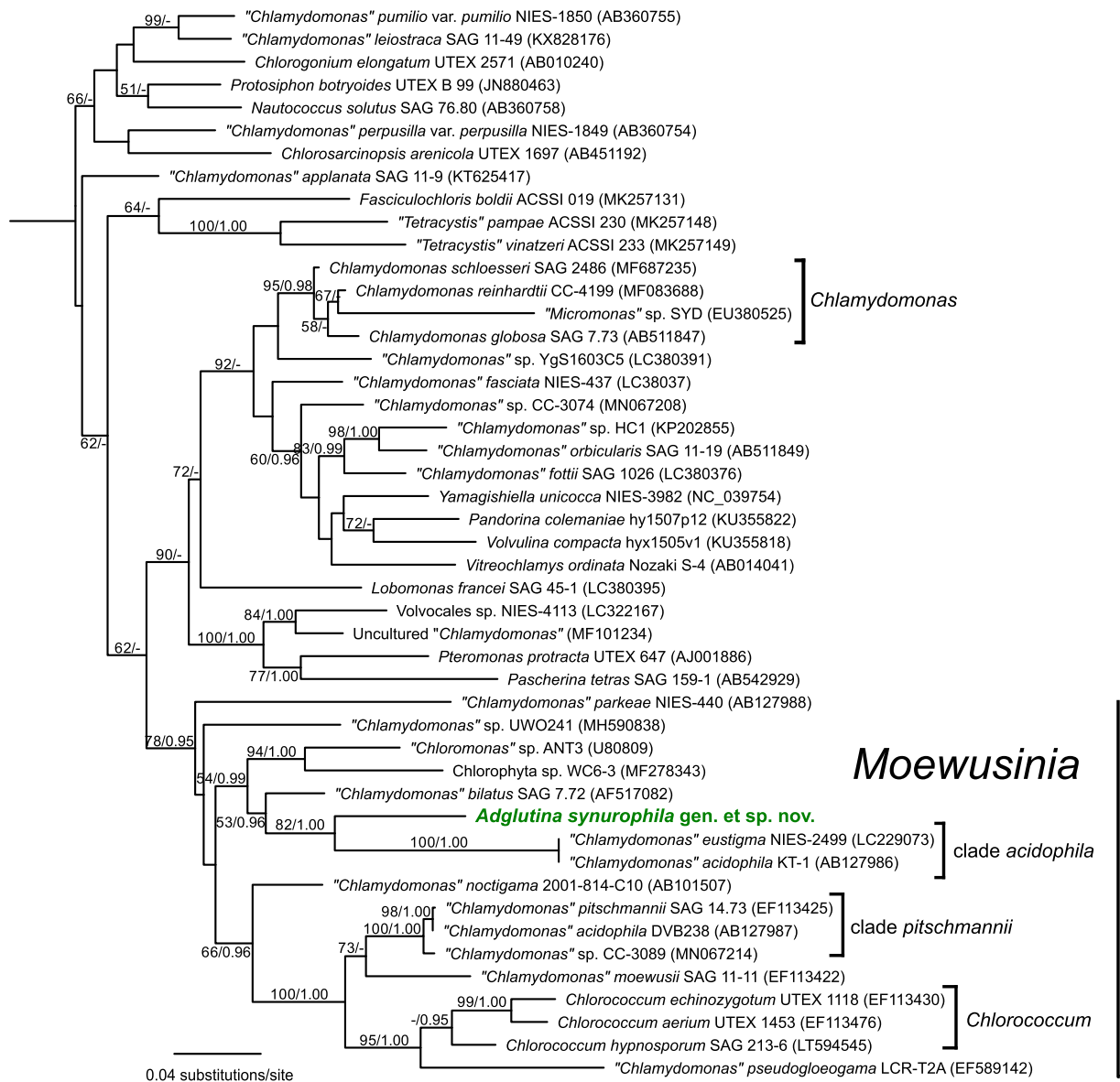


Figure 4. Maximum likelihood phylogenetic tree of the order Volvocales (Chlorophyceae) based on *rbcL* gene sequences. The new genus and species described in this study is highlighted in green. Support values are shown near the nodes as follows: ML bootstrap (>50)/Bayesian posterior probability (≥ 0.95). The sequence from *Tetraselmis striata* SAG 41.85 (Chlorodendrophyceae) is included as an outgroup.

MF484114) detected in Netherlands. It differed from *A. synurophila* strains S163/S165 and S182 by 32 and 31 nucleotides, and had eight and nine single-nucleotide deletions, respectively. The ITS2 secondary structure of the studied strains showed a typical eukaryotic architecture and motifs: four major helices, the third being the longest and the fourth the shortest, helix II containing the 'U-U' mismatch, and a 'UGG' motif present upstream to the apex of helix III. One hemi-CBC (a mutation of one of the two paired nucleotides while maintaining the nucleotide bond) between S163/S165 and S182 was pre-

sent in helix II (A was exchanged for G in S182). The terminal loop of the same helix contained the single-nucleotide (A) insertion. The second nucleotide substitution (A was exchanged for C in S182) appeared in the central loop downstream of helix IV ([Supplementary Material Fig. S2](#)).

Clingy Nature and Host Preferences

To test whether *Adglutina* sticks to *Synura* in laboratory conditions, and whether *Adglutina* prefers its native host *S. petersenii* over other species, we

designed a simple infection experiment with nine different *Synura* strains, representing six separate species: *S. petersenii* (four strains), *S. borealis*, *S. curtispina*, *S. glabra*, *S. macropora*, and *S. hibernica* (see Methods for strain names). After five days of co-culturing strain S163 with the selected *Synura* species, it was observed that *Adglutina* clung to five out of the nine tested strains. All four strains of *S. petersenii* and the single tested strain of *S. borealis* were infected by the green alga. Multiple *Adglutina* cells were observed either resting within *Synura* colonies, or trying to manoeuvre their way inside by first clinging to the peripheral cells of the colony (Supplementary Material Fig. S3). The adhesion seemed to take place through both flagella and papilla. If colonies were broken up, *Adglutina* did not stick to the single *Synura* cells, suggesting that the green alga prefers colonies which are in good physiological condition.

Discussion

Adglutina: a New Genus of Volvocales

We isolated and cultured three strains of green biflagellates representing a novel phylogenetic lineage within the *Moewusinia* phylogroup (Volvocales, Chlorophyceae). Our phylogenetic analyses of 18S rRNA and *rbcL* genes showed that this lineage branches off closest to “*Chlamydomonas*” microalgae inhabiting acidic freshwaters (note that bona fide *Chlamydomonas* species, typified by *Cd. reinhardtii*, belong to the *Reinhardtinia* clade, Fig. 3). Comparison of the ITS2 secondary structures revealed only minor differences (no CBCs) among the three studied strains supporting their assignment to a single species, herein described as *Adglutina synurophila* gen. et sp. nov. Morphologically, the new taxon is recognisable by having small oval to ellipsoidal cells, two flagella of variable length, a low keel-shaped papilla, a cup-shaped chloroplast lacking a pyrenoid, and a lateral ellipsoidal eyespot with a variable position. The observed peculiar form of asexual reproduction including the formation of buds (Fig. 1K–N) has previously been observed in other members of Volvocales, including *Microglena* (phylogroup *Monadinia*; Demchenko et al. 2012) and *Ostravomonas* (syn. *Paludistella*, phylogroup *Chloromonadinia*; Susanti et al. 2020). Since sexual mating was not detected, the observed thick-walled globular cells likely represent asexual resting cells rather than zygotes.

The most striking attribute of *Adglutina* is that it is commonly found attached to or living within *Synura* colonies. The nature of the close interaction between the two algae is unclear. *Adglutina* could protect itself from predation or infection by hiding in the *Synura* colonies while doing no harm to the golden-brown alga. Indeed, being covered by an armour of silica scales, *Synura* could deter some of the native *Adglutina* enemies that prefer to snack on the smaller green cells. Interestingly, the distantly related *Cd. reinhardtii* was found to be shielded from algicides produced by bacteria when embedded and surrounded by the resistant fungal mycelium of *Aspergillus nidulans* (Krespach et al. 2020). Though it is not known whether *Synura* is resistant to particular toxic metabolites, avoiding direct interaction with algicidal bacteria or allochemical molecules produced by other microalgae by using *Synura* as a shield, could be a feasible strategy for *Adglutina* as well. Another reasonable type of intimate association could potentially be parasitism, where *Adglutina* actively harms *Synura* by, for example, overpopulating their colonies and thus reducing available light for photosynthesis. However, experimental studies are needed to investigate either of these speculations.

Regardless of the nature of its interaction with *Synura*, *Adglutina* seems to represent a widespread freshwater lineage, molecularly confirmed to occur in Europe (including Portugal, Spain, and Estonia for the strains studied here) and North America (*Chlamydomonas* sp. BogD6/3T-6w; Fawley et al. 2004). However, no details on the behaviour and morphology of the BogD6/3T-6w strain have been published, and it is no longer available for comparison with our isolates (pers. commun. with Karen and Marvin Fawley). There is evidence for an even broader distribution of *Adglutina*-like microalgae (i.e., green biflagellates attached to *Synura* colonies) found, for example, in New Zealand (Supplementary Material Fig. S4; photo credits to Manaaki Whenua). This suggests that the interaction between the two algae is indeed not accidental, but could represent an important ecological relationship, potentially influencing the occurrence and abundance of both microalgae and overall community structure. Our simple co-culturing experiment confirmed the clingy nature of *Adglutina*, as colonies of all four tested strains of *S. petersenii* (the native host) were invaded by it (Supplementary Material Fig. S3). However, the additional invasion of *S. borealis* colonies suggests that *Adglutina* is proba-

bly not strictly specific to a particular *Synura* species. The colonial nature of *Synura*, which allows *Adglutina* to penetrate and firmly attach, is likely the key factor for the successful epibiotic functioning of *Adglutina*. For example, we observed that green cells morphologically very similar to *Adglutina* also cling to other colony-forming chrysophytes, *Uroglenopsis* and *Urostipulosphaera* (Ochromonadales) (Supplementary Material Fig. S5). Similar epibiotic interactions are not rare but rather little studied and poorly understood, despite the fact they have been noticed and reported multiple times before (examples are referred to below).

Morphologically, *Adglutina* differs from the closest presently known relative, *Cd. acidophila*, by a set of features. Like *Adglutina*, *Cd. acidophila* has a cup-shaped chloroplast, however, it is not lobed and is often situated at the side. Notably, *Cd. acidophila* possesses a large pyrenoid surrounded by a starch envelope, while a pyrenoid is apparently absent in *Adglutina*. No prominent papilla is visible in *Cd. acidophila* when observed through light microscopy (Ettl 1983; Negoro 1944), while a low papilla was detected in *Adglutina*. Finally, flagella are 1.5–2.0 times the body length in *Cd. acidophila*, while flagella in mature cells of *Adglutina* are typically shorter than the cell. The phylogenetic concept of *Cd. acidophila* is somewhat ambiguous due to the unravelled chimeric nature of the 18S rDNA sequence of the *Cd. acidophila* strain (OU 030/a) isolated from the type locality (Nakada et al. 2019). Hence, the identity of *Cd. acidophila* should be formally re-investigated in the future, considering that sequences assigned to *Cd. acidophila* also fall within the ‘clade *pitschmannii*’ (Fig. 3). Furthermore, the ‘clade *acidophila*’ also encompasses an acidophilic strain NIES-2499, identified as *Cd. eustigma*. In contrast to *Cd. acidophila*, *Cd. eustigma* was described as having a thick cell wall, an extremely large eyespot, and a large truncated cone-shaped papilla (Ettl 1983). The genome of the NIES-2499 strain has been sequenced, revealing molecular bases of adaptations for life in acidic environments, proving a true acidophilic nature, considering that similar features were detected in other acidophilic green and red algae (Hirooka et al. 2017). The *rbcl* sequences of *Cd. eustigma* NIES-2499 and *Cd. acidophila* KT-1 are identical (Fig. 4), suggesting either the conspecificity or misidentification of the two algae. Regardless, the phylogenetic distance from the ‘clade *acidophila*’ together with morphological and ecological differ-

ences (living in non-acidic freshwaters and exhibiting epibiotic behaviour) clearly establish *Adglutina* as a taxonomic entity separate from the ‘clade *acidophila*’.

Comparison with Morphologically and Behaviourally Similar Species

From the standpoint of the traditional classification of chlamydomonad-type algae, *Adglutina* would most likely be assigned to the pyrenoid-lacking genus *Chloromonas* (Ettl 1983). However, considering the suite of unique morphological features encompassing the shape of the chloroplast and papilla, the shape and position of the eyespot, and the flagella length, we could not reasonably attribute *Adglutina* to any of the previously described *Chloromonas* species. For example, *Ch. adhaerens*, described as adhering to the copepod *Cyclops* by means of a papilla, also has a cup-shaped chloroplast and flagella the length of the body. However, it differs from *Adglutina* by having a broad saddle-like indented papilla. *Chloromonas anuraeae* was also found to firmly adhere to the rotifer *Keratella* by means of its flagella. Like *Adglutina*, *Ch. anuraeae* has a cup shaped chloroplast, divided on one side into two basal lobes by a deep wedge-shaped incision reaching almost to the posterior, a low keel-shaped papilla, and flagella shorter than the cell. However, in contrast to *Adglutina*, *Ch. anuraeae* has a small dot-shaped eyespot located in the upper part of the cell, and a posterior nucleus. Even though they are morphologically and behaviourally highly comparable, attribution of the strains studied here to the species ‘*anuraeae*’ is problematic. On the other hand, it is possible that *Ch. anuraeae* is closely related to *A. synurophila*, and could represent its sister species. The conspecificity or relatedness of the two species can only be tested once similar green flagellates have been isolated from *Keratella*. Other clingy freshwater *Chloromonas* species include, for example, *Ch. squalens*, described from the mucilage of the green filamentous alga *Spirogyra*, and *Ch. rodhei*, found in the mucilage of the rotifer *Conochilus*. Both species differ from *A. synurophila* morphologically, in the case of *Ch. squalens* primarily in cell shape, since it possesses very ellipsoidal to cylindrical cells, while *Ch. rodhei* possesses a low hemispherical papilla and has flagella more than twice the body length. Additional examples of green biflagellates with similar adhering behaviour include certain pyrenoid-

containing *Chlamydomonas* sensu lato species, such as *Cd. epibiotica*, found living either in the mucilage or on the surface of various planktic microalgae and cyanobacteria (*Microcystis*, *Fragillaria*, *Melosira* or *Pandorina*), and *Cd. olifanii* adhering to the rotifer *Brachionus*. The former species has spherical or spherical-ovoid cells and the latter species notably possesses a low truncated-cone-shaped papilla. Other examples also encompass *Cd. dinobryonis*, described as living mostly in empty *Dinobryon* loricae, and *Cd. rattuli*, found attached to rotifers or to the outer layer of the mucilage of various other planktic algae (Ettl 1983). The two latter species differ from *Adglutina* not only by the presence of a pyrenoid but also by possessing a lateral plate-shaped chloroplast and lacking papillae. Most importantly, *Cd. chrysomonadis* was described by Fott (1957) as clinging to various planktic Chrysophyceae representatives, such as *Synura*, *Mallomonas* and *Dinobryon*. Even though behaviourally extremely compatible, it differs from *Adglutina* by having a trough-shaped chloroplast with a small pyrenoid and lacking an obvious papilla. Molecular data are missing for the species discussed, and their phylogenetic placement within Volvocales remains unclear. Nevertheless, the specific morphological features of *Adglutina* together with being found attached to *S. petersenii* colonies corroborate the phylogenetic conclusions, suggesting the recognition of a novel previously undescribed species.

Generic Taxonomy Within *Moewusinia*

Numerous phylogroups of Volvocales (Nakada et al. 2008) are awaiting re-classification of their constituent taxa using modern methods. Within the *Moewusinia*, some major efforts have already been devoted to better organise the known biodiversity. First of all, *Dangeardinia* was resurrected to accommodate several former *Chlamydomonas* species and currently encompasses three sequenced species. The genus is well distinguished within the group not only phylogenetically (Fig. 3) but also by possessing a chloroplast with large apical and basal parts and a tubular connecting part, and two to four pyrenoids (Nakada et al. 2016). Two novel genera, *Alvikia* and *Eubrownia*, were established to partially resolve the problem of the polyphyly of the genera *Chlorococcum* and *Tetracystis*, respectively, with *Tetracystis* finally considered a heterotypic synonym of *Chlorococcum* and recommended for permanent abandonment by transferring its species to

other genera (Watanabe and Lewis 2017). The morphologically similar genus *Spongiococcum* differs from *Chlorococcum* by possessing a different type of pyrenoid and producing slightly different zoospores. Its sister genus *Actinochloris* contains an asteroid chloroplast with a central pyrenoid, and multiple nuclei; unique features within the *Moewusinia* group. *Axilosphaera*, the sister genus of *Eubrownia*, characteristically develops open spaces around the central pyrenoid. Starting from *Alvikia*, all the six genera discussed have non-motile vegetative cells, contain pyrenoids, and reproduce by both autospores or aplanospores (though this is not clear in *Actinochloris*) and zoospores (Watanabe and Lewis 2017). These genera are phylogenetically intermixed among “*Chlamydomonas*” taxa, suggesting repeated evolutionary switches from motile to non-motile vegetative cells. The remaining freshwater genus *Ophila* matches the chlamydomonad appearance and life cycle, and is primarily associated with the egg masses of the yellow-spotted salamander *Ambystoma maculatum* (Kim et al. 2014). By describing *Adglutina*, we have established a novel phylogenetically and ecologically well-defined genus within the phylogroup *Moewusinia*. As evident from phylogenetic analyses (Fig. 4), additional genus-level taxonomical changes and novelties within the group are expected in the future.

Formal Taxonomy

We follow the set of rules and recommendations of the International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code) (Turland et al. 2018). As the historically established family-level classification of the order Volvocales does not correspond to the phylogenetic relationships within the group (Nakada et al. 2008), we do not formally place the new genus *Adglutina* into a family and leave it as *incertae sedis* in Volvocales.

Phylum: Chlorophyta

Class: Chlorophyceae

Order: Volvocales *incertae sedis*

Adglutina Barcytė, Pusztai, Škaloud & M. Eliáš gen. nov.

Description: Cells oval or broadly ellipsoidal with two equal flagella of variable length. Cell wall smooth with a low keel-shaped papilla. Two contractile vacuoles in the anterior half of the cell. Chloroplast cup-shaped without pyrenoids. Nucleus single, of variable position. Eyespot of variable

shape and position. The genus represents a separate phylogenetic lineage within *Moewusinia* (*Xenovolvox*, Volvocales sensu Nakada et al. 2008) based on 18S rDNA and *rbcl* gene phylogenies, and thus excludes morphologically similar taxa belonging to other phylogroups.

Etymology: The name comes from the Latin verb *adglutino* meaning “to stick, glue”.

Type species: *Adglutina synurophila* sp. nov.

Adglutina synurophila Barcytè, Pusztai, Škaloud & M. Eliáš sp. nov. Fig. 1C–N

Description: Cells ovoid or broadly ellipsoidal, rounded at the base, 4.0–11 µm long and 2.0–8.0 µm wide. Two equal flagella shorter than, equal to or longer than the cell. Cell wall thin and smooth, papilla low and keel-shaped, sometimes indistinct. Two apical contractile vacuoles. Chloroplast cup-shaped, composed of two to four main parts, no pyrenoids. Eyespot ellipsoidal to triangular, located in the lateral middle, slightly anterior or posterior of the chloroplast, often concealed. Nucleus central, anterior, or slightly posterior. Asexual reproduction by two or four zoospores. Sexual reproduction not observed. Firmly adheres to *Synura* colonies.

Holotype (designated here): The type strain S163 is permanently cryopreserved in a metabolically inactive state under the accession number CAUP G 1501-CRYO at the Culture Collection of Algae at Charles University (CAUP) in Prague, Czech Republic.

Isotype (designated here): TEM block CAUP G 1501-ISOTYPE at the Culture Collection of Algae at Charles University (CAUP) in Prague, Czech Republic.

Reference strains (ex-types): The living cultures of S163 have been deposited as CAUP G 1501 at the Culture Collection of Algae at Charles University (CAUP) in Prague, Czech Republic, and as CCALA 1136 at the Culture Collection of Autotrophic Organisms (CCALA) in Třeboň, Czech Republic.

Gene sequences: OL310960 (18S rDNA), OL310961 (ITS2 rDNA), OL310463 (*rbcl*)

Type locality: Pateira de Fermentelos, Portugal

Etymology: *phila* means “loving” in Greek, referring to the *Synura*-loving nature of the species.

Methods

Isolation and cultivation: Three strains of green biflagellates, S163, S165 and S182, were isolated from *Synura petersenii* colonies in samples (S163.B5, S165.G3, S182.E6) from the water bodies of Pateira de Fermentelos, Portugal (40° 33' 21.456" N 8° 30' 31.5" W;

water temperature 7.5 °C, pH 7.9, conductivity 535 µS/cm), Encor das Conchas, Spain (41° 56' 21.948" N 8° 2' 3.192" W; water temperature 7.1 °C, pH 6.0, conductivity 56 µS/cm), and Keerijärv, Estonia (58° 19' 48.576" N 26° 28' 5.556" E; water temperature 8.8 °C, pH 7.4 °C, conductivity 308 µS/cm), respectively. Single colonies of *S. petersenii* that contained green epibionts were picked up by the use of a pipette and transferred to microplates filled with Bold's Basal Medium (BBM; Bischoff and Bold 1963), a cultivation medium not suitable for chrysophytes but highly preferred by the majority of green algae. When green biomass was predominant, clonal strains of the target organism were isolated stochastically through serial dilution in 96-well microplates. The wells were observed under NIB-100 inverted biological microscope (Nanjing Jiangnan Novel Optics Co., Ltd, Nanjing, China) to ensure the presence of the single cells. The three strains were maintained in culture in BBM with natural day/night cycles and at room temperature. The newly established strains are housed in the working collection at the Department of Biology and Ecology, University of Ostrava, and are available upon request.

DNA isolation, PCR, and sequencing: Total genomic DNA from the strains was extracted using a Geneaid Genomic DNA Mini Kit (Plant) (New Taipei City, Taiwan) following the manufacturer's instructions. Nucleotide sequences of the 18S rDNA were amplified and sequenced using primers NS1 and 18L (Hamby et al. 1988). Internal transcribed spacer 2 (ITS2) was amplified and sequenced with two sets of primers, nr-SSU-1780-5' Algal and nr-LSU-0012-3' Algal (Piercey-Normore and DePriest 2001) and ITS1 and ITS4 (White et al. 1990). A partial sequence of the chloroplast *RuBisCO* large subunit gene (*rbcl*) was amplified and sequenced with the primers *rbcl*1F and *rbcl*23R (Hoham et al. 2002). All PCR runs were done using 2x PCR BIO Taq Mix Red master mix (PCR Biosystems, London, UK). Cycling parameters for nuclear markers included an initial denaturation at 95 °C for 5 min, 35 amplification cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and elongation at 72 °C for 3 min, followed by a final elongation at 72 °C for 10 min. Post-cycling, samples were held at 4 °C. Cycling parameters for the plastid marker included an initial denaturation at 95 °C for 10 min, 35 amplification cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, elongation at 72 °C for 2 min 30 sec, followed by a final elongation step at 72 °C for 10 min. The obtained PCR products were purified with a Thermo Scientific GeneJET PCR Purification Kit. All amplicons were directly Sanger-sequenced at Macrogen (Amsterdam, Netherlands). Forward and reverse reads were combined using SeqAssem ver. 07/2008 (Hepperle 2004). Sequences were deposited in GenBank under the accession numbers OL310960 (18S rDNA), OL310961–OL310962 (ITS2 rDNA), and OL310463 (*rbcl*).

Microscopy: The three studied strains were observed using an Olympus BX53 (Tokyo, Japan) microscope. Micrographs were captured using an Olympus DP73 (Tokyo, Japan) digital camera. Cells were measured using the Olympus micro-imaging software cell-Sens v1.6 (Tokyo, Japan).

For DNA staining, cells were fixed for 30 min in 4% PFA in PBS at room temperature. The fixative was washed out with distilled water and cells were air-dried. Finally, the cells were dehydrated in a series of 50%, 80% and 100% ethanol for 3 min each. They were air-dried and mounted with ProLong® Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (or DAPI, Life Technologies). The prepared slides were observed with the same microscope under UV excitation.

For transmission electron microscopy (TEM), the strain S163 was centrifuged and immersed in a cryoprotectant (20% BSA in cell medium) and immediately frozen with a Leica HPM100 high-pressure freezer (Leica Microsystems, Vienna, Austria). The cells were freeze substituted in 2% OsO₄ and 100% acetone using an automatic freeze substitution system (Leica EM AFS2). The sample

was then washed three times in 100% acetone followed by a gradual embedding in EMBED-812 resin at room temperature (resin:acetone – 1:2, 1:1, and 2:1 for one hour each) with a final overnight embedding in 100% resin. Finally, the sample was embedded into a fresh degassed resin and polymerised at 60 °C for 48 hours. Thin sections were cut on a Reichert-Jung Ultracut E ultramicrotome and stained using uranyl acetate and lead citrate. Sections were examined and photographed using a JEOL JEM-1011 (Tokyo, Japan) electron microscope, equipped with a Veleta camera and the iTEM 5.1 software (Olympus Soft Imaging Solution GmbH).

Constructions of alignments and phylogenetic analyses:

Newly obtained nucleotide sequences were BLASTed (Altschul et al. 1997) to search for the closest relatives deposited in the GenBank database. The retrieved sequences were used to build two main datasets for phylogenetic analyses. The first one contained 177 18S rDNA sequences of the main Volvocales (Chlorophyceae) phylogenetic lineages, including a comprehensive representation of the *Moewusinia* phylogroup. The second dataset contained 48 *rbcl* sequences of the closest relatives revealed by the phylogenetic analysis of the 18S rDNA. Nucleotide sequences were aligned using MAFFT version7 (Katoh and Standley 2013) employing the E-INS-I option. The generated separate 18S rDNA and *rbcl* multiple sequence alignments were trimmed with TrimAL v1.2 (Capella-Gutiérrez et al. 2009) with the *–automated1* option, using a heuristic method to decide which is the best automated method to trim the alignment. The final 18S rDNA alignment had 1795 aligned positions, and the final *rbcl* alignment was composed of 1026 aligned positions. The best nucleotide substitution model for the 18S rRNA dataset was searched with ModelFinder (Kalyaanamoorthy et al. 2017) using the BIC criterion as implemented in IQ-TREE version 2.1.1 (Minh et al. 2020), with the TIM2+F+R7 model selected as best fitting the dataset. The analysis was further continued with the reconstruction of the maximum likelihood (ML) phylogenetic tree with 1000 ultrafast bootstrap replicates. The *rbcl* dataset was partitioned by codon position using PartitionFinder2 (Lanfear et al. 2017) with *linked* branch lengths and the *greedy* search choice employed to find optimal models for the first, second and third codon positions, following the BIC. The ML phylogenetic inference was conducted using IQ-TREE with the partitioned dataset and the chosen models applied (TIM+I+G for the first, TIMEF+I+G for the second, and GTR+I+G for the third codon positions), including 1000 ultrafast bootstrap replicates. The Bayesian inference (BI) was conducted using MrBayes 3.2.7. (Ronquist et al. 2012). Two runs with four chains of Markov chain Monte Carlo (MCMC) iterations for 2,000,000 (18S rDNA) and 1,000,000 (*rbcl*, partitioned dataset) generations were performed under the GTR+G+I model, with trees sampled every 100 generations, and the first 25% of trees discarded as burn-in. Stationarity and convergence of the runs were checked with Tracer v1.7.2 (Rambaut et al. 2018). Phylogenetic trees were visualised using Fig-Tree v.1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>), and post-processed with Inkscape 0.91 (Free Software Foundation, Inc., Boston, USA).

ITS2 secondary structures: ITS2 sequences of the three studied strains were annotated using the ITS2 database (<http://its2.bioapps.biozentrum.uni-wuerzburg.de/>). They were used for a BLAST search for high-scoring sequences. ITS2 secondary structures were predicted using RNAstructure 5.7 (Reuter and Mathews 2010). The folded structures were compared and manually modified in 4SALE 1.7 (Seibel et al. 2006, 2008). The sequence and structure alignment was built using the ClustalW algorithm implemented in 4SALE. Visual representation of the structures was prepared with VARNA 3.9 (Darty et al. 2009) and modified with Inkscape 0.91.

Infection experiments: Nine *Synura* strains representing six separate species, *S. petersenii* (R39, R41, S102.CZ, and IR38B), *S.*

borealis (S58.C7), *S. curtispina* (SAG 29.92), *S. glabra* (S32.2), *S. macropora* (S71.B4), and *S. hibernica* (IEB11) were infected with the green biflagellate isolate S163 and co-cultured for five days in eight replicates each in a volume of a well of 24-well cell culture plates (SPL Life Sciences, Pocheon, Korea). The infection was considered successful when the green alga was found inside the majority of *Synura* colonies.

CRedit authorship contribution statement

Dovilė Barcytė: Conceptualization, Investigation, Visualization, Writing – original draft. **Martin Pusztai:** Writing – review & editing. **Pavel Škaloud:** Conceptualization, Resources, Funding acquisition. **Marek Eliáš:** Supervision, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.protis.2022.125858>.

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