Charles University

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Study programme: Ecology

Dovilė Barcytė

Diversity of microalgae from extreme habitats: linking phylogeny and ecology

Diverzita mikrořas z extrémních habitatů: propojení fylogeneze a ekologie

Doctoral thesis

Supervisor: doc. RNDr. Linda Nedbalová, Ph.D.

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Prohlášení

Prohlašuji, že tato disertační práce je mým původním dílem, výsledkem mého vlastního úsilí a spolupráce s uvedenými spoluautory. Uvedla jsem všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného titulu na této nebo jiné instituci.

v Praze, červen 2019

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Dovilė Barcytė

This doctoral thesis is based on the following papers:

I. **BARCYTĖ D** & NEDBALOVÁ L (2017) *Coccomyxa*: a dominant planktic alga in two acid lakes of different origin. *Extremophiles* **21**: 245–257

II. **BARCYTĖ D** & HODAČ L (2019) *Watanabea acidotolerans*: A new trebouxiophyte lineage (Chlorophyta) inhabiting low pH environments from Europe to South America. *Phycological Research* **67**: 120–127

III. **BARCYTĖ D**, NEDBALOVÁ L, CULKA A, KOŠEK F & JEHLIČKA J (2018) Burning coal spoil heaps as a new habitat for the extremophilic red alga *Galdieria sulphuraria*. *Fottea* **18**: 19–29

IV. **BARCYTĖ D**, ELSTER J & NEDBALOVÁ L (2018) Plastid-encoded *rbc*L phylogeny suggests widespread distribution of *Galdieria phlegrea* (Cyanidiophyceae, Rhodophyta). *Nordic Journal of Botany* **36**: e01794

V. **BARCYTĖ D**, HODAČ L, NEDBALOVÁ L & ELSTER J (2018) *Chloromonas arctica* sp. nov., a psychrotolerant alga from snow in the High Arctic (Chlamydomonadales, Chlorophyta). *International Journal of Systematic and Evolutionary Microbiology* **68**: 851–859

VI. **BARCYTĖ D**, HODAČ L, NEDBALOVÁ L & ELSTER J (2018) *Chloromonas svalbardensis* n. sp. with insights into the phylogroup *Chloromonadinia* (Chlorophyceae). *Journal of Eukaryotic Microbiology* **65**: 882–892

VII. **BARCYTĖ D**, HODAČ L & NEDBALOVÁ L (unpublished manuscript) Overlooked diversity with terrestrial lifestyle within the predominantly freshwater and snow phylogroup *Chloromonadinia* (Volvocales, Chlorophyceae). Under review in *European Journal of Phycology*

VIII. **BARCYTĖ D**, PILÁTOVÁ J, MOJZEŠ P & NEDBALOVÁ L (unpublished manuscript) The Arctic green alga *Cylindrocystis* (Zygnematophyceae, Streptophyta) is genetically and morphologically diverse with an effective accumulation of polyphosphate. Under review in *Journal of Phycology*

Appendices:

IX. **BARCYTĖ D**, HODAČ L & NEDBALOVÁ L (2017) *Lunachloris lukesovae* gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), a novel coccoid green alga isolated from soil in South Bohemia, Czech Republic. *European Journal of Phycology* **52**: 281–291

X. **BARCYTĖ D**, FOTT J & NEDBALOVÁ L (2019) A molecular approach to identification of protonemata helps assess biodiversity of extremely acidic freshwaters. *Limnology* **20**: 225–231

Contents

Abstract

The diversity of microalgae from extreme habitats is far from being fully explored and understood. This is due, in part, to limited sampling efforts along with complex algal taxonomy and systematics. In particular, little investigated extremely acidic (pH < 3.0) and polar habitats might veil novel, undescribed species. This doctoral thesis aimed to study algal strains isolated from acidic or acidic and hot, and cold environments using a polyphasic approach. It focused on coccoid (*Coccomyxa* and *Watanabea*), monadoid (*Chloromonas* and its relatives) and saccoderm (*Cylindrocystis*) green microalgae from the classes Trebouxiophyceae, Chlorophyceae, and Zygnematophyceae, respectively. In addition, coccoid red algae (*Galdieria*) from the class Cyanidiophyceae were covered. The main research methods included light and transmission electron microscopy combined with Sanger sequencing. Nuclear 18S rDNA and plastid *rbc*L sequences were used for construction of phylogenetic trees for taxonomic position evaluation. Comparisons of secondary structure models of the highly variable ITS2 rDNA molecular marker served for a more detailed genetic relationship estimation among close relatives.

Results have shown that *Coccomyxa* and *Watanabea* are present in extremely acidic lakes and that acidity and increased availability of phosphorus may have a significant role in determining their dominance and distribution in freshwater ecosystems (**papers I, II**). A new habitat – a burning coal spoil heap – was discovered and described for the extremophilic red alga *Galdieria sulphuraria* (**paper III**). Meanwhile, a new isolate of *Galdieria phlegrea* from an acidic but non-thermal habitat suggested wider distribution and higher ecological versatility of the species than previously thought (**paper IV**). Two new cold-tolerant *Chloromonas* species from the High Arctic were described (**papers V, VI**). In addition, *Chloromonas* was shown to be paraphyletic and temperature was suggested as the main speciation factor (**paper VI**). Little explored terrestrial lifestyle provided further evidence that terrestrial vs aquatic lifestyle could have significantly influenced evolutionary diversification of the *Chloromonadinia* phylogroup and a new genus *Ostravamonas* was proposed (**paper VII**). The first culture-based molecular and morphological/cytological data of the Arctic *Cylindrocystis* were obtained (**paper VIII**). In addition, a new trebouxiophyte lineage *Lunachloris* isolated from a common terrestrial habitat was described (**paper IX**), and moss protonemata occuring in extremely acidic lake were identified to the species level using standard molecular markers (**paper X**).

To conclude, this doctoral thesis revealed the novel diversity of eukaryotic microorganisms in extremely acidic and cold habitats. It showed that diversity of extreme environments is highly underestimated in the traditional understanding of species. Finally, it demonstrated that closely related algal strains, and species, are found in similar extreme environments irrespective to their geographic distance.

Abstract in Czech

Diverzita mikrořas z extrémních habitatů není zcela prozkoumaná, což je zřejmě způsobeno omezeným úsilím, které bylo dosud věnováno sběru vzorků z často nehostinných prostředí, spolu s komplikovanou taxonomií a systematikou řas. Zejména v málo prozkoumaných extrémně kyselých (pH < 3.0) a polárních habitatech se mohou vyskytovat nové, dosud nepopsané druhy. Cílem této disertační práce bylo polyfázické studium řasových kmenů z extrémních stanovišť s nízkým pH, s nízkým pH v kombinaci s vysokými teplotami a z polárních oblastí. Práce je zaměřena na kokální zelené řasy z třídy Trebouxiophyceae (rody *Coccomyxa* a *Watanabea*), monadoidní zelené řasy z třídy Chlorophyceae (*Chloromonas* a příbuzné rody) a saccodermní zelené řasy z třídy Zygnematophyceae (rod *Cylindrocystis*). Dále se zabývá kokálními ruduchami z třídy Cyanidiophyceae (rod *Galdieria*). Hlavní metody výzkumu získaných řasových kmenů zahrnovaly světelnou a transmisní elektronovou mikroskopii a Sangerovo sekvenování jaderných a chloroplastových molekulárních markerů. Jaderné (18S rDNA) a plastidové (*rbc*L) sekvence byly použity na rekonstrukci fylogenetických stromů a na vyhodnocení taxonomického postavení studovaných organismů. Porovnání modelů sekundární struktury jaderné ITS2 rDNA jako vysoce variabilního molekulárního markeru sloužilo pro detailnější odhad genetických vztahů mezi blízce příbuznými organismy.

Výsledky získané v rámci disertační práce ukázaly, že se zelené řasy z rodů *Coccomyxa* a *Watanabea* vyskytují v extrémně kyselém sladkovodním prostředí a že kyselost vody a zvýšená dostupnost fosforu mohou hrát důležitou roli v jejich sezónní dominanci a obecném geografickém rozšíření (**články I, II**). Dále byl objeven a popsán nový typ habitatu – hořící uhelná halda, kde se vyskytuje extremofilní ruducha *Galdieria sulphuraria* (**článek III**). Nově byl druh *Galdieria phlegrea* zjištěn v kyselé mezofilní lokalitě, což naznačuje jeho širší rozšíření a větší ekologickou verzatilitu ve srovnání s dřívějšími údaji (**článek IV**). Z vysoké Arktidy byly popsány dva nové druhy rodu *Chloromonas* tolerující nízké teploty (**články V, VI**). Vlastní fylogenetické studie potvrdily, že rod *Chloromonas* je parafyletický a teplota byla navržena jako hlavní faktor ovlivňující speciaci (**článek VI**). Nově zjištěný výskyt zástupců skupiny *Chloromonadinia* v málo prozkoumaných terestrických habitatech potvrdil předpoklad, že její evoluční diverzifikaci ovlivňuje terestrický respektive akvatický způsob života (**článek VII**). V rámci téže publikace byl navržen nový rod zelených monadoidních řas *Ostravamonas* (**článek VII**). Pro spájivé řasy z rodu *Cylindrocystis* byla získána vůbec první molekulární, morfologická a cytologická data založená na kulturách izolovaných z Arktidy (**článek VIII**). Dále se ukázalo, že se v běžných terestrických habitatech stále skrývají nepopsané linie zelených kokálních řas (například rod *Lunachloris* ze třídy Trebouxiophyceae, **článek IX**). Standardní molekulární markery se také osvědčily při druhové identifikaci protonemat mechů rostoucích v extrémně kyselém jezeře (**článek X**).

Lze shrnout, že tato disertační práce přispěla k odhalení dosud nepopsané diverzity eukaryotických mikroorganismů v extrémně kyselých a v chladných habitatech. Z předložené práce dále vyplývá, že v extrémních prostředích je diverzita, pokud jde o tradiční chápání druhu, silně podceněná. Konečně výsledky práce ukázaly, že se blízce příbuzné taxony vyskytují v podobných typech extrémních prostředí, i když jsou z geografického hlediska izolované.

Introduction

Extreme habitats and extremophiles

There is no perfect definition for an **extreme habitat**, however, they are usually understood as environments that exhibit physical (e.g., temperature, radiation, pressure) and/or geochemical (e.g., pH, salinity, desiccation, toxic metals) extremes that create unvafourable or challenging conditions to most life forms (Rothschild & Mancinelli, 2001). Two types of extreme environments can be distinguished: **stable** and **unstable** in terms of seasonal and diurnal variation (Elster, 1999). However, the term 'extreme environment' is generally associated with stable or prolonged extreme conditions rather than with periodically varying environments, such as, for example, polar rivers, streams or wetlands.

Extreme habitats can occur naturally, e.g., active volcanic geothermal sites or deserts, and be man-made, e.g., acid mine drainage (AMD). These harsh and at first glance uninhabitable environments can surprise by unique and/or unexpected life. Accordingly, they have become analogues for astrobiology (Garcia-Lopez & Cid, 2017; Martins *et al*., 2017). For example, biodiversity studies have been conducted in the extremely dry Atacama Desert in Chile (Azua-Bustos *et al*., 2012; Crits-Christoph *et al*., 2013), extremely acidic Tinto River in Spain (Amaral-Zettler *et al*., 2002; Amils *et al*., 2011), extremely cold and dry McMurdo Dry Valleys in Antarctica (de la Torre *et al*., 2003) or Arctic and Antarctic pack ice (Brinkmeyer *et al*., 2003), including permanently cold, dark and high-pressure subglacial Lake Vostok located at the southern Pole of Cold (Rogers *et al*., 2013).

Organisms that are capable of growing and reproducing in extreme conditions (regarded as such by human standards) are called **extremophiles** (from the Latin *extremus* meaning "extreme" and Greek *philos* meaning "loving"). Extremophiles can be divided into two broad categories: **obligate extremophiles** which require extreme conditions for optimal growth and metabolism, and **facultative extremophiles** or **extremotolerant** organisms which can withstand various abiotic stresses though growing optimally at 'normal' conditions (Rampelotto, 2013). Some extremophiles can be exposed to or require multiple stressors and are, accordingly, called **polyextremophiles**. Extremophiles can also be described on the basis of conditions they live under. For example, **acidophiles** thrive in environments with low pH values (generally lower than 5.0). **Extreme acidophiles** have pH optima < 3.0 (Johnson, 2007), and are found, for instance, in marine volcanic vents, solfataric fields and springs, or in AMD environments. On the contrary, **alkaliphiles** prefer pH values above neutral (significantly higher than 8.0), found in soda lakes and soda deserts, or in highly alkaline groundwaters (Grant *et al*., 1990). **Thermophiles** live at relatively high temperatures

(generally > 40 °C), while **psychrophiles** depend on low temperatures (lower than 15 °C) (Morita, 1975). **Halophiles** thrive in environments with high salt concentrations (generally several times higher than that of the seawater, i.e. 35 g L^{-1}), such as brine springs and solar salterns, coastal splash zones or tide pools, and soda lakes (Oren, 2008; Ventosa, 2004). **Xerophiles** can grow and reproduce in environments with a low water activity, such as hypersaline lakes or extremely dry deserts (Grant, 2004).

Extremophiles include members of all three life domains, namely, Bacteria, Archaea and Eukarya. Prokaryotic microorganisms are usually the dominant biotic constituents of the extreme environments. Though to a lesser extent, microscopic eukaryotes (e.g., algae, fungi, protozoa, tardigrades) are present there as well. Due to different nature of the main taxonomic ranks, boundaries of their tolerable extremes are also different (Fig. 1). For example, the temperature maximum reported for the archea is 122 °C (Takai *et al*., 2008), while the upper temperature limit for the eukaryotes is twice as low (Tansey & Brock, 1972).

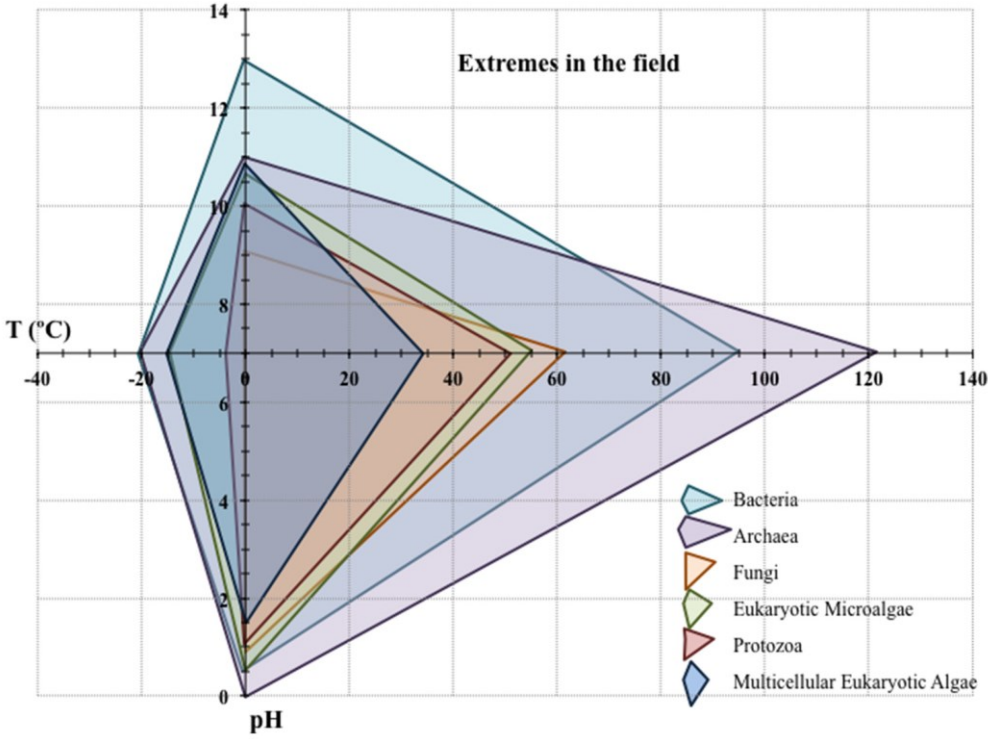

Figure 1. Temperature and pH ranges of different taxonomic groups. Taken from Reese *et al*. (2014).

Life in extreme conditions or survival "at the edge" is coupled with both short-term or long-term adaptations and strategies (Reese *et al*., 2014). Phenotypic (morphological, physiological and behavioural) and genomic (e.g., horizontal gene transfers, chromosome

shattering, etc.) plasticity is crucial for life in extreme environments (Chevin & Hoffmann, 2017; Hirooka *et al*., 2017; Ho & Zhang, 2018; Li *et al*., 2014; Zhang *et al*., 2016).

This doctoral thesis deals with microalgae isolated from **acidic**, **acidic and hot**, and **cold** habitats. Hereafter, just relevant terrestrial and freshwater extreme environments are introduced.

Acidic habitats

Acidic environments (pH < 5.0) are widely distributed in the lithosphere. They occur due to natural and anthropogenic reasons, such as natural release of hydrogen ions (H^+) and acids (e.g., peatlands, forest soils), or natural and antrophogenic atmospheric acid deposition, mostly acid rain (Hendrey *et al*., 1976; Schindler, 1994; Nedbalová *et al*., 2006). Extremely acidic (pH < 3.0) habitats are less common. They have two major origins: one associated with volcanic or geothermal activities, and the other with metal or coal mining, including AMD (Hrdinka *et al*., 2013; **paper III**). In the first case, acidity is generated by either biological oxidation of elemental sulfur (S^0) in terrestrial environments or by dissolution of volcanic gases in aquatic environments. In the second case, acidity is produced by accelerated oxidation of sulfidic minerals, mostly pyrite (FeS_2), coupled with the activity of sulfur-oxidizing bacteria and archaea, e.g., Tinto River (**paper IV**) (Johnson, 1998).

Hydrogen ion activity (pH) controls metal speciation which, in turn, affects their solubility, mobility, availability and toxicity. A small shift in pH can increase or decrease concentrations of heavy metals. For example, aluminium (Al) becomes more soluble and potentially more toxic to biota when acidified because it shifts to the toxic Al^{3+} form (Gensemer & Playle, 1999). Therefore, organisms found in acidic environments are not only exposed to the low pH but also to the high concentrations of various toxic metals, especially Al, since it is the third most abundant crustal element. Moreover, Al forms an inorganic complex with phosphorus (Gensemer & Playle, 1999) what can lead to oligotrophication of a water body (Kopáček *et al*., 2000). In addition, AMD lakes are also usually rich in iron and sulfate (Hrdinka *et al*., 2013). Finally, extremely acidic freshwaters are limited by dissolved inorganic carbon necessary for photosynthesis (Gross, 2000; Lessmann *et al*., 2000).

Overall, the high H^+ influx, toxic water chemistry and frequent nutrient limitation make acidic habitats extreme environments for the majority of life forms.

Hot habitats

Hot environments emerge naturally where volcanic exhalations heat up soils, mud holes or surface waters, forming either acidic solfataric fields, or neutral to slightly alkaline hot

springs. Meanwhile, non-volcanic hot habitats arise where geothermal activity heats up the underground water and rises it to the earth's surface. Anthropogenic hot habitats include smouldering coal spoil heaps (**paper III**) and hot outflows from geothermal power plants (Stetter, 1999).

Apart from high temperature (up to 100 °C), organisms found in hot habitas are also exposed to uncommon composition of minerals (especially iron minerals, such as ferric hydroxides and FeS_2) and gases (e.g., H_2S), pH (ranging from 0–3 or 7–10), redox potential and salinity (Stetter, 1999).

Arctic habitats

The Arctic is a large and heterogenenous area located at the northernmost part of the Earth (Fig. 2). The arctic climate is generally characterized as cold, dry and windy. Winters are long with a low amount or absence of sunlight, while summers are short with long days. Based on environmental and biological characteristics, the Arctic can be divided into the Low Arctic and the High Arctic (Thomas *et al*., 2008). The High Arctic encompasses northern fringes of the continental ladmasses and Arctic Ocean archipelagoes, e.g., Svalbard (Fig. 2). The region is characterized by permanent ice and snow and vegetation is limited to a thin discontinuous cover of tiny flowering plants, mosses and lichens. Permafrost (or permanently frozen ground) is one of the main restricting factors limiting the presence of woody plants at high latitudes (Crawford, 2008).

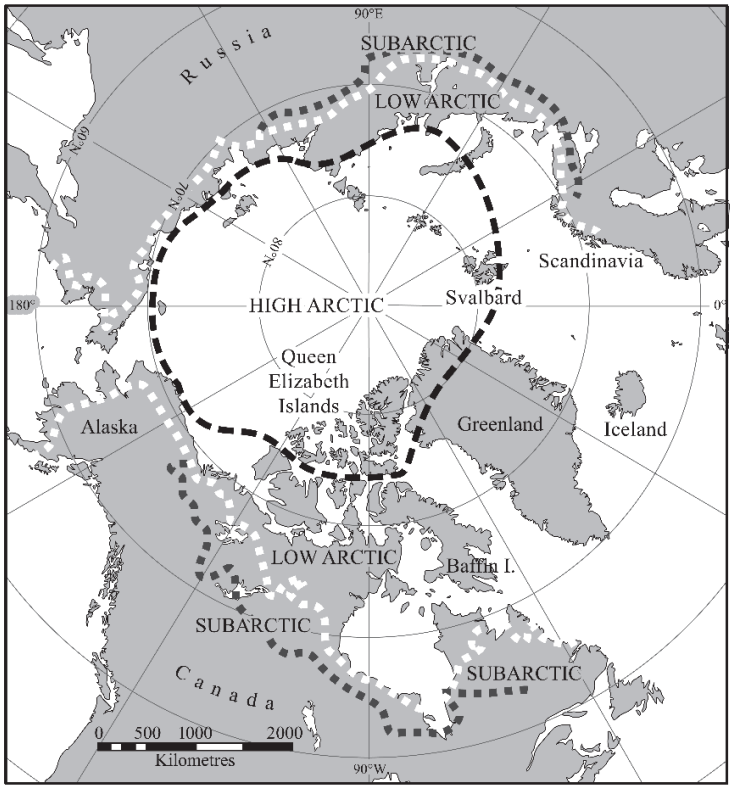

Figure 2. Biogeographical zones of the Arctic. From Thomas *et al*. (2008).

The Arctic possess numerous stress factors, the most obvious being low temperature. Apart from directly induced stress and damages to organisms (Mazur, 1984), low temperature also regulates water availability. For example, during winter, liquid-phase water is locked up in as snow and ice and is inaccessible to organisms. Arctic soils are poorly developed and unstable (Tedrow & Cantlon, 1958). Annual freeze-thaw cycles and other periglacial processes deform soils and affect composition and function of their communities (Kumar *et al*., 2013). Due to low soil temperature, short vegetation season, strong seasonal fluctuations and especially occurrence of permafrost, nutrient cycling is also slow, resulting in the limitation of essential elements, in particular nitrogen and, to some extent, phosphorus (Stark, 2007). However, sometimes nutrients can be abundant, e.g., close to seabird colonies (Ziółek & Melke, 2014). Increased levels of photosynthetically active radiation (PAR; 400–700 nm) and ultraviolet-B radiation (UV-B; 280–320 nm) reaching Arctic affect organisms at different levels, ranging from molecular and physiological damage at the single cell level to levels of populations, communities and ecosystems (Dahms *et al*., 2011).

Arctic non-marine ecosystems include **periglacial and terrestrial habitats** (rock, permafrost, soil), **glacial habitats** (snow, glacial ice, cryoconites, subglacial ice and sediments, subglacial lakes) and **inland waters** (lakes, streams, rivers) (Thomas *et al*., 2008). These habitats are dominated by and contain diverse microorganisms, including photosynthetic microalgae (Charvet *et al*., 2012a, b; Leya 2004; Lutz *et al*., 2016, 2018; Rippin *et al*., 2018; **papers V–VIII**).

Due to geographic isolation and harsh environmental selection, Arctic provides an excellent model system to study microbial diversity, ecology and biogeography.

Algae in extreme environments

Microalgae are a remarkably diverse group of organisms that have colonized and occupied a wide variety of niches, including extreme environments. Species of all major taxonomic groups have adapted to survive or even thrive in harsh environmental conditions. For example, cyanobacteria are well known for living in hot springs or hypersaline and alkaline lakes (Dadheech *et al*., 2013; Oren, 2015). Red algae (Rhodophyta) from the class Cyanidiophyceae are famous for thriving in acidic and hot environments (Miyagishima *et al*., 2017; Reeb & Bhattacharya, 2010; **paper III**). Green algae from the divisions Chlorophyta and Streptophyta include snow and ice specialists, respectively (Cvetkovska *et al*., 2017; Lutz *et al*., 2018). The green algal order Volvocales (Chlorophyceae) encompasses some of the best studied extremophiles falling within the *Moewusinia* (e.g., acidophilic *Chlamydomonas acidophila*, psychrophilic *Chlamydomonas* sp. UWO 241), *Dunaliellinia* (e.g., halophilic

Dunalielia salina and acidophilic *Dunaliella acidophila*) and *Chloromonadinia* (e.g., psychrophilic *Chloromonas nivalis*) phylogroups (Nakada *et al*., 2008). On the other hand, organisms adapted to the same extreme conditions can be found across different evolutionary groups (Fig. 3). For example, the aforementioned green alga *Chlamydomonas acidophila* (Archaeplastida) is often found together with the euglenophyte *Euglena mutabilis* (Excavata) is extremely acidic and metal-rich freshwaters (Hargreaves & Whitton, 1976; **paper I**).

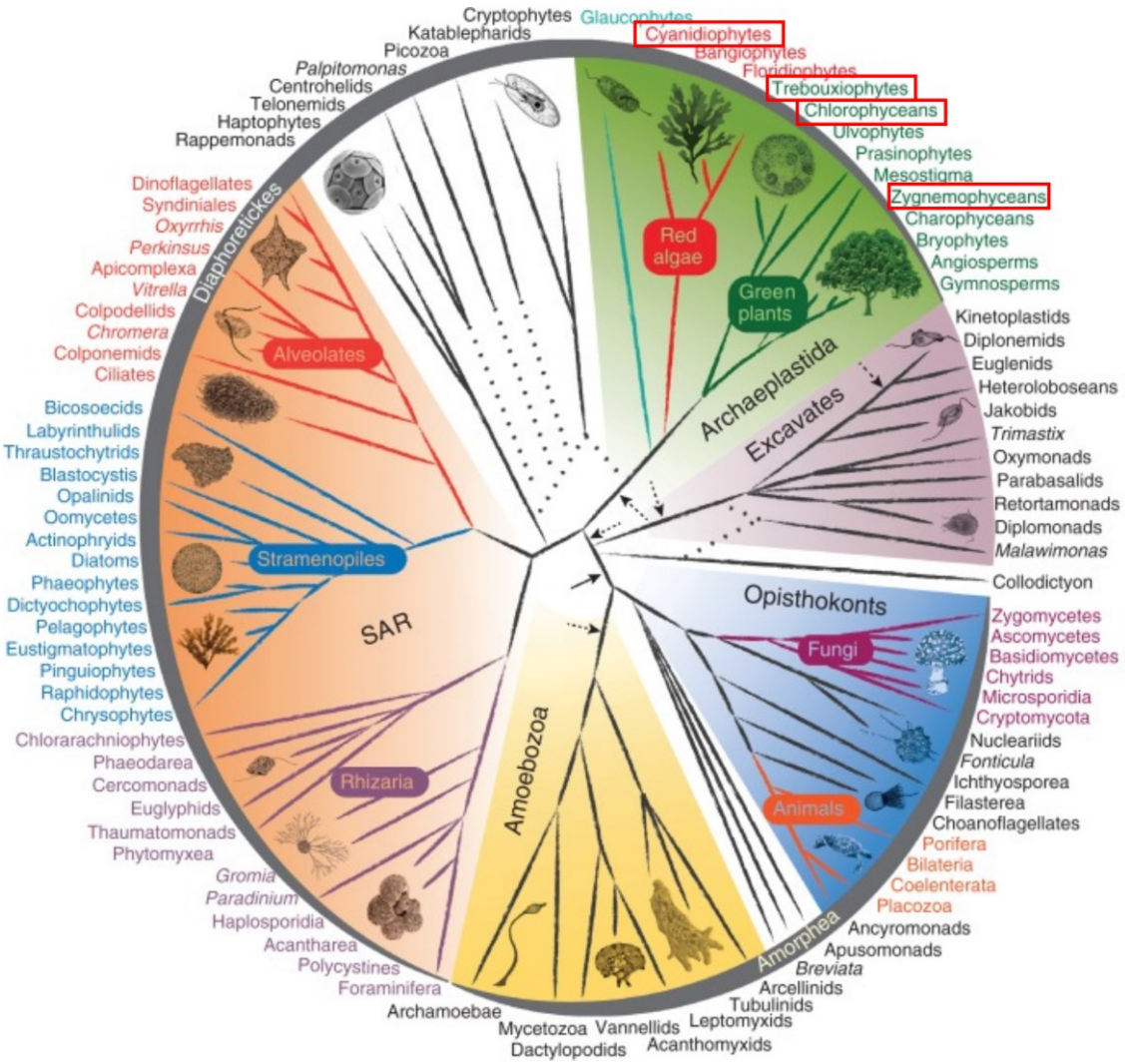

Figure 3. The eukaryotic tree of life. Studied taxonomic groups are marked with red rectangles. Taken from Burki (2014).

With a few notable exceptions, the studies of extremophilic eukaryotic microorganisms are highly neglected in comparison to their prokaryotic counterparts. For example, the biology of extremophilic algae has been reviewed and summarized just in several chapters and books (Elster, 1999; Elster *et al*., 2001; Kuroiwa *et al*., 2017; Reeb & Bhattacharya, 2010; Seckbach, 2007) or review papers (Cvetkovska *et al*., 2017; Pushkareva *et al*., 2016).

Nevertheless, algae living in various extreme environments are being increasingly studied using molecular tools. For example, the current biodiversity studies employing molecular data have focused on highly acidic lakes, rivers and soils (Amaral-Zettler *et al*., 2002; **papers I–IV**), dry deserts (Cardon *et al*., 2008; Fučíková *et al*., 2014), Antarctic lakes (Nedbalová *et al*., 2017), Arctic and Antarctic soil crusts (Rippin *et al*., 2018; **paper VIII**), snowfields and glaciers (Lutz *et al*., 2016, 2018; **papers V, VIII**), or cold wet hummock meadows (**paper VI**) fed by meltwater (**paper VII**). However, due to limited sampling efforts, diversity of microalgae in extreme environments is far from being fully explored and understood.

This doctoral thesis focuses on green algae from the classes Trebouxiophyceae and Chlorophyceae (Chlorophyta), Zygnematophyceae (Streptophyta), and red algae (Rhodophyta) from the class Cyanidiophyceae (Fig. 3).

Algae in acidic (and hot) habitats

Trebouxiophyceae

The green algal class Trebouxiophyceae encompasses unicellular (e.g., *Chlorella*, *Stichococcus*), filamentous (e.g., *Geminella*) and multicellular (*Prasiola*) organisms. Though majority of them comprise morphologically simple coccoid forms. Trebouxiophytes constitute free-living and symbiotic algae, occuring in a wide range of environments: from (aero)-terrestrial to aquatic (Leliaert *et al*., 2012). Despite their simple and uniform morphology, the 'little green balls' are genetically highly diverse (Malavasi *et al*., 2016; **papers I, II)**, including little know phylogenetic lineages (e.g., *Lunachloris*, **paper IX**).

Trebouxiophytes have been reported multiple times from extremely acidic environments. For example, *Viridiella fridericiana* was isolated and described from the soil surface (pH < 1.5) near sulfurous exhalations at Mefite d'Ansanto in Italy (Albertano *et al*., 1991). *Pumiliosphaera acidophila* (formerly *Auxenochlorella protothecoides* var. *acidophila*) was found and described from an acidic volcano stream and soil nearby (pH ~ 2.0) at Pozzuoli Solfatara, Italy (Darienko & Pröschold, 2015). Unidentified but phylogenetically closely related alga was reported from Tinto River (pH ~ 2.5) in Spain (Amaral-Zettler *et al*., 2002) and Nymph Creek (pH ~ 2.7, temperature ≤ 39 °C) in Yellowstone National Park, USA (Ferris *et al*., 2005). Other trebouxiophytes described from acidic habitats include new species of the genus *Coccomyxa*, namely, *C. onubensis* from the aforementioned Tinto River (Fuentes *et al*., 2016) and *C. silvae-gabretae* from the glacial atmospherically acidified Plešné Lake (pH ~ 5.0) in Czechia (**paper I**). A new genus and species *Autumnella lusatica* was proposed form acidic lignite pit lakes (pH 3.3–4.5) in Germany (Ulrich & Röske, 2018). In addition, *Watanabea* sp. was reported from the acidic volcanic Caviahue Lake (pH < 3.0) in Argentina

(Beamud *et al*., 2010; Perdozo *et al*., 2001). The closely related alga was later rediscovered in pit Lake Hromnice (pH ~ 2.6) in Czechia and classified as a new species *W. acidotolerans* (**paper II**).

Trebouxiophytes found in extremely acidic habitats but formerly described from pH neutral environments include *Parachlorella kessleri* isolated from a mesothermal acidic pond (pH 2.5–2.8, temperature 30–35 °C) in Argentina (Juárez *et al*., 2011) and *Coccomyxa elongata* from Hromnice Lake (**paper I**). Of note, numerous *Coccomyxa* strains have been isolated from pH low soils (pH 2.0–2.8) in Italy (Albertano *et al*., 1990). Moreover, a trebouxiophyte strain designated as *Pseudochlorella* sp. was isolated from the AMD (pH 2.13, temperature 14.5 °C) in Nagano Prefecture, Japan (Hirooka *et al*., 2014). The authors demonstrated that the alga had optimal pH 3.0–5.0 and temperature 20–25 °C but was capable of growing at pH 2.0 and at 32 °C.

To sum up, different taxa of the class Trebouxiophyceae are found and can be successful in extremely acidic habitats and their diversity is pending further samplings and investigations.

Cyanidiophyceae

Cyanidiophyceae (Cyanidiophytina, Rhodophyta) represents an ancient group of asexual unicellular polyextremophiles, found in acidic (pH 0.5–4.0) and high temperature (up to 56 °C) environments. The class accommodates the single order Cyanidiales, two families Cyanidiaceae and Galdieriaceae, and eight species belonging to the genera *Cyanidioschyzon*, *Cyanidium* and *Galdieria* (Melora *et al*., 1981; Yoon *et al*., 2016).

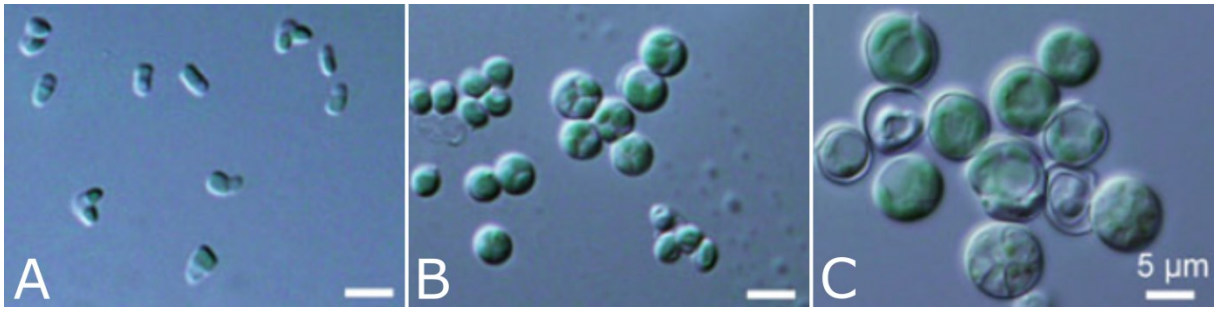

Figure 4. A – *Cyanidioschyzon*, B – *Cyanidium*, C – *Galdieria*. Taken and modified from Miyagishima *et al*. (2017).

The genus *Cyanidioschyzon* is represented by the single species, *Cs. merolae*, characterized by oval to club-like shape, lack of a cell wall, the Golgi apparatus and vacuoles. The alga reproduces by binary fission and is extremely small (Fig. 4A). In contrast, *Cyanidium* and *Galdieria* are spherical, contain cell walls, dictyosomes and vacuoles, and

reproduce by autospores (Fig. 4B, C). They differ from each other in cell size and number of autospores produced (Melora *et al*., 1981; Albertano *et al*., 2000).

Based on ecology, two species of *Cyanidium* are recognized. *Cyanidium caldarium* occurs in acidic and high temperature conditions, while all neutrophilic and mesophilic *Cyanidium* strains were assigned to the species *C. chilense* (Ciniglia *et al*., 2017). The genus *Galdieria* consists of five species. The most widespread and commonly reported one, *G. sulphuraria*, exhibits a clear biogeographical diversification (Ciniglia *et al*., 2014; **paper III**). The two Russian morphospecies, *G. partita* and *G. daedala* (Sentsova, 1991), fall within the clade of *G. sulphuraria* (Pinto *et al*., 2003; Ciniglia *et al*., 2004). *Galdieria phlegrea* was recognized on the basis of ecology and molecular phylogenetics (Pinto *et al*., 2007). It was known exclusively from Italy, until the recent discoveries in Czechia (**paper III**), Spain (**paper IV**) and Turkey (Iovinella *et al*., 2018). Finally, the cosmopolitan *G. maxima* is awaiting a taxonomic reclassification since this species is not a part of the *Galdieria* clade and is more related to *Cyanidioschyzon* (Ciniglia *et al*., 2004; 2014).

The knowledge of Cyanidiales distribution include records from Spain (Moreira *et al*., 1994), Azores (Gross & Gross, 2002), Italy (Ciniglia *et al*., 2004, Yoon *et al*., 2006), Japan and New Zealand (Toplin *et al*., 2008), Chile (Azua-Bustos *et al*., 2009), USA (Skorupa *et al*., 2013), Iceland (Ciniglia *et al*., 2014) and Taiwan (Hsieh *et al*., 2015). This worldwide but discontinous distribution could be determined not only by the lack of suitable habitas supporting their lifestyle but also by limited sampling efforts. Therefore, it is reasonable to assume that cyanidiophycean algae may be present in other parts of the world, especially, in remote volcanic oceanic islands or high background radiation areas (**Barcytė, unpublished data**).

Discoveries of cyanidiophycean microalgae in acidic but non-thermal (Gross *et al*., 2002; Hsieh *et al*., 2015; **paper IV**) and thermal but non-acidic (Iovinella *et al*., 2018) environments pointed to broader ecological boundaries of the class Cyanidiophyceae and indicated that one extreme could be enough to maintain the life of these enigmatic microalgae previously considered as thermoacidophilic.

Same as the distribution and ecology, the biodiversity of Cyanidiales is also very little undersood. Simple and uniform morphology of cyanidiophycean algae may veil cryptic species (**paper IV**).

Other taxonomic groups

The green algae from the class Chlorophyceae are also successful in extremely acidic environments. For example, *Chlamydomonas* (*Cd*.) *acidophila* has been reported from acidic

freshwaters around the world (Gerloff-Elias *et al*., 2005; Hargreaves & Whitton, 1976; **paper I**). Originally, the species has been described from the volcanic Katanuma Lake (pH ~ 1.5) in Japan (Negoro 1944). A volvocalean flagellate strain, identified as *Cd. eustigma*, was isolated from mosses growing in seeps of the AMD in an abandoned sulfur mine in Japan. The genome of this organism revealed molecular bases of adaptations for life in extremely acidic conditions (Hirooka *et al*., 2017). However, *Cd. eustigma* and *Cd. acidophila* are conspecific and both now await a taxonomic reclassification (**Barcytė, unpublished data**). One more flagellate encountered in the extremely acidic habitats includes *Dunaliella* (*D*.) *acidophila*. The alga was first isolated from acid waters (pH 1.0) in Soos Nature Reserve, Czechia and was formerly classified as *Spermatozopsis acidophila* (Kalina, 1965). Later, *D. acidophila* was rediscoverd in acid soils (pH 0.6–1.5) and sulfur springs (pH 0.8–1.5) in Italy (Albertano *et al*., 1981). However, in comparison to *Cd. acidophila*, *D. acidophila* is less frequently reported species and little is known about its genetic diversity (Assunção *et al*., 2012).

Streptophytic green algae, e.g., *Klebsormidium* (Klebsormidiophyceae) are also commonly encountered in low-pH freshwater and terrestrial ecosystems (Škaloud *et al*., 2014). Other genera such as *Zygogonium* and *Zygnemopsis* (Zygnematophyceae) have been reported from limnoterrestrial habitats acidified either by the AMD or microbial activity (Amaral-Zettler *et al*., 2002; Kleeberg *et al*., 2006; Whitton & Diaz, 1981). Desmids (Mesotaeniaceae and Desmidiaceae) are usually found in naturally acidic freshwaters, such as peat bogs (Coesel & Meesters, 2007; Štěpánková *et al*., 2012).

Apart from the green algae, the planktic algal flora of acidic lakes is also dominated by chrysophytes (e.g., *Ochromonas*, *Chromulina*), cryptophytes (e.g., *Cyathomonas*, *Cryptomonas*), dinophytes (*Gymnodinium*, *Katodinium*) and euglenophytes (e.g., *Euglena mutabilis*, *Lepocinclis* sp.) (Beulker *et al*., 2003; Lessmann *et al*., 2000; Nedbalová *et al*., 2006). DeNicola (2000) reviewed diatoms found in acidic environments and suggested a threshold between pH 4.5 and 3.5 below which many species of diatoms are eliminated. The true inhabitats of highly acidic waters include members of the genera *Achnanthes*, *Eutonia*, *Frustulia*, *Nitzschia* and *Pinnularia* (DeNicola, 2000).

Algae in Arctic habitats

Six main ecological types of microalgae can be distinguished in the Arctic habitats: snow, glacier, freshwater, soil, limnoterrestrial and aerophytic algae (excluding marine algae). However, there are no clear boundaries between the main groups and same or similar taxa can be found in a variety of Arctic ecosystems. For example, common Arctic soil taxa of the class Trebouxiophyceae, e.g., *Koliella*, *Raphidonema*, *Stichococcus* and *Coccomyxa*, or yellow-

green algae (Xanthophyceae) of the genera *Heterococcus* or *Xanthonema*, are often encountered in snowpacks or on glaciers (Stibal & Elster, 2005). Or opposite, snow and glacier algae from the genera *Chloromonas* and *Cylindrocystis*, respectively, can be found in the upper soil layer (**Barcytė, personal observations**).

The true snow algae live actively at temperatures close to 0 °C and are easily recognized by staining snow in green, red, orange or yellow colour. They are psychrophiles with a number of short-term and long-term adaptations (Cvetkovska *et al*., 2017). The green algae *Chlamydomonas nivalis* and *Chloromonas nivalis* (Chlorophyceae) are the main constituents of the cryoflora worldwide. They are recognized by red spherical cells or ribbed elongated cells, respectively (Remias *et al*., 2010). Snow cryoflora of the High Arctic was studied by Leya (2004). The author used culture-dependent approach, enabling morphological, physiological and molecular characterization of the collected microalgae. Lutz *et al*. (2016) studied biogeography of the Arctic red snow algae using culture-independent high-throughput sequencing based on a short fragment of the 18S rRNA gene. The obtained data contradicted the previous knowledge (Leya, 2004) by showing that snow algae are cosmopolitan and of low diversity. Meanwhile, Segawa *et al*. (2018) employing 18S-ITS2 rDNA long-read strategy by Sanger sequecing revealed that snow algae comprise both cosmopolitan and endemic taxa.

Apart from psychrophilic species, snow can also be inhabited by psychrotolerant microorganisms (Leya, 2004). In particular, the genus *Chloromonas* encompasses both cold-adapted and cold-tolerant species (Hoham *et al*., 2002). Many studies employing molecular phylogenetic methods have been done on this particular genus (Buchheim *et at*., 1997; Pröschold *et al*., 2001; Hoham *et al*., 2002; Matsuzaki *et al*., 2012, 2014, 2018, 2019). However, *Chloromonas* stills stays little understood in terms of diversity and ecological speciation. The most recent data have shown the diversification of the Arctic psychrotolerant *Chloromonas* (**papers V, VI**) and extended sampling efforts could uncover additional new *Chloromonas* lineages unique to the High Arctic (**Barcytė, unpublished data**).

The glacier algae found on melting glaciers and ice sheet surfaces encompass *Cylindrocystis brebissonii* var. *cryophila*, *Mesotaenium berggrenii* and *Ancylonema nordenskioeldii* (Remias *et al*., 2011; Lutz *et al*., 2018). Systematically, they all belong to the traditional family Mesotaeniaceae of the green algal order Zygnematales within the class Zygnematophyceae (Streptophyta). However, due to difficulties in keeping these algae in cultures, there is no data regarding their morphological and molecular diversity.

Arctic biological soil crusts are mostly dominated by filamentous cyanobacteria (Pushkareva *et al*., 2015), while limnoterrestrial habitats, such as, seepages, streams or littoral

zones, accomodate an enormous diversity of diatoms (Bacillariophyceae) (Pinseel *et al*., 2017) or zygnematophytes (Pichrtová *et al*., 2018). However, they also contain chlorophytes due to their widespread nature and input by snowmelt (e.g., *Chloromonas reticulata*; **paper VII**). Plankton of Arctic lakes are mostly dominated by chrysophytes, dinoflagellates and cryptophytes (Charvet *et al*., 2012a, b). Arctic aerophytic algae thrive on or in the exposed rock surfaces, bone remnants, driftwood or man-made objects. The most common representatives constitute trebouxiophycean algae, such as, *Stichococcus* or *Chlorella*-like species, and cyanobacteria, e.g., *Phormidium* (Raabová *et al*., 2016; **Barcytė, personal observations**).

Aims of the thesis

This thesis aimed to gain deeper insights into diversity of microalgae in selected extreme environments using a polyphasic approach (light and transmission electron microscopy combined with molecular phylogenetics) by:

1. finding out whether algal strains newly isolated from extreme habitats are phylogenetically distinct from their 'normal' counterparts (**papers I, II**)
2. testing if newly isolated strains support the previously uncovered biogeographical and ecological patterns of *Galdieria* microalgae (**papers III, IV**)
3. evaluating taxonomy and unravelling the phylogenetic structure of the widespread genus *Chloromonas*, and getting to know if that structure is reflected in the ecology of its members (**papers V–VII**)
4. providing the first culture-based molecular and morphological data of the Arctic *Cylindrocystis* (**paper VIII**)

Outline of the papers

This thesis consists of eight main papers (**I–VIII**) and two appendices (**papers IX–X**). The first four papers (**I–IV**) focus on microalgae isolated from acidic or acidic and hot habitats, whereas papers **V** and **VI** relate to microalgae found in cold environments. Paper **VII** combines diversity of microalgae isolated from 'extreme' (acidic and cold) and 'normal' terrestrial and freshwater habitats. The last main paper (**VIII**) turns back to the little explored Arctic habitats. The first appendix (**paper IX**) deals with algal divesity as well, though not in the extreme environment. Meanwhile, the second appendix (**paper X**) focuses on the molecular identification of moss protonemata occuring in extremely acidic lake.

Paper I investigates the taxonomic position and phylogenetic relationships of the dominant planktic algae in two acidic and metal-rich lakes of different origin in Czechia. Hromnice Lake (pH < 3.0) was formed as a consequence of the mining of pyritic shales (Hrdinka *et al*., 2013), while Plešné Lake (pH ~ 5.0) was acidified by air pollution (Nedbalová *et al*., 2006). Both lakes are relatively rich in phytoplankton biomass due to an increased input of phosphorus and low grazing pressure but poor in species. The dominant phytoplankton microalgae in Hromnice Lake and Plešné Lake were previously referred to as *Coccomyxa* sp. and *Monoraphidium dybowskii*, respectively (Hrdinka *et al*., 2013; Nedbalová *et al*., 2006). However, phylogenetic analyses (18S and ITS2 rDNA) computed in **paper I** confirmed that both algae were a part of the genus *Coccomyxa* (Trebouxiophyceae, Chlorophyta). *Coccomyxa* from Hromnice Lake was identified as *C. elongata*, which is generally known as a freshwater species (Malavasi *et al*., 2016), though is not commonly reported one. Meanwhile, *Coccomyxa* from Plešné Lake was described as a new species *C. silvae-gabretae*. This study served as the first evidence that representatives of the genus *Coccomyxa* are capable of becoming dominant primary producers in the extreme environment of acid lakes with the increased supply of phosphorus. In addition, both species demonstrated morphological differences (cell shape or cell wall thickness) under natural and laboratory conditions, confirming the phenotypic plasticity of the genus *Coccomyxa* (Darienko *et al*., 2015). The ability to change the morphology may help the cells of *Coccomyxa* to survive harsh conditions in the aforementioned acidic lakes.

Paper II continues the diversity studies in the Lake Hromnice with a description of *Watanabea acidotolerans* sp. nov. (Trebouxiophyceae, Chlorophyta). The alga was isolated from the surface of a stone found at the bottom near the shore of the lake. The most characteristic feature of the genus *Watanabea* is the production of two types – spherical and ellipsoidal – cells, which morphologically resemble other trebouxiophycean taxa. Such algal

'mimicry' may thus result in a low number of reports of the *Watanabea* microalgae. *Watanabea acidotolerans* morphologically and ultrastructurally resembled the *Watanabea* type species, *W. reniformis* (Hanagata *et al*., 1998). However, it differed from another *Watanabea* species, *W. borysthenica* (Darienko & Pröschold, 2018), by having a pyrenoid not surrounded by starch grains. The same naked pyrenoid was documented in the authetic strain of the *Watanabea* type species, though it was regarded as the pyrenoid-less (Hanagata *et al*., 1998). Consequently, this study changed the morphological perception of the genus *Watanabea*. Furthermore, *W. acidotolerans* from Hromnice Lake and another *Watanabea* isolate from a naturally acidic Caviahue Lake in Argentina were identical in their plastid *rbc*L sequences, implying a possibly worldwide distribution of the newly designated *W. acidotolerans* lineage. Both lakes have similar water chemistry in terms of heavy metals and increased concentration of phosphorus (Beamud *et al*., 2010; Pedrozo *et al*., 2001). The increased availability of phosphorus probably overcompensates the unfavourable acidic conditions for the acidotolerant coccoid green microalgae (e.g., **paper I**). In Caviahue Lake, *Watanabea* was reported as a planktic alga (Beamud *et al*., 2010), while in Hromnice Lake *Watanabea* was found as an epilithic species and has never been reported in the phytoplankton (Hrdinka *et al*., 2013). This suggests that *W. acidotolerans* is capable of adapting its life startegy to better fit in the extreme environment of acidic lakes. It is likely that *W. acidotolerans* might be rediscovered in other phosphorus-rich acidic freshwaters.

Paper III investigates a unique acidic and hot habitat – a burning coal spoil heap – for the possible traces of extremophile colonization. Such peculiar post-mining sites can accommodate diverse microorganisms (Kirby *et al*., 2010), including thermoacidophilic microalgae. Heřmanice dump situated near the city of Ostrava, Czechia is one of the last remaining buring coal spoil heaps in Europe. Residuals of trees, i.e. stumps, bark or branches (pH 2.0–3.0), located next to fuming vents (surface temperature 50–55 °C), were found to be covered by blue-green mats of the red alga *Galdieria*. Phylogenetic analyses (*rbc*L and calmodulin) of the six isolates revealed that the population of *Galdieria* from Ostrava belonged to the continental European lineage of the cosmopolitan species *Galdieria sulphuraria*. Accordingly, Italian populations of *Galdieria* were considered as the potential source of the Czech *G. sulphuraria*. This is the first record of *G. sulphuraria* in central Europe and in such type of anthropogenic habitat. In addition, **paper III** suggested that colonization of new habitats by cyanidiophycean algae can be rather quick because environmental conditions of Heřmanice spoil heap that could accommodate thermoacidophilic *Galdieria* have been formed relatively recently. For comparison, *Galdieria* strain CCALA 965 (Culture Collection of Autotrophic Organisms), isolated from the acidic Soos Nature

Reserve in Czechia (Gross *et al*., 2002), was also investigated and confirmed to belong to the species *G. phlegrea*, until **paper III** known just from Italy.

Paper IV continues the study of *G. phlegrea* distribution by investigating a new *Galdieria* isolate from the extremely acidic (pH ~ 2.5) Tinto River in Spain and relating this discovery to the previous knowledge about the distribution and ecology of this enigmatic microalga. The Tinto River is located in the Iberian Pyrite Belt and its acidity is generated by both: the AMD, and chemolithoauthotrophic microorganisms accelerating the process of oxidation of the sulfidic minerals. The river is an exceptional acidic environment because its diversity is dominated by eukaryotic microorganisms (Aguilera, 2013; Amaral-Zettler *et al*., 2002). The occurrence of cyanidiophycean algae in Tinto River has been reported previously (Gross & Gross, 2001; Gómez *et al*., 2011; Moreira *et al*., 1994). However, no molecular data for clear taxonomic conclusions or biodiversity assessment were accessible. The *rbc*L-based phylogeny showed that the newly studied Tinto River alga was closely related to *Galdieria phlegrea* strains originating from various extreme habitats in Czechia, Italy and Turkey, suggesting its wider distribution and higher ecological versatility than previously thought. The results have also indicated that *G. phlegrea*, and then possibly also other cyanidiophycean algae, are not as restricted to strongly acidic and hot microhabitats as previously believed. These speculations were confirmed by Iovinella *et al*. (2018). In addition, **Paper IV** suggested that *G. phlegrea* may harbour hidden biodiversity and that extended sampling could uncover cryptic species.

As much as acidic, extremely cold habitats are also dominated by microorganisms. Since two-thirds of our planet is cold, the diversity of psychrophiles and psychrotolerant microorganisms is an important research direction. Although green microalgae are frequently reported from the polar regions, their diversity still stays poorly investigated and rarely discussed.

The phylogroup *Chloromonadinia* is a part of the Volvocales order within the green algal class Chlorophyceae (Chlorophyta). It was established based on its largest and representative genus *Chloromonas* (Nakada *et al*., 2008). However, diversity of *Chloromonadinia* microalgae is still highly underestimated owing to limited number of strains available and complex taxonomic history of the genus *Chloromonas*. Furthermore, the phylogenetic structure of the *Chloromonadinia* is also neither clear, nor robust. Thus, additional new isolates of *Chloromonas*-like flagellates are intriguing and valuable for various phycological studies, including algal evolution, ecology, physiology and biogeography.

Chloromonas is a common inhabitat of various Arctic ecosystems, detected by multiple methods, including culture-based (Borchhardt *et al*., 2017; Leya, 2004) and culture

independent techniques (Lutz *et al*., 2016; Rippin *et al*., 2018). The following two papers (**V, VI**) deal with the new green photosynthetic microflagellates isolated from cold habitats in the High Arctic. Subsequently, **paper VII** extends the morphological, phylogenetic and ecological knowledge of the *Chloromonadinia* microalgae by investigating two new *Chloromonas* strains of temperate and polar origin and re-investigating three authentic '*Chlamydomonas*' strains described from Europe and New Zealand.

Paper V studies a new cold-tolerant *Chloromonas* strain isolated from a snow sample taken on a glacier in Svalbard. Phylogenetic analyses based on the nuclear 18S rRNA and plastid *rbc*L genes revealed that the new strain was nested within the psychrotolerant *Chloromonas* clade where it formed an independent lineage. Comparisons of secondary structure models of the highly variable ITS2 rDNA marker and a set of unique morphological traits supported the recognition of a distinct species identity of the studied microalga. Consequently, the investigated strain was described as a new species *Chloromonas arctica*. After the pioneering work of Leya (2004), this study represents the first attempt to look more deeply into the diversity of the Arctic volvocalean flagellates using a combination of both culture-based observations and molecular phylogenetics. In addition, **paper V** suggested that diversity of volvocalean microalgae from cold environments is not yet thoroughly explored and that Arctic *Chloromonas* may veil a number of novel, possibly endemic, species yet to be discovered and described.

Paper VI characterizes an additional new species, *Chloromonas svalbardensis*, isolated from a wet hummock meadow in Svalbard. The same polyphasic approach was employed, distinguishing the newly proposed taxon among the other previously described *Chloromonas* species. In addition, **paper VI** re-evaluates the genus *Chloromonas* by highlighting problems in classifying cold-adapted and cold-tolerant species within the framework of the *Chloromonadinia*. Analyses of the 18S rRNA and *rbc*L genes of the phylogroup demonstrated the paraphyletic origin of the genus *Chloromonas* with three main genetically, morphologically and ecologically well-defined clades. Phylogeny was revealed to be consistent with ecology, especially in terms of temperature. For example, *Chloromonas* clade 1 encompassed mesophilic and psychrotolerant organisms that likely do not form snow blooms though are commonly found in snow (e.g., *Chloromonas arctica*, **paper V**). Clade 2 encompassed psychrophilic organisms forming snow blooms, whereas *Chloromonas* clade 3 included mesophilic algae only. Such ecological circumscription of the main clades explained better the previous data showing that *Chloromonas*-like algae isolated from snow are spread within two clades (Hoham *et al*., 2002). Consequently, it was also suggested that the genus

Chloromonas would be understood just as a part of the psychrotolerant clade 1, a subclade containing the *Chloromonas* type species, *Cr. reticulata*.

Paper VII (unpublished manuscript) focuses on the terrestrial members of the *Chloromonadinia*. The study originated by the isolation of a new volvocalean flagellate strain from tree bark on the aforementioned Heřmanice coal spoil heap (**paper III**). The new isolate matched well the morphological description of the species *Chlamydomonas chlorostellata* described form the acidic Tekoa soil in New Zealand (Flint & Ettl, 1966). Their close relationship was also confirmed by a similar cell ultrastructure and 18S rDNA and *rbc*L phylogenies. However, the comparison of ITS2 rDNA secondary structures of both strains yielded three compensatory base changes (CBCs), suggesting that the two isolates should be assigned to separate species. Nevertheless, **paper VII** argues that the popular ITS2/CBC species concept (Coleman 2000, 2009) alone is not enough to delineate species without additional supporting data, and the two strains were considered as the members of the single species. Futhermore, **paper VII** re-examined the authenic strains of the terrestrial species *Chlamydomonas chlorostellata* var. *gracillima* and the freshwater species *Chlamydomonas meslinii*, which were confirmed to be a part of the *Chloromonadinia* phylogroup. Based on the results, species relationships and circumscriptions within the phylogroup were discussed with the proposal of a new genus *Ostravamonas* (including two new species combinations *O. chlorostellata* and *O. meslinii*) and *Chloromonas gracillima* comb. nov. *Ostravamonas chlorostellata* represents the first known members within the *Chloromonadinia* isolated from acidic terrestrial habitats. For comparison and taxonomic implications, a new strain of *Cr. reticulata* isolated from a meltwater stream in the High Arctic was also studied, confirming the psychrotolerant nature and worldwide distribution of the species. This study provided additional evidence that the genus *Chloromonas* accommodates both terrestrial and freshwater species from temperate and polar regions. Finally, **paper VII** suggested that besides temperature, terrestrial vs aquatic lifesyle could have significantly influenced the diversification of the *Chloromonadinia*, and that little explored terrestrial habitats could veil numerous undescribed *Chloromonas*-related microalgae.

Paper VIII (unpublished manuscript) provides the first culture-based observations of the genus *Cylindrocystis* from the High Arctic (Svalbard). Seven new strains with *Cylindrocystis*-like morphology were newly isolated and compared using molecular (*rbc*L and 18S rDNA), morphological (light, confocal laser scanning microscopy) and cytological (Raman microscopy) data. The results have shown that the Arctic *Cylindrocystis* accommodates genetically and morphologically highly diverse microorganisms with terrestrial and freshwater lifestyle. The origin of the new isolates was not monophyletic, confirming the

previously uncovered complex phylogenetic structure of the genus *Cylindrocystis* (Gontcharov & Melkonian, 2010; Hall *et al*., 2008). The new isolates clustered within two statistically well-supported phylogenetic clades and four distinct lineages. The uncovered genotypes (e.g., five different genotypes on the 18S rDNA level) were also different in their morphology. Especially, size and shape of the cells along with chloroplast morphology seemed to corroborate the molecular variability. It was shown that the diversity of the Arctic *Cylindrocystis* is not restricted just to the single species of *C. brebissonii*, but contains a larger number of unrecognized or yet undescribed species. Similar genotypes (especially freshwater) were found to be widely distributed, showing a polar-temperate biogeography. The new polar strains isolated from terrestrial habitats clustered together with hot-desert strains, showing a high ecological versatility of *Cylindrocystis*-like microalgae. Furthermore, the increased accumulation of polyphosphate under laboratory conditions was detected within the chloroplasts of the Arctic *Cylindrocystis* strains, suggesting an effective luxury uptake of phosphorus. The polyP grains occupying most of the plastic volume could help the cells of *Cylindrocystis* to survive and even thrive in the harsh polar regions.

Paper IX describes a new genus and species *Lunachloris lukesovae*. The alga was isolated in 1987 from a wet meadow in South Bohemia, Czechia and preserved in CCALA under strain number 307. Formerly, the isolate was referred to as *Coccomyxa* cf. *gloeobotrydiformis* and was thus studied with the *Coccomyxa* strains from the acidic lakes (**paper I**). However, molecular phylogenetic analyses based on the 18S rDNA and *rbc*L revealed that the strain CCALA 307 formed a distinct sister lineage to *Neocystis* and *Prasiola* clades within the Trebouxiophyceae. Generally, minute soil algae tend to disperse over long distances and are expected to be common (Hodač *et al*., 2016). However, *Lunachloris* has not been detected before, even by culture-independent environmental sequencing. Thus, *Lunachloris lukesovae* might represent an example of a rare or rarely accessible species. **Paper IX** showed that the biodiversity of terrestrial microalgae in temperate regions is also notably unexplored and even 'common' habitats can contain new, as yet unkown species. In addition, this study emphasized the importance of culture collections of microorganisms even in the era of culture-independent biodiversity research, because they may harbour novel and undescribed organisms as well as preserving strains for future studies.

Paper X contributes to biodiversity research in the aforementioned Hromnice Lake (**papers I, II**) by identifying moss protonemata (filametous undifferentiated multicellular structures of bryophytes) and explaining their nature of occurrence. A molecular approach for identification of protonemata was chosen along with sampling of the adult mosses growing on the wet substrate soaked with lakewater. The sequences (chloroplast *rbc*L and mitochondrial

*nad*5) of protonemata matched those of the adults, morphologically identified as *Dicranella* sp. Phylogenetic analysis of the *rbc*L gene showed a sister relationship to *D. heteromalla*, generally known for growing in acidic habitats, and other protonemata occurring in acidic rivers in Japan. The *nad*5-based phylogeny revealed that the studied protonemata belonged to the species *D. cerviculata* and the same taxonomic affiliation was confirmed by the nuclear ITS2 rDNA sequence and its secondary structure. Even though, it is well known that bryophytes invade extremely acidic habitats often just as protonemata, this phenomenon is highly underestimated and rarelly discussed. For example, this is the second study which used molecular tools to identify protonemata in the extremely acidic waters (Higuchi *et al*., 2003). The extreme environment of Hromnice Lake prevents the further development of protonemata which, in turn, are capable of surviving acidic conditions and increased heavy metal concentrations in the prolonged protonemal stage. In addition, **paper X** speculates that protonemata of only acidotolerant moss species, such as *D. cerviculata* or its relatives, can invade extremely acidic freshwaters. Moreover, the sizeable development of protonemata in the lake water may help to colonize the Hromnice Lake shoreline.

Conclusions

This doctoral thesis provided further evidence that extreme environmental conditions (low pH and temperature) drive diversification and speciation of microorganisms. New discoveries of little know microalgae and/or taxonomic descriptions of novel or previously undescribed species in the acidic and cold environments are presented in the **papers I–VIII**. Findings of uncovered eukaryotic diversity contributed new insights into algal ecology and biogeography and showed that closely related strains or species colonize similar extreme habitats irrespective of their geographic distance (**papers II–IV, VII, VIII**).

Microalgae thrive in extreme environments by either being true extremophiles adapted to abiotic stresses (e.g., *Galdieria sulphuraria*; **paper III**) or by being ecologically versatile and tolerant against extremes (e.g., *Coccomyxa*, *Chloromonas*, *Cylindrocystis*; **papers I, V–VIII**). The tolerance to extremes could be explained by the presence of abiotic or biotic benefits (e.g., increased concentrations of nutrients, low competition or predation). Another explanation might be a high spatial heterogeneity of habitats with strong environmental gradients. Such heterogeneity could offer protection against the environmental harshness for extremotolerant microalgae. Phenotypic plasticity (e.g., cell wall thickness; **paper I**), type of reproduction (e.g., asexual reproduction of two types of autospores; **paper II**), or efficient luxury uptake of nutrients (**paper VIII**) likely play significant roles in survival of extremotolerant microorganisms. Survival in hostile habitats such as snow or snowmelt water bodies is further accomplished via active movement towards 'oases' with more favourable conditions (e.g., *Chloromonas*; **papers V–VII**).

Adaptation to low pH environments has evolved multiple times independently within the coccoid microalgae of the green algal class Trebouxiophyceae (Chlorophyta). In this taxonomic group, acidotolerant species represent both species rich (e.g., *Coccomyxa*; **paper I**) and species-poor (e.g., *Watanabea*; **paper II**) monophyletic lineages intermixed within non-extremophilic clades.

The adaptation to acidic and hot habitats is widespread in the red algal class Cyanidiophyceae (**paper III**). However, recent investigations have shown that cyanidiophycean microalgae are ecologically versatile and nonrestricted to polyextremophilic conditions (**paper IV**). They are also capable of long-distance dispersal and rapid colonization of new habitats (**paper III**). New records emphasizing cyanidiophycean occurrence and distribution are expected soon. In addition, they could veil cryptic species diversity (**paper IV**).

The phylogroup *Chloromonadinia* (green algal class Chlorophyceae, Chlorophyta) accommodates ecologically versatile flagellates, including freshwater and terrestrial taxa with psychrophilic, psychrotolerant and acidotolerant lifestyle (**papers V–VII**). The latest phylogenetic studies have shown that psychrophily has evolved just once and separately from the psychrotolerance within the *Chloromonadinia* (**papers V, VI**). The so far poorly explored terrestrial lifestyle has emerged several times independently within the *Chloromonadinia* with only one monophyletic lineage known from acidic environments (*Ostravamonas chlorostellata*; **paper VII**).

The genus *Cylindrocystis* (green algal class Zygnematophyceae, Streptophyta) represents the understudied group of organisms of polyphyletic origin. They have successfully colonized a variety of Arctic habitats, from soil to glacier surfaces. The Arctic *Cylindrocystis* shows a great genetic and morphological diversity awaiting further thoroughful investigations (**paper VIII**).

Ceased gene flow emerging after geographic isolation or niche shift results in infraspecific genetic differentiation in locally distributed species. Such differentiation was detected, for example, in the species *Ostravamonas chlorostellata* using the fast-evolving hypervariable molecular marker ITS2 rDNA (**paper VII**) or in *Galdieria sulphuraria* even using the conservative molecular marker *rbc*L (**paper III**). In contrast, the same genotypes of the widespread species *Chloromonas reticulata* could be detected from the Arctic to the Antarctic (**paper VII**), showing a capability of the effective long-distance dispersal. Similar genotypes of *Cylindrocystis* were detected in both temperate and Arctic habitats (**paper VIII**). However, biogeography and local conditions could have a significant influence on *Cylindrocystis* diversification, especially in terrestrial environments.

Working with monoclonal algal strains, including their isolation from field material and often challenging cultivation under laboratory conditions, and subsequent Sanger sequencing has an important value even in the current era of NGS-based culture-independent biodiversity research. Polyphasic investigations connecting phenotypes (morphology, ultrastructure, physiology) with particular genotypes or phylogenetic lineages, are still essential for understanding of species and so far irreplaceable by culture-independent approach. Culture-based studies as presented in this doctoral thesis represent the standard approch for studying and describing diversity of microorganisms (**papers I–IX**).

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Attached papers

Paper I

BARCYTĖ D & NEDBALOVÁ L (2017) *Coccomyxa*: a dominant planktic alga in two acid lakes of different origin. *Extremophiles* 21: 245–257

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ORIGINAL PAPER

Coccomyxa: a dominant planktic alga in two acid lakes of different origin

Dovilė Barcytė[1] · **Linda Nedbalová**[1]

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Abstract The aim of this study was to reveal the taxonomic position and phylogenetic relationships of the dominant planktic algae in two acid metal-rich lakes of different origin (Hromnice Lake and Plešné Lake, Czech Republic) and to investigate their morphology and ultrastructure under natural and laboratory conditions. Phylogenetic analyses (18S rRNA and ITS-2) revealed that the strain isolated from Hromnice Lake belongs to the species *Coccomyxa elongata*, while *Coccomyxa* from Plešné Lake was described as a new species *C. silvae-gabretae*. It is the first evidence that representatives of this genus are capable of becoming the dominant primary producers in the extreme environment of acid lakes with an increased supply of phosphorus. There were clear differences in cell morphology under different growth conditions, revealing the high phenotypic plasticity of the strains. The ability to change the morphology may help the cells of *Coccomyxa* to survive harsh conditions in the aforementioned acid lakes.

Keywords *Coccomyxa* · Acid lakes · 18S rRNA · ITS-2 · Ecology · Morphology · Phylogeny · Ultrastructure

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✉ Dovilė Barcytė
dovile.barcyte@gmail.com

[1] Department of Ecology, Faculty of Science, Charles University in Prague, Viničná 7, Prague 2 128 44, Czech Republic

Introduction

The acidification of freshwater ecosystems is one of the major environmental and ecological problems of our day. It changes the chemical properties of water and thereby influences the biological structure and composition of communities living there (Hendrey et al. 1976; Hendrey and Wright 1976; Schindler 1994, 1998; Driscoll et al. 2001). Freshwater ecosystems can be anthropogenically acidified either by atmospheric deposition or by acid mine drainage (AMD). Extreme examples of acid lakes are mining lakes which originated in abandoned metal or coal mines (Geller and Schultze 2013).

During acidification, freshwater biota can be influenced directly by changes in water quality or indirectly by the disruption of ecosystem processes (Muniz 1991). What is more, decreases in pH are usually accompanied by increased concentrations of various metals, especially aluminum, which becomes more soluble and, hence, more hazardous to aquatic biota as it shifts to its toxic form (Al^{3+}) under a pH below 5.5 (Gensemer and Playle 1999). In addition, increasing Al concentrations tend to reduce dissolved phosphorus and may lead to an oligotrophication of a water body (Kopáček et al. 2000).

Phytoplankton of acid lakes worldwide is usually dominated by flagellates from various taxonomic groups (Dinophyceae, Chrysophyceae, Chlorophyceae, Cryptophyceae, Euglenophyceae) (Almer et al. 1974, 1978; Lessmann et al. 2000; Nixdorf et al. 2001; Beulker et al. 2003). However, acid mining Lake Hromnice (Western Bohemia) and atmospherically acidified Plešné Lake in the Bohemian Forest (Šumava) are abundant in coccoid green algae with a similar morphology, but without precise taxonomic affiliation. Both lakes are relatively rich in phytoplankton biomass due to the increased input of phosphorus and

low grazing pressure but poor in species (Nedbalová et al. 2006; Hrdinka et al. 2013). The aim of this study was to reveal the taxonomic position and phylogenetic relationships of the dominant planktic algae in the two acid lakes and to compare their morphology and ultrastructure under natural and laboratory conditions.

Materials and methods

Characteristics of the lakes

Plešné Lake

Plešné Lake (48°46′39″N, 13°52′4″E, 1090 m a.s.l.) is situated in the Bohemian Forest (Šumava Mountains) of the Czech Republic. The small lake is of glacial origin with an area of 7.5 ha, a volume of 610,000 m^3, a maximum depth of 18 m and theoretical water residence time of 0.8 years. The lake lies in a steep, forested (Norway spruce), geologically sensitive catchment (67 ha) on a granitic bedrock (Veselý 1994; Vrba et al. 2000). Plešné Lake is a dimictic, mesotrophic lake with anoxia in the hypolimnion during both winter and summer stratification periods and a thermocline depth of 4–5 m. The lake has two surface inlets and two known subsurface inlets (Kopáček et al. 2000, 2006). The shore of Plešné Lake is without aquatic vegetation, the only exception being *Carex* and *Isoetes* in shallow water (Pražáková et al. 2006).

In the early 1960s, Plešné Lake was acidified by air pollution. Strong acidification progressed until the mid-1980s when the pH of the water ranged between 4.4 and 4.7 (Veselý 1994; Kopáček et al. 1998). Since the late 1980s, the lake has started to recover (Veselý et al. 1998; Majer et al. 2003). However, Plešné Lake still remains strongly acidified with inlet water pH < 4.6, surface water pH ~ 5, a depleted carbonate buffering system, sulfate (SO_4^{2-}) as the dominant anion, and increased concentrations of reactive Al (inlet water = 613 μg L^{-1}, surface water = 361 μg L^{-1}) (Kopáček et al. 2002; Nedbalová et al. 2006). Acidification caused the extinction of fish and largely reduced the diversity and biomass of zooplankton in the Bohemian Forest lakes. However, Plešné Lake is unique in its high phytoplankton biomass which comprises 80% of the total planktic biomass. The values of chlorophyll *a* reach up to 24.7 μg L^{-1} (Vrba et al. 2016). The reason for this is a higher inflow of phosphorus (total P = 12.3 μg L^{-1}, soluble reactive P = 9.6 μg L^{-1}) compared with the other lakes (Vrba et al. 2000; Nedbalová et al. 2006). Additionally, the toxic effects of Al on the aquatic biota decreased during the chemical reversal of the lake (Vrba et al. 2003).

The most important component of phytoplankton biomass in Plešné Lake is a green coccoid alga, which was tentatively named according to its morphology *Monoraphidium dybowskii,* and filamentous cyanobacteria (*Limnothrix* sp., *Pseudanabaena* sp.) (Vrba et al. 2000, 2003; Nedbalová et al. 2006). The phytoplankton community also consists of acid-tolerant species of dinoflagellates (*Peridinium umbonatum, Katodinium planum, K. bohemicum, Gymnodinium* sp.); green algae (*Arthrodesmus incus*, *Carteria multifilis*, *Chlamydomonas* sp., *Chlorogonium fusiforme*, *Chloromonas angustissima*, *Koliella corcontica*); cryptomonads (*Cryptomonas erosa*, *C. gracilis*, *C. marssonni*); and chrysomonads (*Bitrichia ollula*, *Dinobryon* spp., *Ochromonas* sp., *Spiniferomonas* sp.). Regarding zooplankton, five species of acid-tolerant rotifers (*Brachionus urceolaris* var. "*sericus*," *Collotheca pelagica*, *Keratella serrulata*, *Microcodon clavus*, *Synchaeta tremula*) and two species of copepods (*Heterocope saliens*, *Acanthocyclops vernalis*) occur in Plešné Lake (Nedbalová et al. 2006). Recently, the predatory copepod *Cyclops abyssorum* was successfully reintroduced into the lake (Kohout and Fott 2006).

Hromnice Lake

Hromnice Lake (49°51′02″N, 13°26′39″E, 380 m a.s.l.) is located in the western part of the Czech Republic. It was formed as a consequence of the mining of pyritic shale, when the pit (approx. 50 m deep, 260 m × 150 m) was spontaneously flooded with acid rock drainage. This anthropogenic lake is the most acidified lake (pH ~ 2.6) in the Czech Republic with an area of 0.974 ha, a volume of 60 980 m^3, and a maximal depth of 14 m. The lake basin is formed by strongly inclined slopes with bare rock and shale debris. The surroundings are densely forested with a large area of arable land to the north of the pit. Hromnice Lake is a permanently meromictic lake with a chemocline lying at a depth of 3–3.5 m and has no surface inlet or outlet. The shore is without aquatic vegetation, the only exception being that protonemata of a moss which was found at the bottom near the shore (Hrdinka et al. 2013).

The extreme chemical composition of lake water is characterized by high concentrations of SO_4^{2-} (up to 6410 mg L^{-1}), Fe (up to 2100 mg L^{-1}), Al (up to 176 mg L^{-1}), and increased concentrations of other heavy metals, e.g., Mn, Ni (up to 4.56 mg L^{-1}), Cu (up to 1.52 mg L^{-1}), Co (0.56 mg L^{-1}), and Pb (0.13 mg L^{-1}). The concentration of phosphorus (PO_4^{3-}) ranges from 0.04 mg L^{-1} at the surface and up to 1.6 mg L^{-1} in the deeper layers (Hrdinka et al. 2013).

Hromnice Lake is poor in phytoplankton species but contains a wealth of biomass. The values of chlorophyll *a* (20–50 μg L^{-1}) are comparable with eutrophic lakes. The most abundant species is a coccoid green trebouxiophycean alga *Coccomyxa* sp. occurring throughout the year along with a flagellate *Euglena mutabilis*. Other flagellates

Table 1 List of strains included in this study with newly deposited GenBank accession numbers

Strain Number	Culture Collection	Species	Habitat	GeneBank Accession Numbers	
				18S rRNA	ITS-2
–	–	*Coccomyxa elongata*	Hromnice Lake	KX809905	KX809914
1095	CCALA	*Coccomyxa silvae-gabretae*	Plešné Lake	KX809909	KX809916
H 101	CAUP	*Pseudococcomyxa simplex*	stock media	KX809907	KX809911
425	CCALA	*Pseudococcomyxa simplex*	hydrogeology bore	KX809906	KX809912
427	CCALA	*Pseudococcomyxa* sp.	pool	KX809908	KX809915
428	CCALA	*Pseudococcomyxa* sp.	thermal spring	KX809910	KX809913

Chlamydomonas acidophila and *Chromulina* sp. were found in high abundance only during certain periods of the year. Regarding zooplankton, only two acid-tolerant rotifers (*Cephalodella* sp. and *Elosa worallii*) were found in this extreme lake (Hrdinka et al. 2013).

Algal strains

Phytoplankton samples from Hromnice Lake were taken in August 2012, samples from Plešné Lake in the summer of 2012 and 2014, and the isolation of *Coccomyxa* strains were done by a serial dilution. The cultures were grown at room temperature in circumneutral Bold's Basal Medium (BBM) (Bischoff and Bold 1963). Three additional *Coccomyxa* strains were taken from the Culture Collection of Autotrophic Organisms in Třeboň (CCALA) and one strain from the Culture Collection of Algae of Charles University in Prague (CAUP), Czech Republic (Table 1). Fresh samples from liquid monocultures were further used for genetic analysis and microscopic observations.

DNA extraction, PCR, and sequencing

Algal cells were disrupted using the bead-beating method: 100 µl of lysis buffer combined with an equivalent amount of glass beads (425–600 µm in diameter) were added in the bead-beating tubes with algal samples and "beaten" for 30 s at 5000 rpm in a bead-based homogenizer *PowerLyzer*® (MoBio, Carlsbad, CA, USA). The samples were immediately placed on ice to minimize the activity of DNAses. The isolation of genomic DNA was done using *Invisorb*® *Spin Plant Mini Kit* (STRATEC Molecular, Berlin, Germany), according to the manufacturer's instructions.

For the amplification of the 18S rRNA gene and internal transcribed spacer 2 (ITS-2), PCR was performed as follows: the 18S rRNA gene was amplified using a eukaryote-specific primer 20F (Thüs et al. 2011) and CH1750R (Hallmann et al. 2013) specific for green algae. The ITS-2 region was amplified using the AL1500af primer (Helms et al. 2001) specific for algae and the eukaryote-specific primer LR3 (Vilgalys and Hester 1990). PCR was performed in a thermocycler *TProfessional Basic* (Biometra, Goettingen, Germany) using the following program for the primer set 20F/CH1750R: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min. For the ITS-2 primer set AL1500af/LR3, an initial denaturation at 95 °C for 5 min, linked to 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 25 s, and a final extension at 72 °C for 5 min was performed.

Aliquots of 2 µL of the PCR products were analyzed by electrophoresis on a 1% agarose gel in a 0.5 TBE buffer (140 V 30 min). The PCR products were purified using *MSB*® *Spin PCRapace* Kit (STRATEC Molecular).

Sequencing reactions were performed using a *BigDye*® *Terminator v3.1 Cycle Sequencing* Kit (Applied Biosystems, Darmstadt, Germany). The 18S rRNA gene was sequenced with the standard sequencing primers 34F, 370R, and 1122F (Pažoutová et al. 2010); 895R, 1422F (Remias et al. 2012); 891F, 1122R, 1422R (Friedl unpubl.); for the ITS-2 sequences primers 5.8SbF (Mikhailyuk et al. 2008) and ITS4 (White et al. 1990) were used. The DNA purification was done using ethanol and precipitated sequencing products were analyzed by the ABI Prism 3100 (Applied Biosystems) automated sequencer at the Experimental Phycology and Culture Collection of Algae at the University of Goettingen (EPSAG).

Phylogenetic analyses

New 18S rRNA and ITS-2 *Coccomyxa* sequences were assembled and edited in the program SeqAssem (Hepperle, 2004). For the alignment, the homologous sequences were retrieved from available public sequence databases using BLAST (Altschul et al. 1990) at NCBI (http://www.ncbi.nlm.nih.gov/). All sequences were imported and stored as a preliminary alignment in BioEdit (Hall 1999). Two separate alignments (18S rRNA and ITS-2) were created and

analyzed independently. The final 18S rRNA alignment was done with MAFFT v. 7 (Katoh and Standley 2013) available online (http://mafft.cbrc.jp/alignment/server/) and adjusted manually with the exclusion of any ambiguous regions. The ITS-2 region was annotated using an online ITS-2 database (Ankenbrand et al. 2015) (http://its2.bio-apps.biozentrum.uni/wuerzburg.de/) and the sequences were aligned with MAFFT under the Q-INS-i strategy.

The best-fit nucleotide substitution models were estimated with Modeltest 3.7 (Posada and Crandall 1998) in conjunction with PAUP* 4.0b10 (Swofford 2003). Based on the Akaike Information Criterion (AIC) (Sullivan and Joyce 2005), the GTR + I + Γ model was selected to best fit the 18S rRNA alignment, while for the ITS-2 alignment the GTR + Γ model was chosen as the best fit. Three types of phylogenetic analyses were used for the alignments: Bayesian Inference (BI), Maximum Likelihood (ML), and Maximum Parsimony (MP).

Bayesian analyses were conducted in MrBayes 3.2.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Two parallel Markov chain Monte Carlo (MCMC) runs for one million generations with one cold and three heated chains were conducted for both alignments using the selected best-fit evolutionary models, with trees sampled every 100 generations. The first 2500 generations were discarded as burn-in. Bayesian posterior probabilities were used to assess clade support. ML analyses were conducted in the software program MEGA 7 (Kumar et al. 2016) using Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar 2000) with search level 5. The reliability of each internal branch was evaluated based on 1000 bootstrap replicates. MP analyses were also conducted with MEGA using the SPR algorithm with search level 2 in which the initial trees were obtained by 10-time randomization of the sequence and 1000 bootstrap replicates. The obtained bootstrap values were used to assess clade support.

The computed phylogenetic trees were visualized using FigTree 1.4.2 (Rambaut 2012) and modified by Inkscape 0.91 (Free Software Foundation Inc., Boston, USA).

Light and electron microscopy

Microscopic observations of the strains were done with a Nikon Eclipse E400 (Nikon Corp., Japan) light microscope. Microphotographs were taken using a Canon EOS D650 (Canon Inc., Japan) digital camera and processed using QuickPHOTO Camera 3.0 software (Promicra, Czech Republic). The same software was used to measure the size (length and width) of the cells.

For transmission electron microscopy (TEM), the cultures of the Hromnice and Plešné strains as well as field samples from Plešné Lake were fixed for 24 h in 2.5% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2), and post-fixed in 2% OsO_4 of the same buffer. Fixed cells were dehydrated through an ascending ethanol and acetone series and embedded in an Araldite and Poly/Bed® 812 mixture. Ultrathin sections were cut with a diamond knife on an Ultracut E ultramicratome (Reichert-Jung, Wien, Austria) and stained using uranyl acetate and lead citrate. The TEM grids were examined with a JEOL 1011 TEM (JEOL Ltd., Tokyo, Japan). Photomicrographs were obtained using a Veleta CCD camera equipped with the image analysis software Olympus Soft Imaging Solution GmbH (Münster, Germany) and later modified by Inkscape 0.91.

Results

Analysis of the molecular data

We have examined two free-living *Coccomyxa* strains isolated from acid lakes in the Czech Republic using both morphological identification and molecular markers. Both strains occupied different phylogenetic positions (Figs. 1, 2) and varied in morphology (Figs. 3, 4). The sequences of the 18S rRNA gene and ITS-2 were deposited in NCBI GenBank with the accession numbers given in Table 1. The additional four *Coccomyxa* strains isolated from different habitats were newly sequenced in this study and their GenBank accession numbers are also shown in Table 1. GenBank numbers for the rest of the taxa included in the phylogenetic analyses are listed in Table S1 in the electronic supplementary material.

BLAST searches confirmed that all newly sequenced strains belong to the genus *Coccomyxa* (Trebouxiophyceae, Chlorophyta). Two separate analyses were done in order to include all available 18S rRNA sequences of *Coccomyxa* from acid freshwaters. Therefore, the 18S rRNA dataset contained 72 sequences including *Elliptochloris* as the closest relative of *Coccomyxa* (Friedl et al. 2007; Eliáš et al. 2008) which, along with *Hemichloris*, formed an outgroup. The alignment consisted of 1713 positions with 1566 constant and 93 parsimony-informative sites. All positions with less than 95% site coverage were eliminated resulting in 1677 positions in the final 18S rRNA dataset for ML and MP analyses. ITS-2 alignment included 65 nucleotide sequences with 418 aligned positions, containing 141 constant and 198 parsimony-informative sites. All sites in the ITS-2 alignment were used to reconstruct the phylogeny.

The obtained phylogenetic trees matched well with the phylogram inferred by Malavasi et al. (2016) and supported many novel lineages recognized by the authors. There were no prominent contradictions in phylogenetic reconstructions between the two datasets, except the position

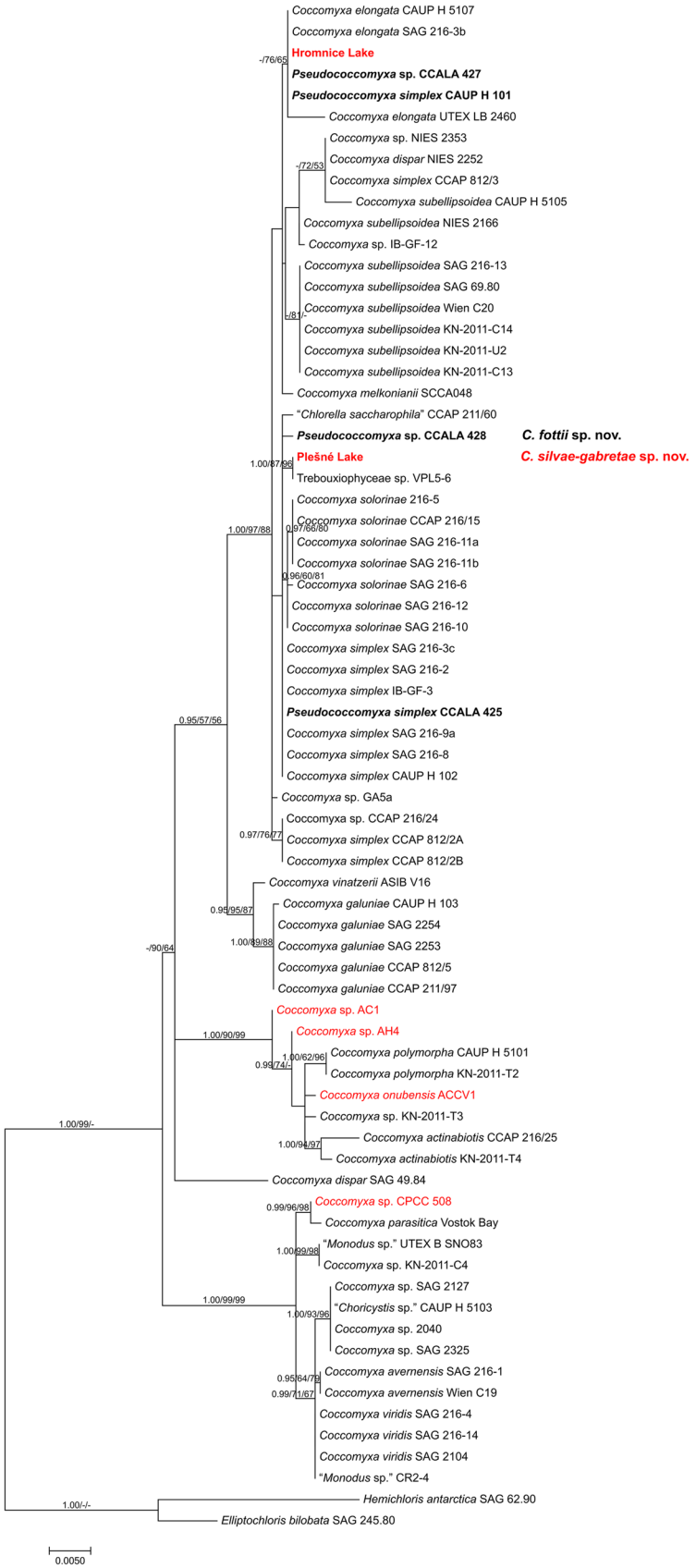

Fig. 1 Phylogenetic tree from the ML analysis of the 18S rRNA gene sequence data. Posterior probabilities ≥0.95 and bootstrap values >50 are mapped to the corresponding internodes (BI probability/ML bootstrap/MP bootstrap). The sequences in *bold* were obtained in this work, sequences in *red* represent isolates from acid freshwaters. Taxonomy follows Malavasi et al. (2016). The *scale bar* represents the expected number of substitutions per site

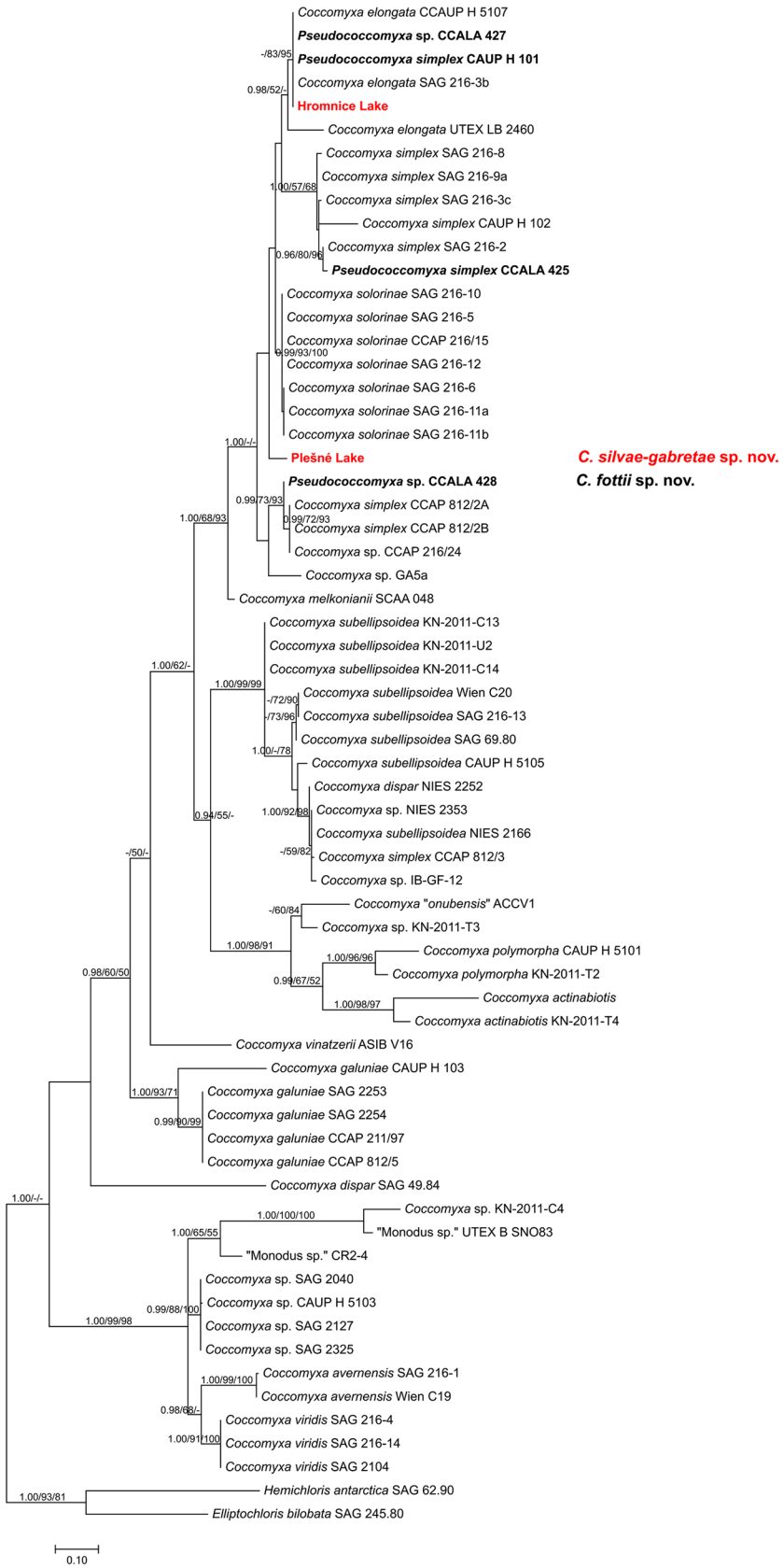

Fig. 2 ITS-2-based ML tree. Posterior probabilities ≥0.94 and bootstrap values >50 are mapped to the corresponding internodes (BI probability/ML bootstrap/MP bootstrap). The sequences in *bold* were obtained in this work, sequences in red represent isolates from two acid lakes. Taxonomy follows Malavasi et al. (2016). The *scale bar* represents the expected number of substitutions per site

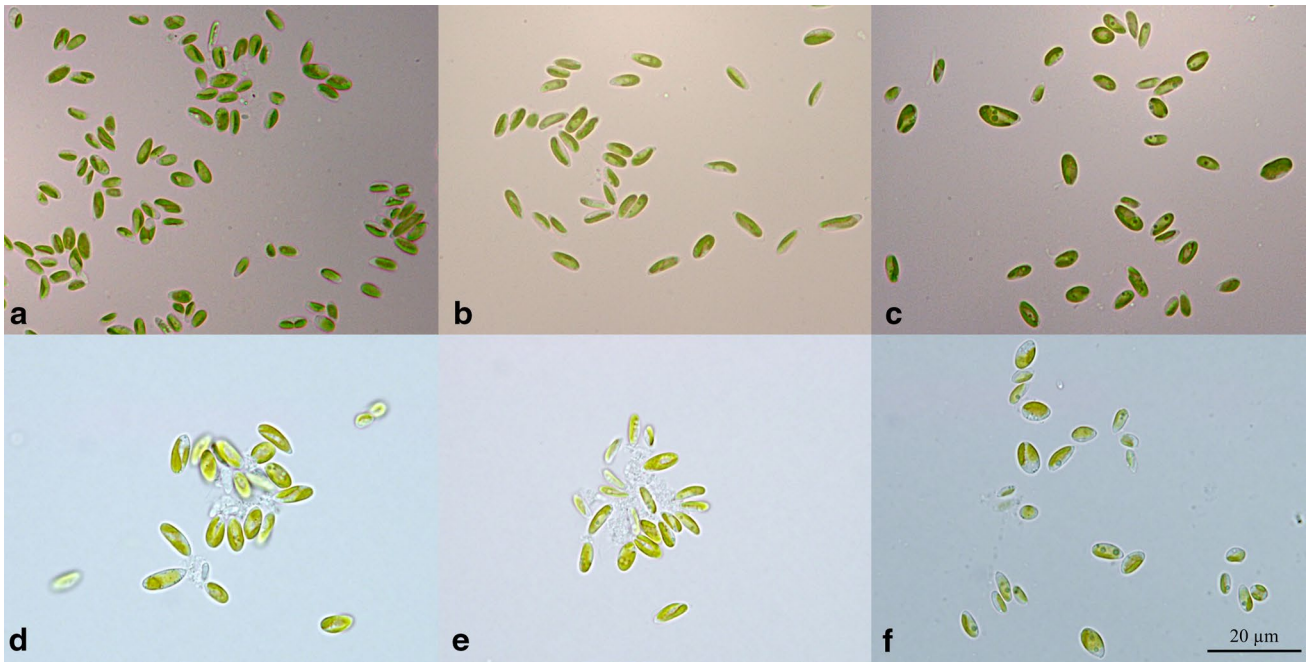

Fig. 3 Morphology of *Coccomyxa* strains included in this study: **a** Plešné Lake; **b** Hromnice Lake; **c** CCALA 428; **d** CAUP H 101; **e** CCALA 427; **f** CCALA 425

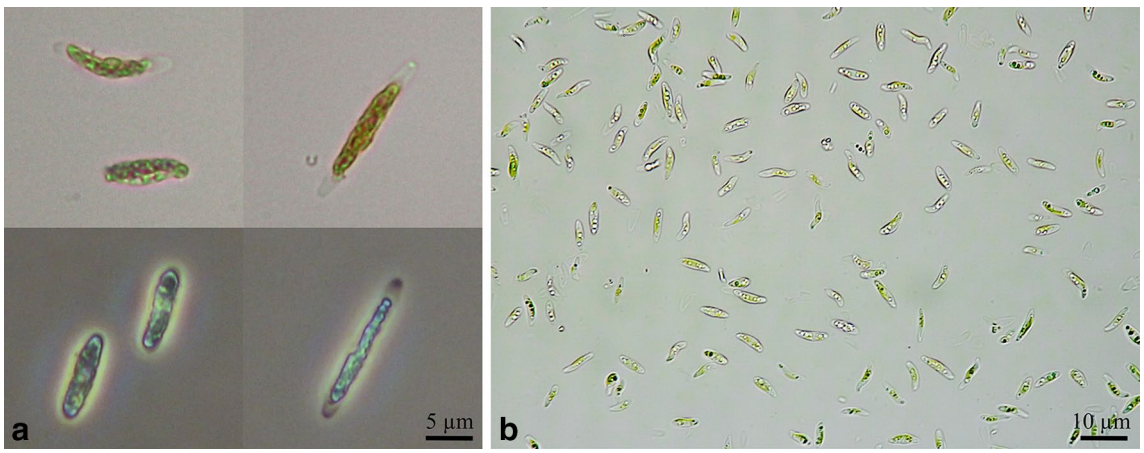

Fig. 4 Field samples from: **a** Plešné Lake; **b** Hromnice Lake

of *Coccomyxa* strains CAUP H 5105 and "*Monodus* sp." CR2-4 (Figs. 1, 2). Moreover, some of the clades did not obtain any statistical support by BI in 18S rRNA analyses (Fig. 1), while in ITS-2 analyses they were well-supported (Fig. 2).

Coccomyxa strains isolated from the acid Hromnice and Plešné lakes were placed within different positions in all of the computed phylogenetic trees. The strain isolated from Hromnice Lake was genetically identical to *Coccomyxa elongata* SAG 216-3b and CAUP H 5107, along with newly sequenced strains *Pseudococcomyxa* sp. CCALA 427 and *P. simplex* CAUP H 101. None of the ITS-2 sequences currently available in GenBank were identical to the *Coccomyxa* sequence isolated from Plešné Lake. Surprisingly, the 18S rRNA sequence of the strain was identical to a sequence designated as Trebouxiophyceae sp. VPL5-6 known from an Antarctic lake, and thus the Plešné strain formed a distinct lineage both in 18S rRNA and ITS-2 analyses (Figs. 1, 2). Strain *Pseudococcomyxa* sp. CCALA 428 also appeared to form an individual lineage in 18S rRNA analysis (Fig. 1). ITS-2 analysis revealed that the strain was most closely related to "clade C" (Malavasi

et al. 2016), containing strains with no species affiliations (Fig. 2). *Pseudococcomyxa simplex* CCALA 425 was confirmed to belong to the '*simplex*' clade.

Morphology and ultrastructure

The morphological investigation of the Hromnice and Plešné strains was done when the exponential phase of monocultures was reached. Both examined strains had typical *Coccomyxa* morphology: cells were elongated, ellipsoidal, thin-walled, and solitary; each cell contained a parietal, lateral in position chloroplast without a pyrenoid. Both strains slightly differed in the cell size/shape (Fig. 3).

The strains taken from the algae culture collections (CCALA 425, 427, 428; CAUP H 101) did not show any peculiar morphological differences from the typical *Coccomyxa* morphology or each other. However, the strains CAUP H 101 and CCALA 427 were slightly elongated, highly resembling the Hromnice strain (Fig. 3b, d, e).

There was a clear difference in the appearance of *Coccomyxa* cells when observed naturally in Plešné Lake compared to those grown under laboratory conditions. The cells from the natural population were unusually elongated, often inclined to one side and covered by mucilage. Interestingly, in most of cases the extension of the cell wall occurred at cell poles, creating elongated spindle-shaped cells (Fig. 4a). None of these features were observed in laboratory conditions. The comparison of natural material and laboratory cultures from Hromnice Lake by light microscope also revealed some morphological differences. The cells from the natural habitat highly varied in their shape and were irregularly curved (Fig. 4b).

The ultrastructure of the Hromnice and Plešné monocultures cultivated for many generations in BBM was virtually identical. The cells contained a single parietal chloroplast with paired thylakoids and starch granules inside the interthylacoidal space. Plastoglobules were also present in both strains. The nucleus and two-to-four mitochondria were visible in the cytoplasm (Fig. 5a, b). In contrast to the laboratory culture, the observation of the Plešné Lake field samples revealed huge, electron dense bodies, which in fact are associated with lipid droplets. What is more, the cells appeared to have a thicker cell wall and were covered by mucilage (Fig. 5c, d).

Coccomyxa silvae-gabretae Barcytė & Nedbalová sp. nov.

Diagnosis Mature vegetative cells are solitary, ellipsoidal, often elongated-oval, highly variable in length, and 4.1–8.2 µm x 2.0–3.3 µm in size. The chloroplast is lateral trough-shaped, occupying half of the cell volume. A pyrenoid is absent and the cell wall is thin. In type locality, the cells are covered by a thick cell wall and the average cell is 11.7 µm long and 2.0 µm wide. Asexual reproduction by 2–4 autospores. Protoplast division is oblique. The autospores are liberated through a rupture of the mother cell wall.

Exact identification is possible only using phylogenetic markers.

Type locality Plešné Lake, Bohemian Forest, Czech Republic.

Holotype The living culture has been deposited at CCALA under number 1095.

Etymology Silva Gabreta is a latin name for Bohemian Forest.

Coccomyxa fottii Barcytė & Nedbalová sp. nov.

Diagnosis Mature vegetative cells are solitary, elongated ellipsoidal, 5.4–9.7 µm × 2.0–3.4 µm in size, and both ends are rounded with one apex often slightly narrower. The cell wall is thin. The chloroplast parietal is trough-shaped, massive, and without pyrenoid. Lipid droplets are present in the protoplast. Reproduction is by 2–4 autospores. Protoplast division is oblique. Liberation of autospores is through the rupture of the mother cell wall.

Exact identification is possible only using phylogenetic markers.

Habitat freshwater.

Type locality thermal spring, Piešťany, Slovakia.

Holotype The strain is available at CCALA under strain number 428.

Etymology The species was named to mark the 40th anniversary of the death of famous Czech phycologist Prof. RNDr. Bohuslav Fott, DrSc. (1908–1976).

Discussion

Phylogeny and morphology

Coccomyxa (Trebouxiophyceae, Chlorophyta) is one of the most widespread known green alga, playing an important role both in terrestrial and aquatic environments. Despite its importance, the actual diversity of free-living *Coccomyxa* has been underestimated for many years, and only recently it was reviewed and summarized for the very first time by Darienko et al. (2015) and subsequently by Malavasi et al. (2016). In contrast to both previous studies which analyzed concatenated SSU and ITS rDNA dataset, we have computed two independent analyses for the 18S rRNA gene and ITS-2. Moreover, we have expanded the dataset from 61 to 72 sequences in the 18S rRNA analysis and to 65 sequences in the ITS-2 analysis as some of the ITS-2 sequences were not available, and we used only complete or almost complete ITS-2 sequences with the 3′ end of 5.8 rRNA and 5′ end of 26S rRNA genes. Our phylogenetic

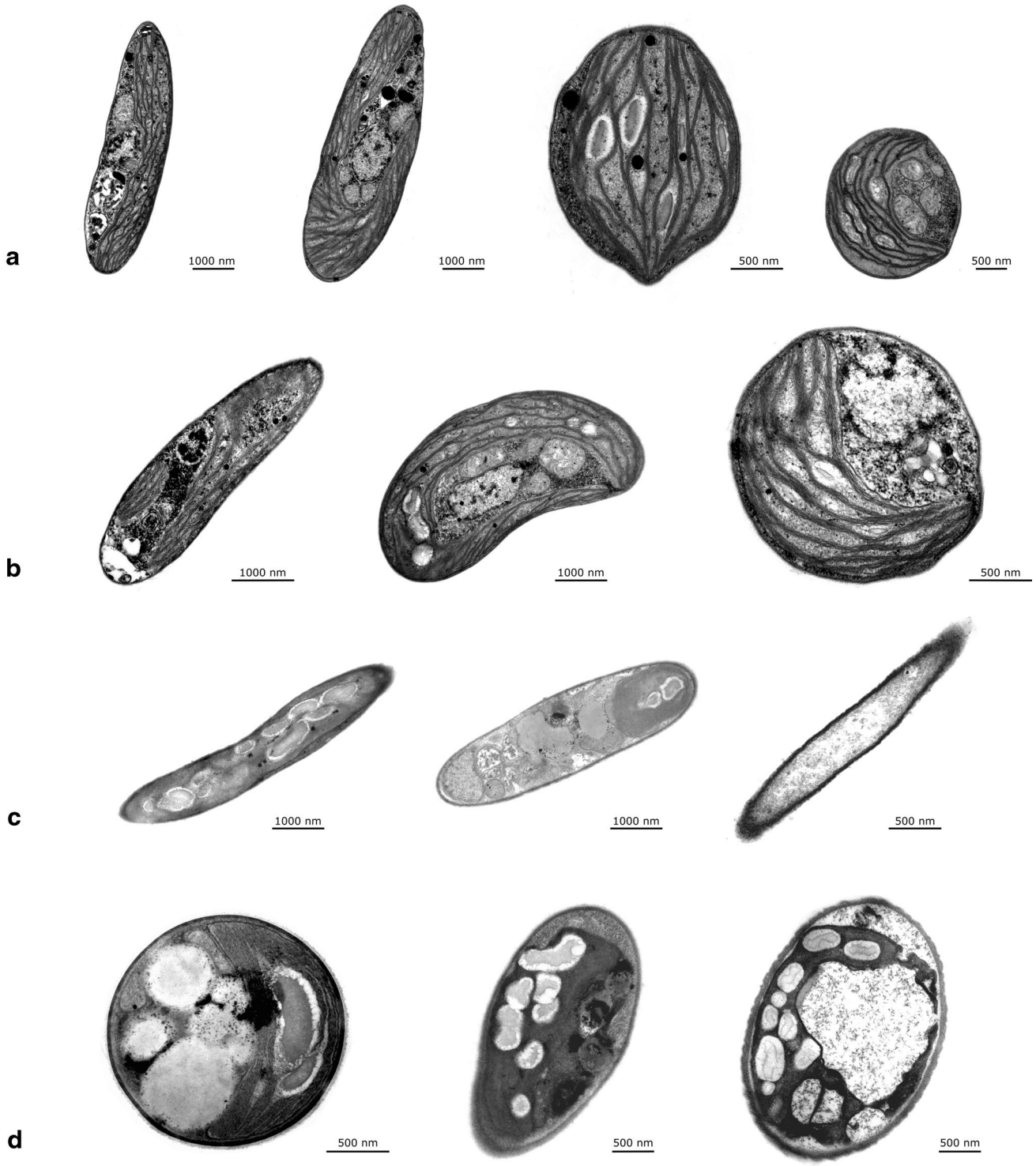

Fig. 5 Longitudinal and cross-sections of the cells: **a** Hromnice Lake strain, **b** Plešné Lake strain, **c** and **d** Plešné Lake field sample: note a thick cell wall

analyses were in agreement with Malavasi et al. (2016) and thus we followed the same taxonomy as proposed by the above-mentioned author. A few contradictions arose when two phylogenetic trees were compared, showing that the phylogeny and taxonomic position of *Coccomyxa* species is still far from being fully understood and an increased taxon sampling should be done for a better resolution of the actual diversity within the genus.

The small size and simple morphology of unicellular autosporic green algae usually lead to taxonomic difficulties and make them unattractive objects for morphological studies. The cells of *Coccomyxa* share very similar characteristics and in most cases the identification to a species level based on morphological features is impossible and, therefore, can only be done using the molecular markers. Moreover, *Coccomyxa* appears to be phenotypically very plastic (Darienko et al. 2015), which makes morphological identification even harder. Thus, a barcoding for the genus is a necessity, and ITS-2 was recently proposed as a suitable marker for such purpose (Darienko et al. 2015).

Based on our phylogenetic analyses, the isolates from two low pH lakes, characterized by high concentrations of various toxic metals, belong to two different *Coccomyxa* species. *C. elongata* (Hromnice Lake) is generally known as a freshwater species, e.g., CCALA 427, or living on wet sandstone rocks. However, this species is not a very commonly reported one. *C. silvae-gabretae* sp. nov. (Plešné Lake) appeared to be closely related only to one strain, VPL5-6, isolated from a lake in the Antarctic Peninsula (De Wever et al. 2009).

The *Coccomyxa* strain isolated from a thermal spring in Slovakia herein was also described as a new species, *C. fottii.* However, is it conspecific to another yet undescribed species (=clade C, Malavasi et al. 2016), or if it really represents an evolutionary lineage of its own, should be better confirmed by an increased number of closely related sequences in future analyses. The representatives of "clade C" seem to prefer freshwater habitats as well (CCAP 812/2A, CCAP 216/24) but also have been isolated from a tobacco plant (CCAP 812/2B).

The cultivation of *Coccomyxa* strains isolated from the Hromnice and Plešné lakes in an artificial circumneutral medium have clearly changed the morphology of the cells, and thus we confirmed that the phenotypic changes of *Coccomyxa* can be induced by direct environmental factors as was also demonstrated by Darienko et al. (2015). The irregular shape of *Coccomyxa* cells from Hromnice Lake could be a direct consequence of the extreme lake environment as well as an actively modified shape by the cells themselves to better survive in a challenging habitat as is known in some cases of bacteria (Young 2006). However, more studies are needed to confirm these speculations. The expansion of the *Coccomyxa* cells in Plešné Lake could be explained by the fact that *Coccomyxa* cells are known to be covered by a cellulosic cell wall containing sporopollenin (Honegger and Brunner 1981; Albertano et al. 1990). Low pH weakens hydrogen bonds in cellulose strands and may result in uncontrolled cell expansion (Gross 2000). The non-typical shape of the *Coccomyxa* cells in Plešné Lake indeed highly resemble another coccoid green alga *Monoraphidium* (Chlorophyceae), which may be notable.

Herein, we conclude that the success of *Coccomyxa* in Hromnice and Plešné lakes may partly be explained by the fact that they are able to adapt their morphology depending on environmental conditions, e.g., a thicker cell wall and/or mucilage layer.

Ecology

The present study confirms that *Coccomyxa* is able to grow in a wide range of habitats including the most extreme ones and that no clear ecological requirements have been noticed for a particular species. The capability of one species to colonize very different habitats indicates a very broad ecological niche of the genus. Being so flexible and adaptable, *Coccomyxa* may have an advantage when conditions become extreme and therefore become the dominant alga. Remarkably, *Coccomyxa* has also been found as a contaminant in flasks with chemical solutions prepared for analytical purposes, in stock solutions used to make media for algal cultivation (Sládečková 1959) and growing in distilled water in the laboratory (Taylor 1965). A strain of *Coccomyxa* (CPCC 508) tolerant to low pH and 100 mM nickel was isolated from the acid- and metal-contaminated Boomerang Lake in Ontario (Verma et al. 2009) (Fig. 1). Another metal-resistant *Coccomyxa* strain (ACCV1) was isolated from the extremely acid (pH 1.7–3.1) Tinto River in Spain (Garbayo et al. 2012) (Fig. 1). In addition to that, Falagán et al. (2014) reported two *Coccomyxa* sequences (AC1, AH4) from two stratified acid pit lakes located in Spain (Fig. 1). Moreover, a new species *C. melkonianii* was recently described from the Rio Irvi river in Sardinia which is also affected by mining activities and characterized by a high content of various heavy metals (Malavasi et al. 2016).

It is interesting to note that *Coccomyxa* is not commonly reported as occurring along with other phytoplankters in bodies of water which are acid. If present, it is one of the most abundant species (Nedbalová et al. 2006; Hrdinka et al. 2013) or even the only one (Falagán et al. 2014). This suggests that acidic conditions may allow and promote the existence of *Coccomyxa* by reducing predation and interspecific competition for nutrients, light, and space. This phenomenon was observed in both Hromnice and Plešné lakes given that both lakes are lacking grazing zooplankton and rich in phosphorus (Hromnice Lake, Hrdinka et al. 2013), or with a relatively high input of phosphorus, but with depleted phosphate in the water column (Plešné Lake, Nedbalová et al. 2006). These lakes offer a suitable habitat for such an opportunistic genus as *Coccomyxa*, which immediately reacts to favorable conditions and quickly exploits the available resources. On the other hand, the occurrence of *Coccomyxa* in acid freshwater locations may actually be more frequent than previously reported. First of all, when not the most abundant species, *Coccomyxa* may

sometimes be overlooked among the other algae because of its small size. Secondly, the small coccoid algae are always difficult to identify and misleading conclusions could have been made about algal composition in one or both acid lakes in the past. For example, some species of *Monoraphidium* have also been reported as occurring in acid lakes in Scandinavia (Almer et al. 1978; Hörnström et al. 1995), as was done for Plešné Lake (Nedbalová et al. 2006). However, their true identity has to be confirmed in the future using molecular markers.

Apart from this, no studies were done summarizing the diversity of *Coccomyxa* regarding acid freshwater environments. Phylogenetic trees of the 18S rRNA gene computed in this study show that the strains capable of living in acid habitats evolved within different *Coccomyxa* species and do not depend on the species per se. Different strains with distinct genetic variation may simply have adapted to the specific environmental conditions they were exposed to. Furthermore, we found out that *Coccomyxa elongata* is capable of inhabiting both acid lakes and laboratory solutions (Hromnice Lake, CAUP H 101) as was never reported before.

Conclusions

The taxonomic position of the dominant planktic alga in Hromnice and Plešné lakes (previously referred as *Coccomyxa* sp. and *Monoraphidium dybowskii*, respectively) was finally revealed. The strain isolated from Hromnice Lake belongs to the species *Coccomyxa elongata*, while the strain from Plešné Lake is a newly described species *Coccomyxa silvae-gabretae*. A new species *Coccomyxa fottii* was also described in this study. The increasing number of recently described *Coccomyxa* species, e.g., Darienko et al. (2015), Malavasi et al. (2016), shows that the molecular diversity of the genus, especially free-living *Coccomyxa*, is as yet under-explored and extensive sampling and studies should be done focusing on the actual diversity and ecology of the genus.

There is no clear trend between the *Coccomyxa* species and their realized ecological niches, and the capability of *Coccomyxa* to live in acid habitats evolved within different *Coccomyxa* species. Strains genetically identical to Hromnice and Plešné strains may be especially successful in extreme conditions. *C. elongata* and *C. silvae-gabretae* can become dominant phytoplankton algae in the extreme environment of acid lakes with an increased supply of phosphorus.

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Strain	Species sensu Malavasi et al. (2016)	Accession Number (18S rRNA)	ITS-2 rDNA availability*
AC1	*Coccomyxa* sp.	KC155323	-
ACCV1	*Coccomyxa* sp.	HE617183	yes
AH4	*Coccomyxa* sp.	KC155324	-
ASIB V16	*Coccomyxa vinatzeri*	HG972994	yes
CAUP H 102	*Coccomyxa simplex*	HE586504	yes
CAUP H 103	*Coccomyxa galuniae*	HE586505	HE586520
CAUP H 5101	*Coccomyxa polymorpha*	HG972979	yes
CAUP H 5103	"*Choricystis* sp."	HG973007	yes
CAUP H 5105	*Coccomyxa subellipsoidea*	HG972974	yes
CAUP H 5107	*Coccomyxa elongata*	HG972981	yes
CCAP 211/60	*Chlorella saccharophila'*	FR865679	-
CCAP 211/97	*Coccomyxa galuniae*	FN298928	yes
CCAP 216/15	*Coccomyxa solorinae*	HG972985	yes
CCAP 216/24	*Coccomyxa* sp.	FN298927	yes
CCAP 216/25	*Coccomyxa actinabiotis*	HW267776	yes
CCAP 812/2A	*Coccomyxa simplex*	HG972992	yes
CCAP 812/2B	*Coccomyxa simplex*	HG972993	yes
CCAP 812/3	*Coccomyxa simplex*	HG972972	yes
CCAP 812/5	*Coccomyxa galuniae*	HG972995	yes
CPCC 508	*Coccomyxa* sp.	AM981206	-
CR2-4	"*Monodus* sp."	HE586519	yes
GA5a	*Coccomyxa* sp.	AB917140	yes
IB-GF-12	*Coccomyxa* sp.	KM020052	yes
IB-GF-3	*Coccomyxa* sp.	KM020053	-
KN-2011-C13	*Coccomyxa subellipsoidea*	HE586510	yes
KN-2011-C14	*Coccomyxa subellipsoidea*	HE586511	yes
KN-2011-C4	*Coccomyxa* sp.	HE586508	yes
KN-2011-T2	*Coccomyxa polymorpha*	HE586514	yes
KN-2011-T3	*Coccomyxa* sp.	HE586515	yes
KN-2011-T4	*Coccomyxa actinabiotis*	HE586516	yes
KN-2011-U2	*Coccomyxa subellipsoidea*	HE586517	yes
NIES 2166	*Coccomyxa subellipsoidea*	AGSI00000000	yes
NIES 2252	*Coccomyxa dispar*	HG972973	yes
NIES 2353	*Coccomyxa* sp.	HG972971	yes
SAG 2040	*Coccomyxa* sp.	HG973004	yes
SAG 2104	*Coccomyxa viridis*	HG973003	yes
SAG 2127	*Coccomyxa* sp.	HG973005	yes
SAG 216-1	*Coccomyxa avernensis*	HG972999	yes
SAG 216-10	*Coccomyxa solorinae*	HG972986	yes
SAG 216-11a	*Coccomyxa solorinae*	HG972983	yes
SAG 216-11b	*Coccomyxa solorinae*	HG972984	yes
SAG 216-12	*Coccomyxa solorinae*	HG972987	yes
SAG 216-13	*Coccomyxa subellipsoidea*	KF673374	AY328523
SAG 216-14	*Coccomyxa viridis*	HG973002	yes
SAG 216-2	*Coccomyxa simplex*	HG972989	yes
SAG 216-3b	*Coccomyxa elongata*	HG972980	yes

SAG 216-3c	*Coccomyxa simplex*	HG972990	yes
SAG 216-4	*Coccomyxa viridis*	HG973001	yes
SAG 216-5	*Coccomyxa solorinae*	HG972982	yes
SAG 216-6	*Coccomyxa solorinae*	HG972988	yes
SAG 216-8	*Coccomyxa simplex*	HG972991	yes
SAG 216-9a	*Coccomyxa simplex*	FN298926	yes
SAG 2253	*Coccomyxa galuniae*	HG972996	yes
SAG 2254	*Coccomyxa galuniae*	HG972997	yes
SAG 2325	*Coccomyxa* sp.	HG973006	yes
SAG 245.80	*Elliptochloris bilobata*	HG972969	yes
SAG 49.84	*Coccomyxa dispar*	HG972998	yes
SAG 62.90	*Hemichloris antarctica*	HG972970	yes
SAG 69.80	*Coccomyxa subellipsoidea*	HG972977	yes
SCCA048	*Coccomyxa melkonianii*	KU696488	yes
UTEX B SNO83	"*Monodus* sp."	HE586506	yes
UTEX LB 2460	*Coccomyxa simplex*	HE586524	yes
Vostok Bay	*Coccomyxa parasitica*	LN879479	-
VPL5-6	Trebouxiophyceae sp.	FJ946891	-
Wien C19	*Coccomyxa avernensis*	HG973000	yes
Wien C20	*Coccomyxa subellipsoidea*	HG972975	yes

*complete or almost complete sequence available

Paper II

BARCYTĖ D & HODAČ L (2019) ***Watanabea acidotolerans*****: A new trebouxiophyte lineage (Chlorophyta) inhabiting low pH environments from Europe to South America.** ***Phycological Research*** **67: 120–127**

Authors' contributions:

DB obtained all the data, LH analyzed the molecular data; DB and LH jointly wrote the paper

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Watanabea acidotolerans: A new trebouxiophyte lineage (Chlorophyta) inhabiting low pH environments from Europe to South America

Dovilė Barcytė [1,2]* **and Ladislav Hodač** [3]

[1]Department of Zoology, National Museum, Prague 9, Czechia, [2]Department of Ecology, Faculty of Science, Charles University, Prague 2, Czechia and [3]Department of Systematics, Biodiversity and Evolution of Plants (with Herbarium), Georg-August-University of Göttingen, Göttingen, Germany

SUMMARY

Here we studied a new *Watanabea* strain CAUP H 8901-CRYO isolated from an extremely acidic (pH 2.6) pit lake in Czechia. We used morphological, ultrastructural and molecular data to ascertain its taxonomic position and phylogenetic relationships within the genus *Watanabea.* The morphology of the studied strain matched the formal *Watanabea* description, including its most characteristic feature, the production of two types of autospores. The new isolate morphologically and ultrastructurally resembled the *Watanabea* type species, *W. reniformis.* However, it differed from *W. borysthenica* by having a pyrenoid not surrounded by starch grains. Molecular phylogenetic analyses of nuclear 18S rDNA and plastid *rbc*L showed that the strain CAUP H 8901-CRYO formed an independent lineage within the genus *Watanabea.* Furthermore, analyses of nuclear ITS2 secondary structure demonstrated an additional side loop coiling from the helix III, the unique feature of CAUP H 8901-CRYO, not present in the authentic strain of *W. reniformis* SAG 211-9b. The ITS2 secondary structures of the two strains also differed by one compensatory base change (CBC) and a number of additional nucleotide substitutions. The new acidotolerant strain and another *Watanabea* isolate from a naturally acidic lake in Argentina were identical in their plastid *rbc*L sequence. Consequently, here we proposed a new species, *Watanabea acidotolerans*, to accommodate a lineage of microalgae with a possible worldwide distribution and tolerance to low pH habitats.

Key words: Acidotolerant, algae, phylogeny, Trebouxiophyceae, *Watanabea*.

INTRODUCTION

Watanabea Hanagata, Karube, Chihara et Silva is a little known genus of green algae (Trebouxiophyceae, Chlorophyta) found in freshwater and terrestrial ecosystems (Hanagata *et al*. 1998; Fučíková *et al*. 2014; Song *et al*. 2016). The genus is nested within the *Watanabea* clade encompassing a number of morphologically simple but phylogenetically and ecologically highly diversified taxa with predominantly terrestrial lineages from temperate and tropical regions (Neustupa *et al*. 2013; Fučíková *et al*. 2014; Song *et al*. 2016; Procházková *et al*. 2018).

The type species of the genus *Watanabea*, *W. reniformis* (authentic strain SAG 211-9b), was established based on morphological differences from other *Chlorella*-like microalgae (Hanagata *et al*. 1998). Its most characteristic feature is the formation of two types of cells, which had not been reported for any other member of the *Watanabea* clade. The latest molecular phylogenetic studies demonstrated that the genus *Watanabea* contains several unidentified organisms (Song *et al*. 2016; Procházková *et al*. 2018) and that new isolates could significantly contribute towards better understanding of the genus. For example, a new species *W. borysthenica* Darienko and Pröschold (authentic strain SAG 2550) was recently proposed to accommodate two new epilithic and edaphic *Watanabea* strains (Darienko & Pröschold 2018).

This study presents a survey of a new epilithic *Watanabea* strain CAUP H 8901-CRYO isolated from an extremely acidic pit lake in Czechia. We aimed to ascertain its taxonomic position and phylogenetic relationships within the genus *Watanabea* using a polyphasic approach, encompassing light and electron microscopy with a combination of three molecular markers (18S rDNA, *rbc*L and ITS2 rDNA).

MATERIALS AND METHODS

The strain CAUP H 8901-CRYO investigated in this study was isolated from the surface of a stone found at the bottom near the shore of the extremely acidic (pH 2.6) Hromnice Lake in Czechia (49°51′02.5″ N, 13°26′39.3″ E) in May 2017. A scraped-off biofilm was transferred to a 24-well microplate filled with circumneutral (pH 7) Bold's Basal Medium (BBM; Bischoff & Bold 1963). The microplate was placed in a Q-Cell 200 incubator (PolLab, Wilkowice, Poland) at 20°C with a continuous light of 20–30 μmol m^{-2} s^{-1} intensity. Growth of microalgae was observed using an inverted phase contrast Nikon Diaphot 200 microscope (Nikon Corp., Tokyo, Japan) and serial dilution was used to separate different species. The same procedure was carried out numerous times to obtain the monoclonal strain of the targeted organism. Finally, the isolate

*To whom correspondence should be addressed.
Email: dovile.barcyte@gmail.com
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was transferred to an Erlenmeyer flask and cultivated under the aforementioned conditions. To test the acidotolerant nature of the alga, the strain CAUP H 8901-CRYO was cultivated in BBM and Allen's *Cyanidium* medium (Allen 1959; Watanabe *et al*. 2000) with pH adjusted to 2.6 by H_2SO_4.

The strain CAUP H 8901-CRYO was studied under an Olympus BX43 light microscope (LM) (Olympus Corp., Tokyo, Japan). Microphotographs were taken using an Olympus DP27 digital camera. The Olympus micro imaging software cellSens v1.15 was used to obtain morphometric measurements of the alga. Transmission electron microscopy (TEM) was performed as described in Barcytė *et al*. (2017).

Genomic DNA was extracted and polymerase chain reactions (PCRs) were done as previously described (Barcytė *et al*. 2017). 18S-ITS1-5.8S-ITS2 rDNA region was amplified using primer pairs NS1 (White *et al*. 1990)/18L (Hamby *et al*. 1988), AL1500af (Helms *et al*. 2001)/LR3 (Vilgalys & Hester 1990) and nr-SSU-1780-5'Algal/nr-LSU-0012-3'Algal (Piercey-Normore & Depriest 2001). A partial fragment of a ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbc*L) was amplified with primers rbcL1F and rbcL23R (Hoham *et al*. 2002). PCR products were purified with ethanol and sequenced by Macrogen (Amsterdam, the Netherlands) in both directions with the same primers. Internal primers 1122F (5′-GGC TGA AAC TTA AAG GAA TTG-3′) and 1122R (5′-CAA TTC CTT TAA GTT TCA GCC-3′) (T. Friedl, unpublished) were also applied for the 18S rDNA sequencing.

Two separate multiple sequence alignments were computed and best-fit models of nucleotide substitution were evaluated in the same manner as published in Barcytė *et al*. (2017). A maximum-likelihood (ML) phylogenetic analysis for the 18S rDNA alignment (GTR+Γ+I model) was computed in the software RAxML 7.0.4 (Stamatakis *et al*. 2008). The ML phylogenetic analysis for the *rbc*L dataset was conducted using the program Garli 2.01 (Zwickl 2006) under the three nucleotide substitution models (GTR+Γ for 1st codon position, JC+I for 2nd codon position and GTR + Γ + I for 3rd codon position). Bayesian inference (BI) was computed in MrBayes 3.2.1 x64 (Ronquist *et al*. 2012) applying the aforementioned models corresponding to both datasets. The final trees were visualized using FigTree v1.4.2 (Rambaut 2007).

The internal transcribed spacer 2 (ITS2) sequence of the strain CAUP H 8901-CRYO was annotated using the ITS2 database (Koetschan *et al*. 2012). The ITS2 secondary structure was computed, compared and drawn as described in Barcytė *et al*. (2017).

RESULTS

Growth, morphology and ultrastructure

The investigated strain CAUP H 8901-CRYO grew well in circumneutral BBM. It also reproduced in the tested acidic conditions, however, the growth rate was considerably slower based on the direct cell counting. Two main types of coccoid cells were observed – ellipsoidal and spherical – occurring at the same time in the life cycle of the alga (Figs 1,2). The different outline of the vegetative cells resulted from a production of two types of autospores, the only reproduction form observed in the alga.

The first type of autospores included cylindrical to narrow ellipsoidal cells formed within a parental cell wall. Typically 8 to 16 such autospores were produced within a single autosporangium. The released young cells were 3.5–8.0 μm in

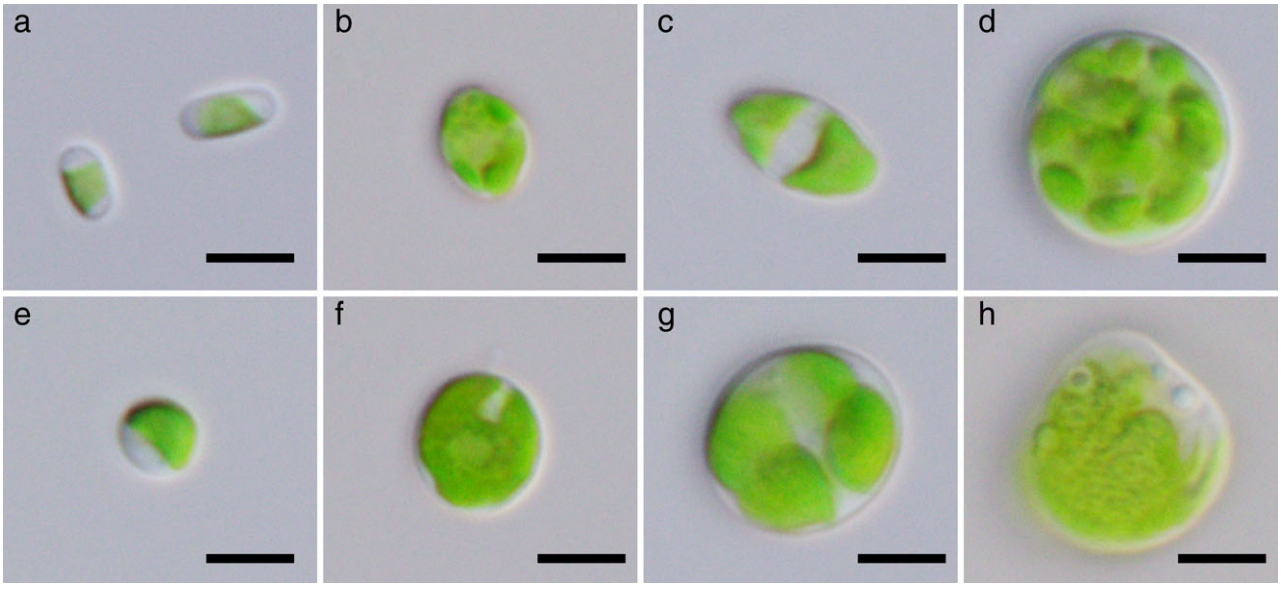

Fig. 1. Morphology of *Watanabea acidotolerans* CAUP H 8901-CRYO. (a) Young ellipsoidal cells with a trough-like chloroplast; (b) mature ellipsoidal cell with a chloroplast occupying most of its volume; (c) mature vegetative cell with a chloroplast composed of two lobes; (d) autosporangium containing ellipsoidal cells; (e) young spherical cell; (f) mature spherical cell with a cup-shaped chloroplast and a pyrenoid matrix; (g) old spherical vegetative cell with a multilobed chloroplast; (h) old spherical cell with one pointed end (pyriform shape). Scale bars = 5 μm. [Color figure can be viewed at wileyonlinelibrary.com]

length and 2.0–4.0 μm in width and had a parietal trough-like chloroplast. The ends of the autospores were rounded, both symmetrically and asymmetrically, and sometimes the cells were slightly curved (Fig. 1a). Mature cells became broad ellipsoidal and were 8.0–12.0 × 3.5–5.0 μm in size. Often, the cells were slightly pointed, causing one end to look broader than the other (a slightly pyriform shape). The chloroplast of aging vegetative cells developed into a cup-shaped with several inclusions or lobes in mature cells (Fig. 1b,c).

Accordingly, the second type of autospores encompassed nearly spherical cells produced in duplets or tetrads, occasionally in sextets. Such hatched cells were up to 5.5 μm in diameter and also had a parietal trough-like chloroplast as their ellipsoidal counterparts (Fig. 1e). The mature cells stayed subspherical, or occasionally were pointed at one pole, and their size reached 6.5–14.0 μm in diameter. Chloroplast of senescent cells became cup-shaped with deep inclusions and occupied most of the cells' volume (Fig. 1f–h).

During light microscopic observations, pyrenoids were not always obvious in the chloroplasts of the studied strain. However, the TEM revealed that the alga had single rather atypical pyrenoid that was angular in shape (Fig. 2b,c). Sometimes thylakoid lamellae entered into the pyrenoid region or crossed it. In young just produced cells, such rudimentary pyrenoids were not observed (Fig. 2a), however, they were prominent in mature cells of both forms (Fig. 2b,c). In senescent cells pyrenoid matrices became smaller and were hardly noticeable among chloroplast inclusions and lobes (Fig. 2d). Apart from chloroplast, the cells' volume was occupied by nucleus, mitochondria and vacuoles (Fig. 2c,d). A cell wall was smooth and trilaminar in ultrastructure. Remnants of parental cell walls were commonly seen in the aging cultures (Fig. 2c). Sometimes these remnants still contained one or two autospores that did not escape the parental carcass during the release of the cells. Both types of old cells contained a number of lipid droplets that were usually located in the periphery or at one pole of the cells. Noteworthy, in circumneutral BBM the exponentially growing cultures were dominated by ellipsoidal cells since more cells of this particular shape were produced. However, due to suppressed growth rate in acidic conditions the spherical cells prevailed. The reduced proliferation in acidic media was coupled with an increased production of lipid droplets.

Molecular phylogeny and ITS2 rDNA secondary structure

In 18S rDNA phylogenetic analysis, the studied strain CAUP H 8901-CRYO was nested within a highly supported (ML/BI: 100/1.00) monophyletic clade, forming a sister lineage to *Watanabea reniformis* and *W. borysthenica* (Fig. 3). The strains within the newly designed *Watanabea acidotolerans* lineage differed by 0–2 nucleotides from each other, whereas *W. acidotolerans* differed from *W. reniformis* and *W. borysthenica* by 26–34 nucleotides. The 18S rDNA phylogeny supported the monophyletic origin of the three *Watanabea*

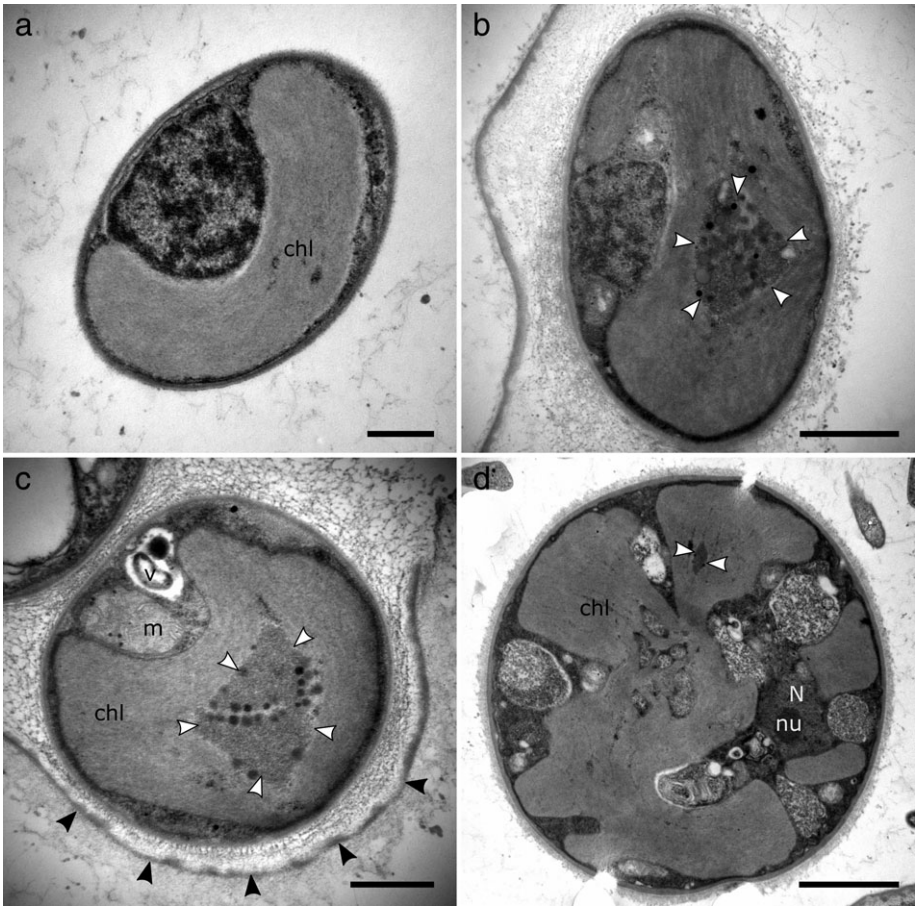

Fig. 2. Ultrastructure of *Watanabea acidotolerans* CAUP H 8901-CRYO. (a) Young ellipsoidal cell with a trough-like chloroplast; (b) mature ellipsoidal cell with a cup-shaped chloroplast containing a pyrenoid matrix (white arrowheads); (c) mature spherical cell with a cup-shaped chloroplast and a pyrenoid-like structure (white arrowheads); (d) old spherical cell with deep chloroplast inclusions. chl, chloroplast; m, mitochondrion; N, nucleus; nu, nucleolus; v, vacuole. Black arrowheads point to mother cell wall remnants. Scale bars a, c = 0.5 μm, b = 1 μm, d = 2 μm.

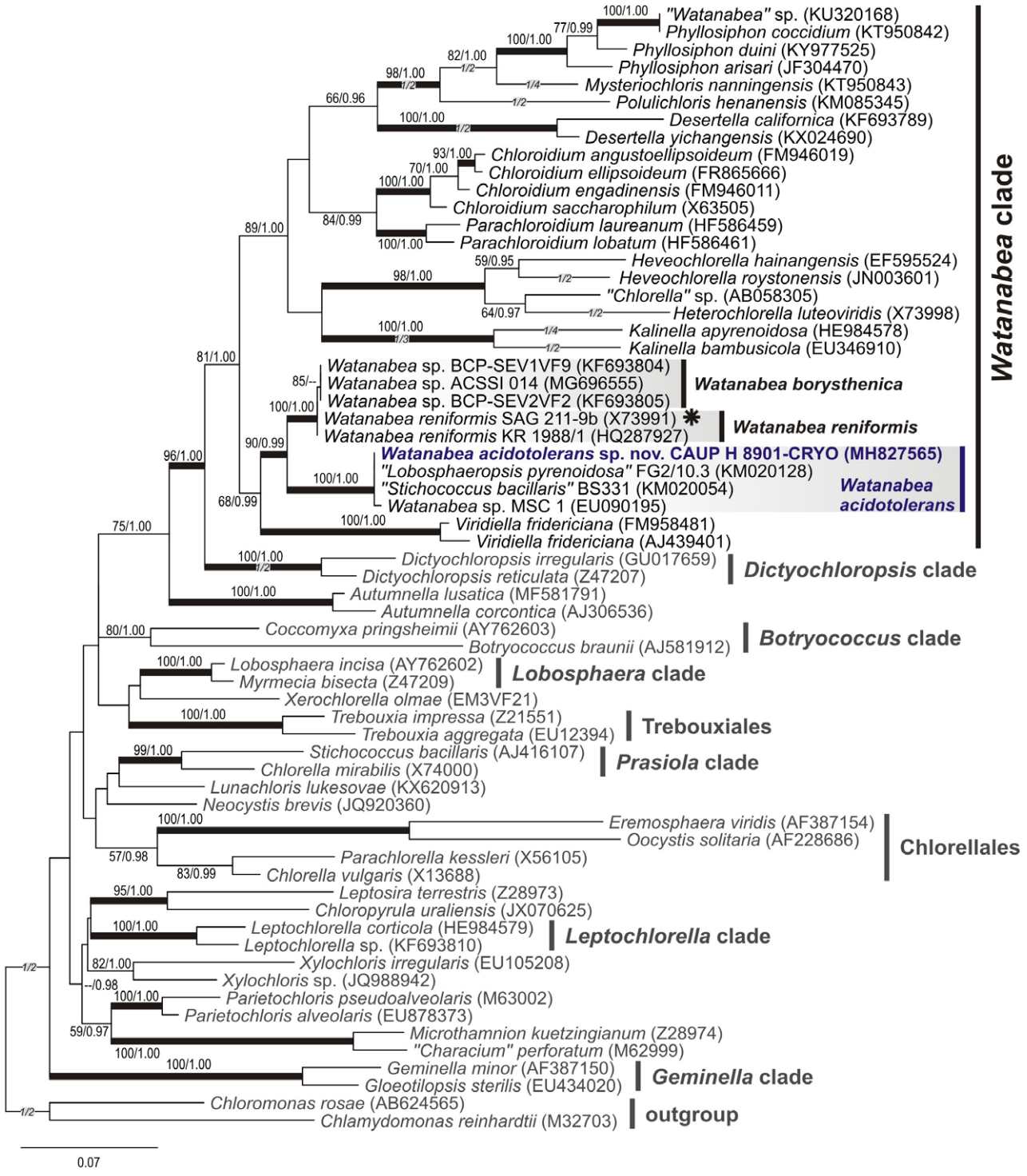

Fig. 3. Maximum-likelihood tree of the class Trebouxiophyceae based on 18S rDNA. Numbers next to branches indicate statistical support values: ML bootstraps and BI posterior probabilities. Thick lines indicate branches with statistical support: ML ≥ 90 / BI ≥ 0.95. Asterisk indicates the authentic strain of *Watanabea* type species, *W. reniformis*. [Color figure can be viewed at wileyonlinelibrary.com]

lineages (ML/BI: 90/0.99). However, the existence of *W. borysthenica* as a phylogenetic species was not supported in the Bayesian analysis (Fig. 3). The monophyly of the genus *Watanabea* was also supported by the *rbc*L phylogenetic tree (Fig. 4). Here, the presence of multiple species within the genus *Watanabea* was better revealed. For example, *Watanabea* sp. BCP-SEV2VF2 (as *W. borysthenica*) was clearly separated from *W. reniformis* SAG 211-9b (Fig. 4), while in 18S rDNA analysis the same strain had an ambiguous phylogenetic placement (Fig. 3). The *rbc*L sequence of the strain CAUP H 8901-CRYO was identical to the *Watanabea* strain CCAP 6091/1 and differed by 38 nucleotides from the

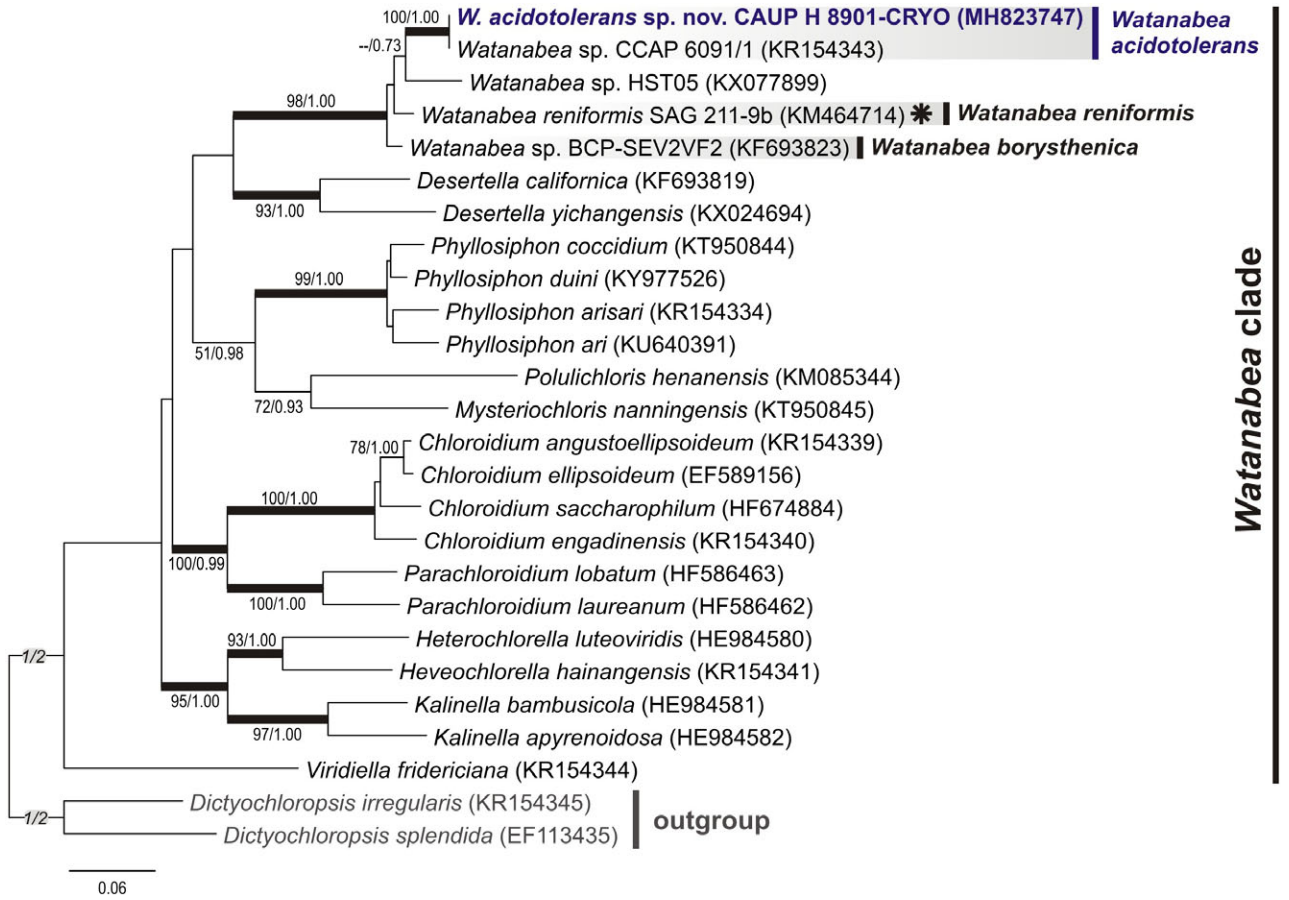

Fig. 4. Maximum-likelihood tree of the *Watanabea* clade based on the *rbc*L marker. Numbers next to branches indicate statistical support values: ML bootstraps and BI posterior probabilities. Thick lines indicate branches with statistical support: ML ≥ 90 / BI ≥ 0.95. Asterisk indicates the authentic strain of *Watanabea* type species, *W. reniformis.* [Color figure can be viewed at wileyonlinelibrary.com]

W. reniformis authentic strain. Besides, the same dataset demonstrated the existence of the possibly fourth epilithic *Watanabea* species (strain HST05).

The new strain CAUP H 8901-CRYO differed from *W. reniformis* SAG 211-9b by 33 nucleotides in the ITS2 primary sequence and by secondary structure, including one compensatory base change (CBC) in helix III and multiple single-strand hemi-CBCs in helices I-III (Fig. 5). The partial sequence of *W. borysthenica* strain ACSSI 014 (MG523285) differed from *W. acidotolerans* by 37 nucleotides and numerous indels. The CAUP H 8901-CRYO with its closest revealed relative strain BS331 shared a unique structural feature, a short side loop coiling from the helix III in the ITS2 secondary structure, what was not present in *W. reniformis* (Fig. 5). In addition, the strains differed from each other by 19 nucleotides, including one hemi-CBC in helix II and three hemi-CBCs in helix III (Fig. 5).

Formal taxonomic description

Watanabea acidotolerans Barcytė & Hodač, sp. nov.

Description. Cells solitary, coccoid of two forms, ellipsoidal and spherical/subspherical. Young ellipsoidal cells 3.5–8.0 × 2.0–4.0 μm in size; mature ellipsoidal cells 8.0–12.0 × 3.5–5.0 μm in size. Diameter of young spherical cells up to 5.5 μm; mature cells up to 14 μm. Chloroplast single, smooth, parietal and with pyrenoid matrix. In young cells the chloroplast is trough-like, while mature cells have a cup-shaped chloroplast with several inclusions or lobes. Cell wall thick. Lipid droplets present. Asexual reproduction via 2–6 spherical or 8–16 narrow ellipsoidal autospores.

Diagnosis. The species differs from the *Watanabea* type species, *W. reniformis*, in nuclear 18S-ITS1-5.8S-ITS2 rDNA (including ITS2 secondary structure) and *rbc*L sequences. A pyrenoid matrix of *W. acidotolerans* differs from *W. borysthenica* in that the former is not surrounded by starch grains.

Holotype. CAUP H 8901-CRYO, a permanently cryopreserved strain at the Culture Collection of Algae of Charles University Prague, Czechia (https://botany.natur.cuni.cz/algo/caup.html). Figure 1 shows the morphology of the authentic strain.

Type locality. 49°51′02.5″ N, 13°26′39.3″ E; Hromnice Lake (Hromnické jezírko), Czechia.

Etymology: The species name refers to the acidotolerant nature of the alga.

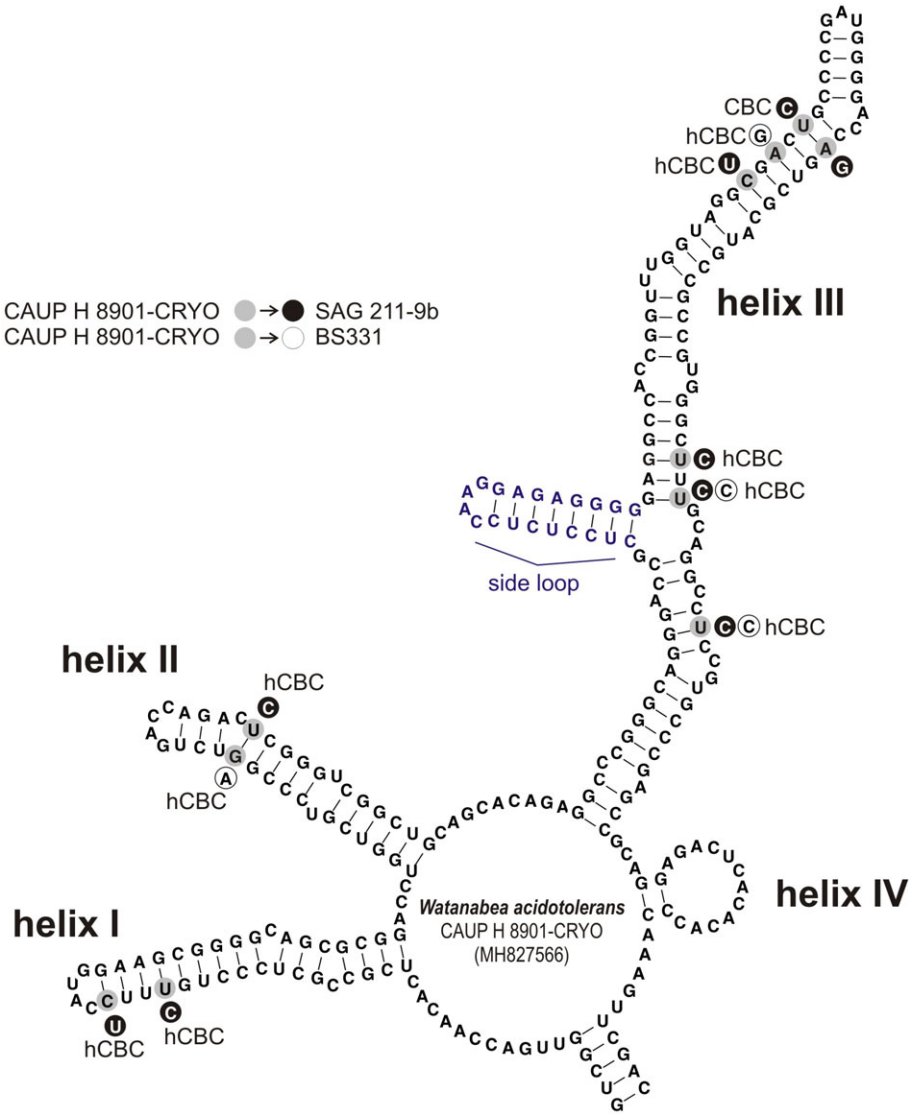

Fig. 5. Secondary structure model of the ITS2 of *Watanabea acidotolerans* CAUP H 8901-CRYO. Nucleotides highlighted in circles mark double-strand compensatory base changes (CBC) and single-strand or hemi compensatory base changes (hCBC). Grey circles highlight base changes between *W. acidotolerans* CAUP H 8901-CRYO and *W. reniformis* SAG 211-9b (black circles) and between *W. acidotolerans* CAUP H 8901-CRYO and BS331 (white circles). The side loop within the helix III is a characteristic feature of the *W. acidotolerans* lineage. [Color figure can be viewed at wileyonlinelibrary.com]

DISCUSSION

The new coccoid green alga isolated from the extremely acidic (pH 2.6) habitat matched the description of the genus *Watanabea*, including cell morphology, ultrastructure and reproduction. Molecular phylogenetic approaches confirmed its identity to the genus *Watanabea* but with considerable molecular differences from *W. reniformis* and *W. borysthenica.* Based on these differences, here we proposed a new species, *W. acidotolerans.*

In comparison to the two described species, *W. acidotolerans* is morphologically more similar to *W. reniformis* than to *W. borysthenica.* For example, *W. borysthenica* has a prominent pyrenoid surrounded by numerous small starch grains and visible under the LM (Darienko & Pröschold 2018). Whereas, the presence of the pyrenoid in *W. reniformis* (Hanagata *et al*. (1998) noted 'the chloroplast stroma, which is not occupied with thylakoid lamellae') and *W. acidotolerans* was confirmed just by the TEM.

Even though *W. acidotolerans* was isolated from the low pH habitat, the alga is not an obligate acidophile because it grew well in BBM with circumneutral pH. It is not surprising because coccoid green algae isolated from extremely acidic environments usually are capable of growing over a wide range of pH (Albertano *et al*. 1991; Fuentes *et al*. 2016). Two *Watanabea* sp. strains, MSC 1 and CCAP 6091/1, were genetically very closely related to the strain CAUP H 8901-CRYO. They were isolated from a volcanic acidic Caviahue Lake in Argentina with similar but less extreme water chemistry (Pedrozo *et al*. 2001; Beamud *et al*. 2010). Both Lake Caviahue and Lake Hromnice exhibit high concentrations of phosphorus (P), which is unique since volcanic lakes along with pit lakes usually are oligotrophic systems. We speculate that the presence of nutrients probably overcompensates the unfavorable acidic conditions for the majority of coccoid green algae and species that are not necessarily fully adapted for life in acidic environments may commonly be found there (e.g., Barcytė & Nedbalová 2017). *Watanabea acidotolerans* was found as an epilithic species in Hromnice Lake and has never appeared in

phytoplankton (Hrdinka *et al*. 2013; Barcytė & Nedbalová 2017), while in Caviahue Lake the alga was reported as a planktic species (Beamud *et al*. 2010). This shows that the species is capable of adapting its life strategy to better fit in the extreme environment of acidic lakes. In addition, Beamud *et al*. (2010) experimentally showed that low availability of NO_3 probably prevents the alga from becoming a dominant primary producer in the Caviahue Lake. However, our simple growth assay suggested that the acidic conditions could be the main restricting factor. Beamud *et al*. (2010) noted that *Watanabea* from Caviahue Lake is 'likely to be classified as a new species, *Watanabea 'caviahuensis*'. However, apart from this brief statement, no formal species description has been provided. In addition, considering the fact that *W. acidotolerans* was detected in two geographically distant lakes, we predict its worldwide distribution; *W. acidotolerans* may be rediscovered in other P-rich acidic freshwaters.

Our phylogenetic analysis supported the phylogenetic independency of *W. acidotolerans* from *W. reniformis* and *W. borysthenica.* However, *rbc*L phylogenetic analysis revealed that there might be additional unresolved species within the genus *Watanabea.* The newly proposed *W. acidotolerans* lineage contained two misidentified strains, i.e. BS331 as *Stichococcus bacillaris* and FG2/10.3 as *Lobosphaeropsis pyrenoidosa* (Fig. 3). Both species are a part of the class Trebouxiophyceae, however, the genus *Stichococcus* Nägeli belongs to the Prasiola clade, while *Lobosphaeropsis* Reisigl is the part of Chlorellales (Fig. 3). The strains CAUP H 8901-CRYO and BS331 were identical in their 18S rDNA, but they exhibited multiple nucleotide substitutions in the ITS2 rDNA. Nevertheless, the conserved ITS2 secondary structure common to both strains supported their conspecificity.

Apart from *Watanabea*, the production of morphologically different autospores has also been reported for the genus *Elliptochloris* Tschermak-Woess. Young ellipsoidal cells of *W. acidotolerans* indeed resemble *Stichococcus* in its cell shape, while chloroplast morphology also shows resemblance to that of *Coccomyxa* Schmidle. Furthermore, mature ellipsoidal cells look similar to *Chloroidium* Nadson, the closely related genus within the *Watanabea* clade. In addition, the spherical cells may also be misidentified for a different species. For example, especially mature spherical cells of *W. acidotolerans* resemble *Myrmecia* Printz or *Lobosphaeropsis* in terms of having a lobed chloroplast and a pyrenoid in the latter genus. Such algal 'mimicry' may thus result in a low number of reports of the genus *Watanabea* since it may be mistaken with other trebouxiophycean taxa mentioned above.

Members of the *Watanabea* clade occupying different ecological niches illustrate how phylogenetically closely related and morphologically similar taxa evolve by specializing to different substrata or physicochemical conditions. Even within *Watanabea* genus itself different phylogenetic lineages represent different life strategies, ranging from terrestrial edaphic and epilithic to aquatic, including extremely acidic waters. Thus, phylogeny and ecology rather than morphology should probably be considered as a primary method for species determination within the *Watanabea* genus and the clade.

ACKNOWLEDGMENT

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Paper III

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DB obtained and analyzed the data, and wrote the paper, LN helped with the sampling and read the draft of the paper, AC, FK and JJ discovered the site and wrote the section "Sampling site"

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Burning coal spoil heaps as a new habitat for the extremophilic red alga *Galdieria sulphuraria*

Dovilė Barcytė[1,*], Linda Nedbalová[1], Adam Culka[2], Filip Košek[2] & Jan Jehlička[2]

[1]*Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2, 128 44, Czech Republic; *Corresponding author e–mail: Dovilė Barcytė, dovile.barcyte@gmail.com*
[2]*Institute of Geochemistry, Mineralogy and Mineral Resources, Faculty of Science, Charles University, Albertov 6, Prague 2, 128 43, Czech Republic*

Abstract: *Galdieria sulphuraria* (Cyanidiales) is a worldwide acclaimed thermoacidophilic red microalga with a limited distribution due to special conditions required for growth and metabolism. Until now, the alga was almost exclusively restricted to acid geothermal environments around the world. However, we have found this species on the surface of a burning coal spoil heap in central Europe. It is the first record of *G. sulphuraria* in this type of habitat. A *rbc*L phylogeny confirmed that the population of this extremophile belongs to the continental European lineage and we consider Italian geothermal sites as a potential source of Czech *G. sulphuraria*. The dispersal of unicellular red microalgae is far from fully understood and the discovery of *Galdieria* in another region of Europe on a relatively newly established anthropogenic site allows us to understand better the distribution patterns and dispersal abilities of this ecologically important algal group. In addition, we have also analyzed the phylogenetic position of *Galdieria* strain CCALA 965 isolated from a highly acidic site without geothermal activity in the Czech Republic and confirmed it to belong to the species *G. phlegrea*, until now known only from Italy.

Keywords: calmodulin, dispersal, ecology, *Galdieria*, *rbc*L, thermoacidophilic algae

Introduction

Unicellular red alga *Galdieria* (Cyanidiales) is a worldwide known extremophile that thrives in acidic (pH 0.5–4) and often high temperature (up to 56 ºC) environments, which are usually associated with geothermally active regions. For example, it is well known from Italy (Gross et al. 1998; Ciniglia et al. 2004; Yoon et al. 2006; Pinto et al. 2007), Yellowstone National Park, USA (Ferris et al. 2005; Skorupa et al. 2013), New Zealand (Toplin et al. 2008) and Iceland (Ciniglia et al. 2014). Rocks and sediments surrounding hot sulphur springs, steaming fumaroles or boiling mud pools are typical habitats for this extremophile (Gross et al. 1998; Gross & Gross 2002; Ciniglia et al. 2004, 2014; Ferris et al. 2005; Yoon et al. 2006; Pinto et al. 2007; Toplin et al. 2008; Skorupa et al. 2013; Hsieh et al. 2015). Although *Galdieria* is found all over the world, its distibution is discontinuous due to special growth and habitat requirements. Therefore, it serves as a good model organism to study dispersal patterns in microorganisms.

Based on molecular phylogenetics, three well supported *Galdieria* species exist: *G. maxima* Sentsova, *G. sulphuraria* (Galdieri) Merola (including two Russian species *G. partita* Sentsova and *G. daedala* Sentsova, described based on morphological differences) and *G. phlegrea* Pinto, Ciniglia, Cascone et Pollio. Contrary to the cosmopolitan species *G. maxima* and *G. sulphuraria*, *G. phlegrea* is known only from Italy where it was found to thrive in highly acidic non–thermophilic sites (Ciniglia et al. 2004, Pinto et al. 2007). Interestingly, before the discovery of the Italian strains of *G. phlegrea*, Gross et al. (2002) also described a non–thermophilic *Galdieria* isolated from a diatomite shield in the National Nature Reserve Soos, Czech Republic (Fig. S1). The phylogenetic analysis of the partial 18S rRNA gene sequence showed that the strain was distantly related to other *Galdieria* species and indicated a possibly new *Galdieria* species from a very acid but non–volcanic habitat (Gross et al. 2002).

Extremely acid environments can originate due to oxidation of sulphide minerals and subsequent formation of sulphuric acid. This can be considered as a natural weathering process, and discharge of acid from mineralized rock complexes containing sulphides or coals is of little importance. However, mining

activities accelerate the rate of oxidation reactions as large masses of sulphide minerals are exposed to the atmosphere. Moreover, oxidation of coal releases heat, which is further amplified by other exothermic reactions, e.g., oxidation of pyrite (FeS_2) which is commonly finely dispersed or accumulated in sedimentary rocks or coal mass. Temperature rises when heat dissipation is insufficient, and instead it accumulates until the ignition point of 300 °C is reached. However, the process of spontaneous combustion is highly complex and it is driven by a number of internal and external factors (KIM 2011). The combustion or thermal decomposition without access of air at temperature up to 1000 °C generates fumes of various composition but they commonly contain H_2O, CO, SO_2, H_2S or NH_3 (TVRDÝ & SEJKORA 1999). Gaseous substances react with each other or with rock substrate to give rise to new mineral phases, mostly soluble sulphates but rarely also uncommon organic minerals (ŽÁČEK & SKÁLA 2015). SO_2 and H_2S are combined with atmospheric oxygen and water to form sulphuric acid which is together with weathering of pyrite the main cause of acid water drainage occurring on burning coal heaps (MATÝSEK & RACLAVSKÁ 1999). Moreover, relatively low–temperature vents through which heat and fumes are released, create unusual habitats which, surprisingly, can be accommodated by acid and heat–loving organisms (KOMÁREK & ROSA 1957).

Coal has been mined for over 200 years in central Europe. The extensive mining activities in Ostrava–Karviná Coal Mining District situated in the upper Silesia started in 1776 and lasted till 1992 when the first shutdowns of underground mines began. The region was the most important resource of bituminous coal in the country. Approximately 1.6 billion tonnes of hard coal was mined and almost 0.65 billion tones of waste rock was extracted during the whole existance of a coalfield. Majority of such waste rock was removed from the site, however, ~ 35% has been deposited in spoil heaps (MARTINEC & SCHEJBALOVÁ 2004). The spoil heap Heřmanice (Fig. 1) situated in the northeastern part of the city of Ostrava (Fig. S1) is among the largest spoil heaps within the Ostrava–Karviná Coal District and is one of the last remaining burning coal spoil heaps in Europe.

We have investigated Heřmanice spoil heap for possible traces of extremophile colonization and we have found *Galdieria*–like alga occupying this unusual habitat. The aim of this study was to confirm the occurrence of *Galdieria* in the Czech Republic using molecular tools and to report a new habitat suitable for the alga. For comparison, we have also studied *Galdieria* strain CCALA 965 isolated from a natural highly acidic non–thermophilic site in the Czech Republic. Based on this comparison, we discuss the ecology, distribution and dispersal of the two *Galdieria* species.

MATERIAL AND METHODS

Sampling site. Heřmanice spoil heap (Fig. 1) was created in 1838 within the first coal mining works in the area. Most of the spoil is derived from the exploitation of Heřmanice mine that was in operation from 1942 to 1993. The volume of deposited material reaches more than 17 million m^3 with the total covered area of 110 ha. The highest point of the heap stands 60 m above ground level with an average height 30 m of the whole heap. The first signs of thermal activity were already observed during the 1950s and 1960s. The current underground fire episode probably began after 1995 and it affected the whole southeast part of the heap. Therefore, the extensive remediation of the area was initiated in 2005 by state authorities.

From the geological point of view, the studied locality lies in the NW part of Upper–Silesian Basin which is filled with clastic Upper Carboniferous sediments along with bituminous coal seams. The exploited coal seams stratigraphically belong to the Ostrava Unit which is a succession of periodically repeated fine to coarse–grained sandstone, siltstone, claystone and coal seams. Therefore, the body of Heřmanice heap consists of considerably heterogeneous material varying in grain size. Siltstones to claystones are predominant, sandstone is less frequent. Primary minerals of unaltered waste rocks and coal include clay minerals, quartz, pyrite and carbonates. In thermally active zones secondary conglomerates and porcelanites may occur. The percentage of the residual combustible material in the form of coal fragments or coal mass particles dispersed in associated rocks varies and most frequently ranges from 12 to 20% (JELÍNEK et al. 2015).

Currently, the site with the most visible effect of underground fires and active fume vents is located on the top of the heap and near slopes (GPS N49°52'01.52", E18°18'59.19") (Fig. 1). Here, current studies focus on detection of new occurrences of unusual minerals (sulphates, organic minerals) and their spectroscopic properties (KOŠEK, unpublished). Very fine–grained coaly material occurs ubiquitous on the surface of the heap and also in the porous spaces of the superficial neoformed sediments. Black clayey matter covers the surface of the heap and rare coarser fragments of shales, graywackes or bituminous coal. Clays represent the most common minerals in the superficial layer (20 cm) of the heap with occasional fragments of charcoal formed by underground heating.

The surface temperature of fume channels ranges between 200 and 500 °C in the center of the fumarole zone. The measurements were carried out by a non–contact infrared thermometer (OS423–LS, OMEGA Engineering). Here trees were removed to prevent the spread of the fire on the surface of the heap. Gypsum has been detected in association with low temperature vents and relict remnants of trees. Sulphur, sal ammoniac (NH_4Cl), mascagnite ($(NH_4)_2SO_4$) and a number of NH_4–, Fe–, Al– and Mg–sulphates were found occasionally as a result of the fume activity. With increasing distance from the fumarole zone center, the temperature of fumes decreases and occurrence of vents is mostly limited to relict remnants of trees. Altered residual birch stumps and remnants of the external ex–woody parts of the trees occur now as humified and carbonified blackish relics. They are mostly friable, moist at the contact with water vapor coming from the subsurface vent.

***Galdieria* strains.** We have found and sampled biomass of

Galdieria from five independent fuming vents located a few meters away from each other (Table S1) in April 2016, while the first biomass from the locality was already brought and freezed in April 2015. The surface temperature of a single vent was measured with a glass thermometer and varied in the range of 50–55 ºC. The alga was growing only on organic material, i.e. tree stumps, bark or branches (Fig. 2). The biomass was easily scratched from a surface with a spatula or a pipette to a collection tube or a whole piece of wood was transferred to a zip plastic bag. The pH of the substrate was measured by soaking wood particles in distilled water and measuring the pH of the solution with a laboratory pH meter (WTW inoLab pH 720, Germany). The results ranged from 2 to 3. Similar pH values were also obtained from a soil extract. Using a small amount of inoculum from the field material, we have established four independent cultures (each from a different spot sampled in 2016) which were grown in a modified Allen's *Cyanidium* medium (Allen 1959; Watanabe et al. 2000) on heating mats TRIXIE (Heimtierbedarf GmbH & Co, Jarplund–Weding, Germany) illuminated with an Exo Terra (Rolf C. Hagen Inc.) porcelain clamp lamp with a glow reflector containing an Exo Terra Intense Basking Spot 100 W bulb. The temperature of the cultivation space varied from 35 to 45 ºC and pH of the media was adjusted to 2.5.

One more *Galdieria* strain isolated from the Czech Republic and preserved at Culture Collection of Autotrophic Organisms (CCALA) under number 965 was also included in this study. The strain was isolated from a mud pool discharging CO_2 in the Soos National Nature Reserve situated in the western part of the Czech Republic not far from town Františkovy Lázně (Fig. S1). A detailed physiological characteristic of this strain was provided by Gross et al. (2002). We cultivated this strain in the aforementioned medium and conditions as well as in Q–Cell incubator (PolLab, Bielsko–Biała, Poland) at 20 ºC.

Morphological characterization. Cell morphology of *Galdieria* strains was investigated using both a Nikon Eclipse E400 light microscope (Nikon Inc., Tokyo, Japan) and Zeiss LSM 880 laser scanning confocal microscope (Zeiss, Jena, Germany) equipped with an Argon laser. We used a 488 nm excitation line collecting emitted light between 499–597 and 611–759 nm. The images were visualized using ImageJ 1.50. For transmission electron microscopy (TEM), *Galdieria* strain from Ostrava was fixed for 24h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and postfixed in 2% OsO_4 in the same buffer. Fixed cells were dehydrated in a graded ethanol series (35%, 50%, 70%, 80%, 96%, 100% for 15 min), transferred to acetone (3×100% for 15 min) and finally embedded in Araldite–Poly/Bed® 812 mixture (Polysciences Inc., Hirschberg an der Bergstraße, Germany). Ultrathin sections were cut on a Reichert–Jung Ultracut E ultramicrotome (Wetzlar, Germany) and stained using uranyl acetate and lead citrate. Sections were examined using a JEOL JEM–1011 electron microscope (JEOL Ltd., Tokyo, Japan). Photomicrographs were obtained using a Veleta CCD camera (EMSIS GmbH, Münster, Germany) equipped with image analysis software Olympus Soft Imaging Solution GmbH (Münster, Germany) and later modified by Inkscape 0.91 (Free Software Foundation Inc., Boston, USA).

DNA isolation and sequencing. Total genomic DNA was extracted from two environmental Ostrava samples (2015 and 2016), four established *Galdieria* cultures and strain CCALA 965. Algal cells were disrupted using glass beads and DNA was isolated with DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). PCR was done using PPP Master Mix (Top–Bio s.r.o., Prague, Czech Republic) in a total volume of 25 µl in a thermocycler GeneTouch (BioER, Hangzhou, China). Ribulose–1,5–bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene was amplified with primers rC475F

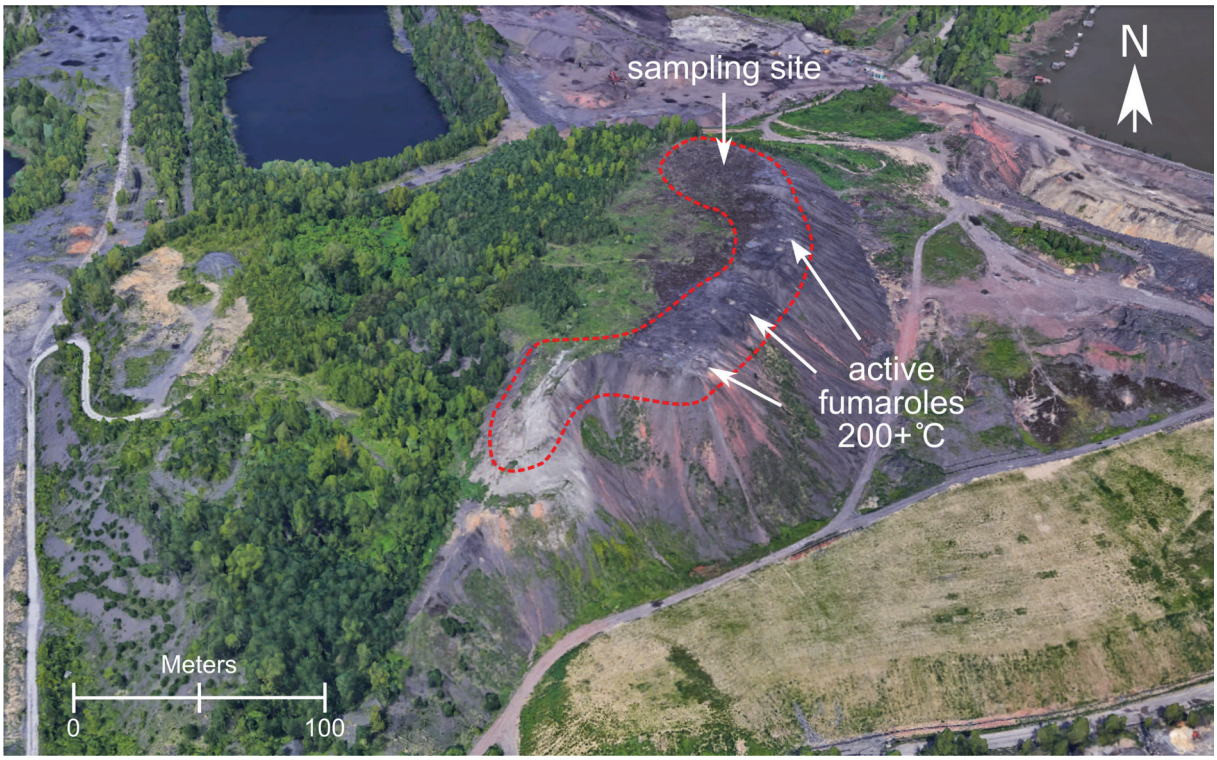

Fig. 1. Sampling site: Heřmanice spoil heap in Ostrava, Czech Republic.

Fig. 2. Sampling site: coal is still burning under the ground (a, b). *Galdiera* was found growing on the tree residuals (c, d, e) where hot fumes were coming out. Image (f) shows *Galdieria* cells from the field sample. Scale bar 20 µm.

and rCR (Ciniglia et al. 2004). A partial fragment of calmodulin gene (*CaM*) of environmental Ostrava sample 2015 and strain CCALA 965 was amplified using primers Calmo 140F and Calmo 510R (Yoon et al. 2006). The PCR products were purified with ethanol and sent to Macrogen (Amsterdam, the Netherlands) for sequencing or cloning and sequencing.

Phylogenetic analyses. The *rbc*L and *CaM* gene sequences were aligned using MAFFT v7 (Katoh & Standley 2013) available online (http://mafft.cbrc.jp/alignment/software/) and refined manually. The best–fit nucleotide substitution model was estimated with Modeltest 3.7 (Posada & Crandall 1998) in a conjunction with a program PAUP* 4.0b10 (Swofford 2003). General time reversible model with a proportion of invariable sites and a gamma–shaped distribution of rates across sites (GTR+I+G) was selected as the best–fit for the *rbc*L dataset based on Akaike information criterion (AIC). For Bayesian inference (BI) the program MrBayes (Huelsenbeck & Ronquist 2001) was used. Two MCMC runs for one million generations each with one cold and three heated chains were conducted with trees sampled every 100 generations. Maximum likelihood (ML) analysis was conducted in MEGA7 (Kumar et al. 2016) with 1000 bootstrap replicates using Nearest–Neighbor–Interchange

(NNI) heuristic method. Maximum parsimony (MP) was computed using the same software and the same number of bootstrap replicates with Subtree–Pruning–Regrafting (SPR) search method. All sites of the alignment were used in the analyses.

For the *CaM* data set of *G. sulphuraria* Hasegawa–Kishino–Yano (HKY)+I+G model was selected as the best–fit by hierarchical likelihood ratio tests (hLRTs) while AIC proposed GTR+I+G model. However, we have choosen to use HKY model to best match the previuous analysis done by Yoon et al. (2006). For *G. phlegrea*, we used HKY+G model as it was proposed by AIC. The models were applied both in ML and BI analyses. One million generations with trees sampled every 100 generations were performed for each Bayesian analysis. Bootstrap analyses with 1000 replicates were conducted with ML and MP statistical methods in MEGA7 with the use of all sites in *G. sulphuraria* case and with partial deletion of missing data in *G. phlegrea* case and SPR search method was used.

Phylogenetic trees were visusalised with FigTree v1.4.2 (Rambaut 2014) and final graphical editing was done in Inkscape v0.91.

Results

Growth and morphology

We succeeded to set up a suitable cultivation unit for *Galdieria* and sustain the growth of the new isolates. Having an optimal growth at about 35 ºC (Gross et al. 2002), the strain CCALA 965 showed a very slow growth while placed at higher temperatures as well as in the incubator at 20 ºC.

Galdieria from Ostrava exhibited a typical Cyanidiales morphology: the cells were spherical with a thick cell wall and a blue–green pyrenoidless chloroplast occupying most of the cell volume (Figs 3a–c, 4). The TEM investigation confirmed the simple morphology of the cells (Fig. 5). Young cells had a cup–shaped chloroplast (Fig. 5a) whilst in older cells the chloroplast was multi–lobed (Fig. 4; 5b,c) and floridean starch was visible in cytosol (Fig. 5b). Strain CCALA 965 had a very similar morphology and the differences, if any, were indistinguishable using the light microscope (Fig. 3d–f).

Phylogenetic analyses

This study contained 65 *Galdieria* sequences of *rbc*L gene together with *Porphyra purpurea* (DQ418738) and *Bangia atropurpurea* (AY119770) as outgroup taxa. Genbank accession numbers of all *Galdieria* sequences included in this study are listed in Table S2. The alignment included 964 bp. The analyzed sequences of *Galdieria* were primarily divided into two strongly supported clades which correspond to two diffrent *Galdieria* species: *G. sulphuraria* and *G. phlegrea*. *G. sulphuraria* encompassed sequences from different parts of the world and clustered into seven main and distinct lineages based on their geographical origin (Fig. 6). Other trees conducted by BI and MP analyses were of a very similar topology except that in MP analysis the sequence DBV 135 known from Mexico was placed between North American and Icelandic lineages. All six sequences (4 strains + 2 environmental samples) from Ostrava coal mining site were identical and fell within continental European lineage of *G. sulphuraria*. Strain CCALA 965 was found out to belong to species *G. phlegrea* (Fig. 6).

Ten *CaM* clones of Ostrava environmental sample 2015 were selected and subjected to sequencing. The size of the obtained sequences varied from 310–312 bp. The clones were aligned with the ones available from Yoon et al. (2006) (Table S2) and the final size of the alignment was 320 positions. The obtained phylogenetic tree (Fig. 7) showed that *Galdieria* from

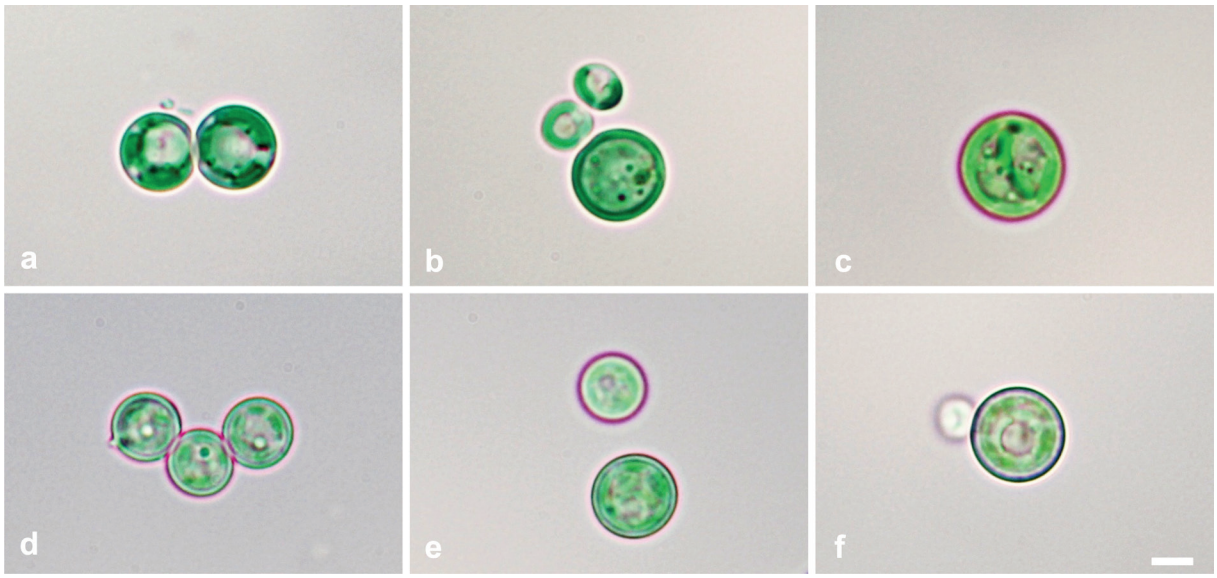

Fig. 3. Morphology of two *Galdieria* species: (a–c) *G. sulphuraria* from Ostrava, (d–f) *G. phlegrea* CCALA 965 from Soos.

Ostrava forms a distinct population rather than being intermixed with the Italian taxa. However, no bootstrap support was obtained for the whole group. Moreover, deep brantching pattern within Ostrava population was uncovered with two clear sub–groups (Fig. 7).

We also sequenced 336 bp of the partial *CaM* gene of the strain CCALA 965 and have aligned it with the sequences (385 bp in length) as used in YOON et al. (2006). Hence, we obtained the phylogenetic tree of almost identical topology (Fig. 8) as in the original paper with few mismatches of little importance. Our analyses supported three *CaM*–based lineages of *G. phlegrea* proposed by YOON et al. (2006). The strain CCALA 965 clustered within *CaM* Group A with six Pisciarelli clones and seven clones of a strain DBV 009 from Viterbo (Fig. 8).

DISCUSSION

Burning coal spoil heaps are extreme habitats in many ways. First of all, the combustion process can mobilize many elements which could be the limiting factor for biodiversity. The content of majority of trace elements in coal seams of Ostrava–Karviná Coal Mining District is considerably lower than the average mean values in hard coal, while the mean concentrations of Ag, Cs, La, Li, Pb, Sr, As and W are about by one order higher than the average mean values for bituminous coals (PEŠEK et al. 2010). Under certain conditions, potentially toxic elements can be mobilized from primary phases or coal and can be incorporated in secondary minerals on the surface. Mineral species containing potentially toxic compounds (As, Se, Pb, F) have been described from burning coal dumps around the world (LAPHAM et al. 1980; ŽÁČEK & SKÁLA 2015). However, no mineralization of this type has previously been observed, neither at Heřmanice dump nor at other burning dumps in this mining district. Therefore, the amount of mobilized toxic elements available for microorganisms in Heřmanice spoil heap can be considered as low and originate mainly from coaly matter. As a result, burning coal spoil heaps can create suitable environment for the thermoacidophilic organisms like *Galdieria sulphuraria*. For this reason, post–mining sites are important anthropogenic habitats by offering secondary habitats for various organisms, including microalgae. Microbiological studies of these sites have mainly focused on acidophilic bacteria (reviewed in KIRBY et al. 2010) as they are the most numerous organisms there. However, in extremely acid and hot mining areas well–adapted Cyanidiales could prevail. Moreover, *G. sulphuraria* has already been reported from a mining area, i.e. Rio Tinto river contaminated by acid mine drainage in southwestern Spain (MOREIRA et al. 1994; GROSS & GROSS 2001). However, until now, there have been no reports of Cyanidiales from burning coal spoil heaps.

Interestingly, *Galdieria* is usually found growing in endolithic or interlithic habitats (GROSS et al. 1998; CINIGLIA et al. 2004; YOON et al. 2006; PINTO et al. 2007), however, in Ostrava site we have found the entire rich population living on the soft substrates as are the residuals of trees. First of all, organic matter can hold water which is crucial for survival and which may be limiting in low water holding capacity having soils of the spoil heaps, especially when the coal is still burning underneath the surface. We have visited the sampling site a day after rain and could easily spot the flourishing *Galdieria* on every vent through which fumes were coming out. Secondly, *G. sulphuraria* is capable

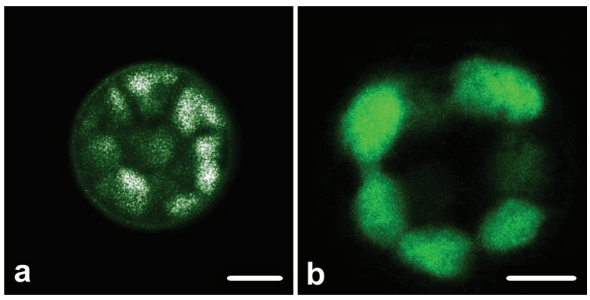

Fig. 4. Multi–lobed chloroplast of *G. sulphuraria* from Ostrava. Scale bars 3 µm.

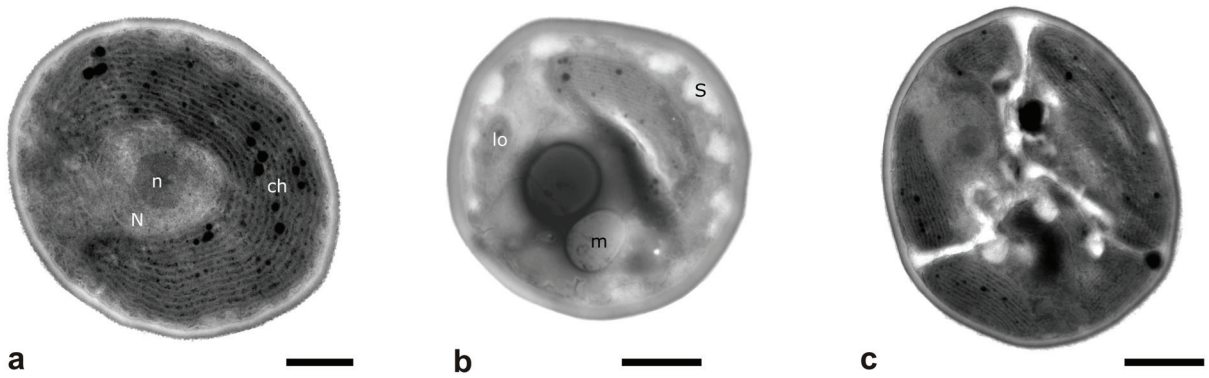

Fig 5. TEM micrographs of Ostrava strain. (a) young cell; (b) old cell; (c) old cell with multi–lobed chloroplast. Abbreviations: (n) nucleus, (nu) nucleolus, (ch) chloroplast, (lo) lobe, (m) mitochondrion, (s) floridean starch. Scale bars a 0.5 µm; b–c 1 µm.

to grow heterotrophically (Gross & Schnarrenberger 1995) and the direct growth on organic substrate could be beneficial for the alga.

Our phylogenetic analyses of *rbc*L gene showed that *Galdieria* from Ostrava coal mining site belongs to the cosmopolitan species *G. sulphuraria*. It is the first record of this species growing in central Europe. Our isolates were closely related to the Italian strains, together forming the continental European lineage of *G. sulphuraria*. A non–thermophilic strain of *Galdieria*, which was also found in the Czech Republic (Gross et al. 2002), herein referred to as CCALA 965, was shown to belong to the species *G. phlegrea* until now known only from Italy (Pinto et al. 2007) and this affiliation was confirmed by both *rbc*L and *CaM* phylogienies. Accordingly, we have shown that this species is not endemic to Italy and could be found elsewhere. However, we are not sure if the population of this alga is still existing in the Soos Nature Reserve, which is the original locality of CCALA 965.

Microscopic investigation of Ostrava strains did not reveal any new morphological features of the alga. Moreover, *G. sulphuraria* and *G. phlegrea* morphologically look the same and molecular analysis together with habitat preference and growth requirements are thus the primary methods for distinguishing both species. Apparently, *G. phlegrea* prefers dry endolithic sites with extremely low pH (0.5–1.5) and relatively low temperatures (25–38 ºC) (Ciniglia et al. 2004; Pinto et al. 2007), what matches the characteristics of the strain described by Gross et al. (2002). On the contrary, *G. sulphuraria* is found in the environments having a pH up to 4 and temperatures up to 56 ºC. The pH of the samples was strongly acidic (pH ≤ 3) and the average surface temperature of the fuming vents was 52 ºC. However, *Galdieria* was growing a few to a dozen centimeters above the ground (Fig. 2), so the actual temperature the alga was thriving at was arguably lower.

Fig. 6. Phylogenetic tree inferred from maximum likelihood (ML) analysis of *rbc*L gene sequences (with a hidden outgroup). Only Bayesian posterior probabilities ≥ 0.99 and bootstrap values (ML/MP) ≥ 95% are shown. Ostrava sequences from environmental samples are marked with asterisks (*2016, **2015).

Although *Galdieria* is assumed to have a low desiccation tolerance (SMITH & BROCK 1973; GROSS et al. 2002) and a long–distance transport could be fatal to the cells, its inoculum was apparently able to overcome the distance between the probable source population in Italy and the Ostrava site in a rather short period of time with anemochory or zoochory as the most probable dispersal modes (TOPLIN et al. 2008; CINIGLIA et al. 2014). For instance, GROSS et al. (2001) demonstrated that *Galdieria* from São Miguel Island (Azores) was most closely related to the strain from Southern Spain, which is the closest geographical site to the island (GROSS & GROSS 2002). Moreover, the colonization of new habitats is probably rather quick because the current state of the Ostrava site which could accommodate thermoacidophilic *Galdieria* has formed only relatively recently. Regarding propagation within the continent, dispersal in patches may also apply for this extremophile. Compared to its counterparts from Cyanidiales, *Galdieria* is the most flexible and adaptable one (reviewed in YOON et al. 2006) and could be the most sucessful to establish and sustain its populations where its survival requirements are provided. In addition to that, our study supports a biogeographical pattern of *G. sulphuraria* (TOPLIN et al. 2008; CINIGLIA et al. 2014; HSIEH et al. 2015). Moreover, here we demonstrate that it is highly likely that the Italian populations of *Galdieria* could be the potential source of this extremophile within all central Europe if only suitable habitats for this alga were established. In addition, we speculate that only a small amount of inoculum was enough to colonize Heřmanice spoil heap and develop into an independent population based on *CaM* sequences and their rather low variability in comparison with the Italian populations.

G. sulphuraria is the only eukaryotic organism forming visible biomass on the burning coal–waste heap of Heřmanice, however, other species of extremophiles might be expected there as well. For example, we have found and molecularly identified an acidophilic and thermotolerant fungus *Acidomyces acidothermus* (YAMAZAKI, TOYAMA et NAKAGIRI) HUJSLOVÁ

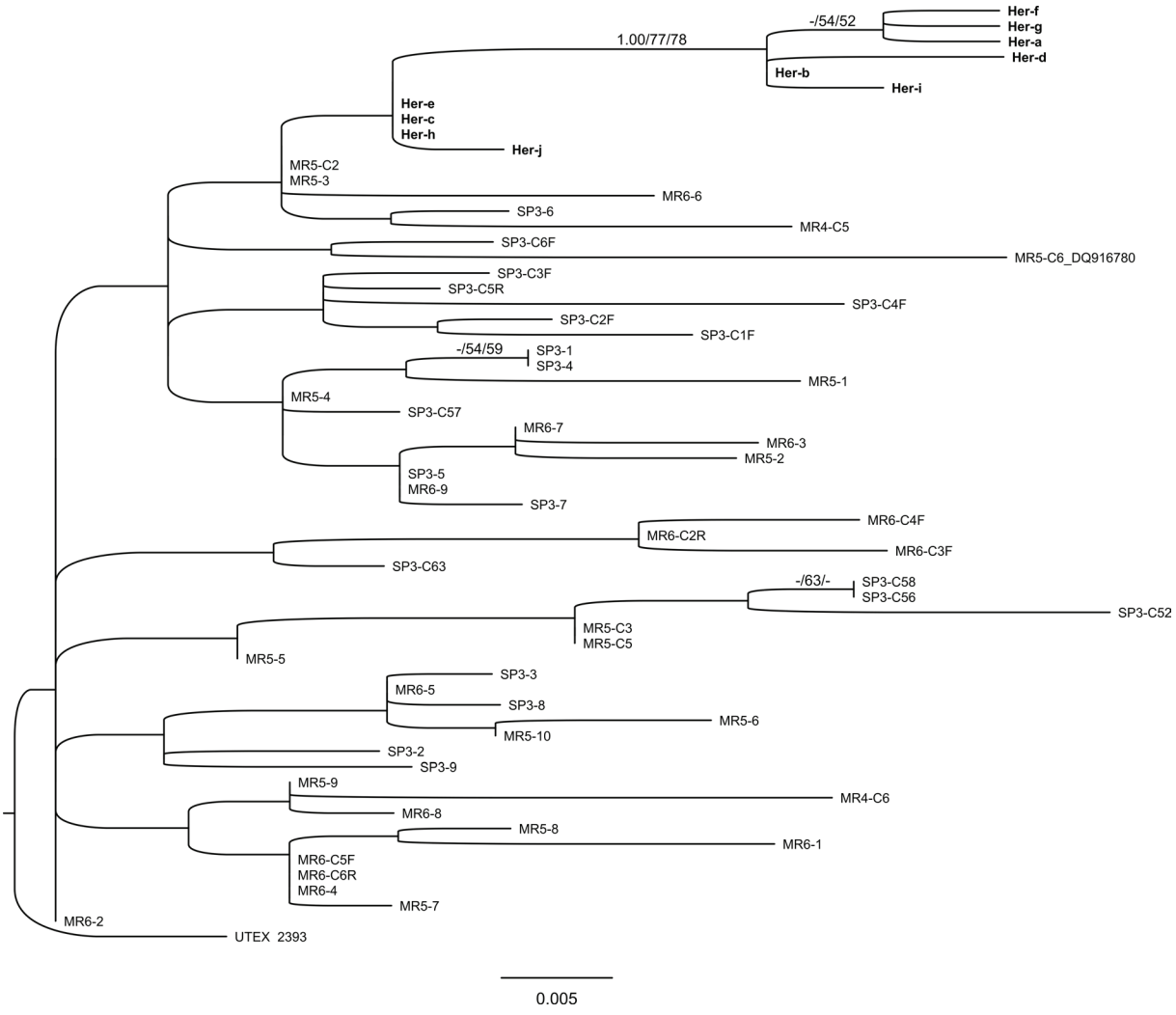

Fig. 7. Phylogenetic tree of *CaM* gene of *G. sulphuraria* inferred from ML analysis. Only Bayesian posterior probabilities ≥ 0.99 and bootstrap values (ML/MP) > 50% are shown**.** New sequences are in bold. MR refers to Monte Rotondo and SP to Sasso Pisano (Larderello, Tuscany, Italy) populations.

et Kolaříκ and acid–and–metal–tolerant green alga *Coccomyxa onubensis* Fuentes, Huss, Montero, Torronteras, Cuaresma, Garbayo et Vílchez so far known only from Rio Tinto river. On the other hand, we have also found and isolated common terrestrial algae such as *Chloroidium saccharophilum* (Krüger) Darienko, Gustavs, Mudimu, Menendez, Schumann, Karsten, Friedl et Pröschold and *Chlorococcum* sp. Meneghini from a fallen tree branch covered by warm fumes. Komárek & Rosa (1957) found cyanobacterium *Hapalosiphon* (formerly *Somerriella*) *cossyrensis* (Borzi) Komárek in the spoil heap in Sokolov region, western Bohemia, Czech Republic, in the years when the coal was burning underneath the ground. Interestingly, this species was originally described from Pantelleria Island, Italy, growing on volcanic rocks under which hot water was flowing down. Consequently, burning coal–waste heaps could accomodate a great deal of unique organisms and further investigation of the site could bring interesting results.

Regarding *G. phlegrea*, it has been assumed that the transportability and adpatability of this alga was even more difficult compared with other *Galdieria* species (Ciniglia et al. 2014). However, the limitation of habitats which could satisfy ecophysiological needs of the alga and the affiliation to certain type of habitats could play even more important driving factor of the distribution of *G. phlegrea*. Soos Nature Reserve is a unique place situated in seismic center of the Cheb Basin where magma moves towards the earth's surface (Bankwitz et al. 2003). The Reserve consits of peat bogs, mineral salt marshes and springs with huge concentrations of different mineral salts. The variety of biotops and habitats in the Reserve was determined not only by specific geological conditions but also by human interventions. Peat was extracted about 70 years ago in the southern part of the Reserve, revealing a layer of diatomaceous earth containing different sulphides, e.g., FeS_2. Due to their oxidation, sulphuric acid has accumulated creating an extremely acid environment (Gross et al. 2002). In the middle and south part of the diatomite shield polygonal soil without vegetation

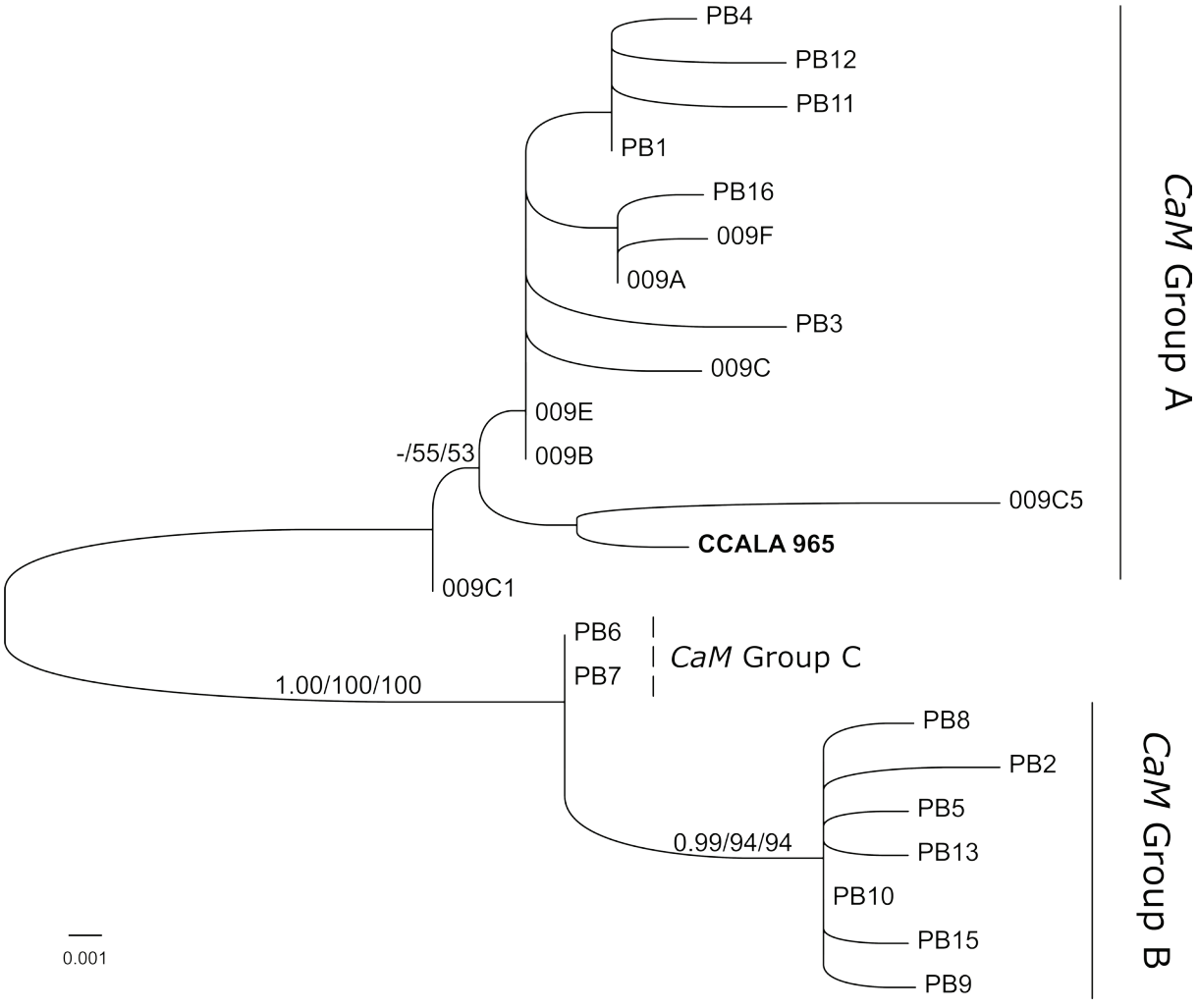

Fig. 8. Phylogenetic tree of *CaM* gene of *G. phlegrea* (CCALA 965) inferred from ML analysis. Only Bayesian posterior probabilities ≥ 0.99 and bootstrap values (ML/MP) > 50% are shown**.** PB refers to Pisciarelli population; number 009 to the strain isolated from Viterbo (Italy). See Yoon et al. (2006).

and extreme acidic pH is prevailing. In some places pH varies even from 0–2. In summer, crystallized salts, e.g., Na_2SO_4, $Na_2CO_3 \cdot H_2O$, $CaSO_4 \cdot H_2O$, NaCl, $FeSO_4$, is a common phenomenon on the surface of the soil (Hájek & Vízdal 1998). *G. phlegrea* was isolated here from a so–called mofette, which is a hole emitting volcanic CO_2. An area of approximately 100 x 80 m of the Soos Nature Reserve is occupied by mofettes, where gas originates from active magma around 30 kilometres under the earth's surface and bubbles through the mud pools. The recent study of mofettes done by Beulig et al. (2015) in the neighbouring area of Soos Nature Reserve showed that they could actually promote the soil colonization by acidophilic microorganisms as mofette soil tends to be more acidic than the adjacent ones. Unfortunately, it is very hard to make any conclusions about the actual colonization of Soos Nature Reserve by *G. phlegrea*, but strains belonging to *CaM* Group A could possibly exhibit a higher tolerance and adaptability for travelling longer distances. However, this could be confirmed or denied only if new strains of the alga were found in the future.

To sum up, in this study we report a new habitat suitable for the thermo–acidophilic red alga *G. sulphuraria* and contribute to the knowledge about yet poorly understood dispersal of Cyanidiales. We put forward that environmental suitability is the most important factor restricting the occurrence of Cyanidiales.

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Supplementary material

the following supplementary material is available for this article:

Table S1. Coordinates of the sampling points.

Table S2. List and accession numbers of sequences analyzed in this study.

Fig. S1. Map of Europe and Czech Republic showing localities where *Galdieria* was found: Soos Nature Reserve and Heřmanice (a part of the city of Ostrava).

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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Table S1. Coordinates of the sampling points.

Strain	**Coordinates**	**Year**
Heřmanice 1	N49 52.121 E18 19.011	2016
Heřmanice 2	N49 52.118 E18 19.022	2016
Heřmanice 3	N49 52.117 E18 19.024	2016
Heřmanice 4	N49 52.117 E18 19.014	2016
Heřmanice 5	N49 52.114 E18 19.012	2016
Heřmanice 6	N49 52.094 E18 19.022	2015

Table S2. List and accession numbers of sequences analyzed in this study.

Strain/clone	Named in NCBI	Species	Locality	Country	Gene	Accesion Number
009A	*Galdieria sulphuraria*	***Galdieria phlegrea***	Viterbo	Italy	*CaM*	DQ916810
009B	*Galdieria sulphuraria*	***Galdieria phlegrea***	Viterbo	Italy	*CaM*	DQ916808
009C	*Galdieria sulphuraria*	***Galdieria phlegrea***	Viterbo	Italy	*CaM*	DQ916807
009C1	*Galdieria sulphuraria*	***Galdieria phlegrea***	Viterbo	Italy	*CaM*	DQ916811
009C5	*Galdieria sulphuraria*	***Galdieria phlegrea***	Viterbo	Italy	*CaM*	DQ916812
009E	*Galdieria sulphuraria*	***Galdieria phlegrea***	Viterbo	Italy	*CaM*	DQ916806
009F	*Galdieria sulphuraria*	***Galdieria phlegrea***	Viterbo	Italy	*CaM*	DQ916809
A12	Uncultured *Cyanidiales*	***Galdieria sulphuraria***	Pisciarelli	Italy	*rbcL*	AY541313
ACUF 398	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Seltun	Iceland	*rbcL*	KC883973
ACUF 464	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Viti	Iceland	*rbcL*	KC883876
ACUF 465	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Viti	Iceland	*rbcL*	KC883877
ACUF 466	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Viti	Iceland	*rbcL*	KC883878
ACUF 467	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Viti	Iceland	*rbcL*	KC883879
ACUF 470	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Seltun	Iceland	*rbcL*	KC883882
ACUF 472	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Seltun	Iceland	*rbcL*	KC883883
ACUF 473	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Seltun	Iceland	*rbcL*	KC883884
ACUF 474	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Seltun	Iceland	*rbcL*	KC883885
ACUF 475	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Seltun	Iceland	*rbcL*	KC883886
B15	Uncultured *Cyanidiales*	***Galdieria phlegrea***	Pisciarelli	Italy	*rbcL*	AY541314
B19	Uncultured *Cyanidiales*	***Galdieria phlegrea***	Pisciarelli	Italy	*rbcL*	AY541315
B20	Uncultured *Cyanidiales*	***Galdieria phlegrea***	Pisciarelli	Italy	*rbcL*	AY541316
C1	Uncultured *Cyanidiales*	***Galdieria phlegrea***	Pisciarelli	Italy	*rbcL*	AY541317
CCALA 965	*Galdieria phlegrea*	***Galdieria phlegrea***	Soos Nature Reserve	Czech Republic	*rbcL*	MF163159
CCALA 965	*Galdieria phlegrea*	***Galdieria phlegrea***	Soos Nature Reserve	Czech Republic	*CaM*	MF163160
CCMEE 5706	*Galdieria sp.*	***Galdieria sulphuraria***	Craters of the Moon	New Zealand	*rbcL*	EF675177
CCMEE 5707	*Galdieria* sp.	***Galdieria sulphuraria***	Waiotapu	New Zealand	*rbcL*	EF675181
CCMEE 5708	*Galdieria* sp.	***Galdieria sulphuraria***	Whaka	New Zealand	*rbcL*	EF675172

Table S2 Cont.

CCMEE 5710	*Galdieria* sp.	***Galdieria sulphuraria***	White Island	New Zealand	*rbcL*	EF675183
CCMEE 5711	*Galdieria* sp.	***Galdieria sulphuraria***	White Island	New Zealand	*rbcL*	EF675173
CCMEE 5712	*Galdieria* sp.	***Galdieria sulphuraria***	Craters of the Moon	New Zealand	*rbcL*	EF675178
CCMEE 5714	*Galdieria* sp.	***Galdieria sulphuraria***	Waimangu	New Zealand	*rbcL*	EF675180
CCMEE 5717	*Galdieria* sp.	***Galdieria sulphuraria***	Rotorua	New Zealand	*rbcL*	EF675176
CCMEE 5718	*Galdieria* sp.	***Galdieria sulphuraria***	Whaka	New Zealand	*rbcL*	EF675179
CCMEE 5719	*Galdieria* sp.	***Galdieria sulphuraria***	Waiotapu	New Zealand	*rbcL*	EF675175
CHJ-4	*Cyanidiales* sp.	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	JQ269635
D15	Uncultured *Cyanidiales*	***Galdieria sulphuraria***	Pisciarelli	Italy	*rbcL*	AY541322
D5	Uncultured *Cyanidiales*	***Galdieria sulphuraria***	Pisciarelli	Italy	*rbcL*	AY541321
DBV 002	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*rbcL*	AY541311
DBV 009	*Galdieria sulphuraria*	***Galdieria phlegrea***	Viterbo	Italy	*rbcL*	AY119768
DBV 011	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Caserta	Italy	*rbcL*	AY541303
DBV 012	*Galdieria sulphuraria*	***Galdieria phlegrea***	Benevento	Italy	*rbcL*	AY541310
DBV 015	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Ischia-fango	Italy	*rbcL*	AY541305
DBV 017	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Solfatara	Italy	*rbcL*	AY541306
DBV 018	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Scarfoglio	Italy	*rbcL*	AY541304
DBV 021	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Vulcano	Italy	*rbcL*	AY541307
DBV 063	*Galdieria sulphuraria*	***Galdieria phlegrea***	Agrigento	Italy	*rbcL*	AY119769
DBV 074	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Java	Indonesia	*rbcL*	AY541308
DBV 135	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Agua-Azul	Mexico	*rbcL*	AY541309
DS1-9	*Cyanidiales* sp.	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	JQ269631
DS2-5	*Cyanidiales* sp.	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	JQ269629
DS3-1	*Cyanidiales* sp.	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	JQ269633
DS5-4	*Cyanidiales* sp.	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	JQ269632
E11	Uncultured *Cyanidiales*	***Galdieria sulphuraria***	Pisciarelli	Italy	*rbcL*	AY541324
E12	Uncultured Cyanidiales	***Galdieria sulphuraria***	Pisciarelli	Italy	*rbcL*	AY541325
Her-a	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163161

Table S2 Cont.

Her-b	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163164
Her-c	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163165
Her-d	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163162
Her-e	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163163
Her-f	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163166
Her-g	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163167
Her-h	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163168
Her-i	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163169
Her-j	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*CaM*	MF163170
Hermanice 1	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*rbcL*	MF163153
Hermanice 2	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*rbcL*	MF163155
Hermanice 3	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*rbcL*	MF163154
Hermanice 4	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*rbcL*	MF163157
Hermanice 5	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*rbcL*	MF163156
Hermanice 6	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Heřmanice, Ostrava	Czech Republic	*rbcL*	MF163158
IPPAS P500	*Galdieria partita*	***Galdieria sulphuraria***	Kamchatka	Russia	*rbcL*	AB018008
IPPAS P508	*Galdieria daedala*	***Galdieria sulphuraria***	Kunashir	Russia	*rbcL*	AY541302
LCATERR-7	*Cyanidiales* sp.	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	JQ269608
LCBCEL-5	*Cyanidiales* sp.	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	JQ269609
LCBTERR-6	*Cyanidiales* sp.	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	JQ269612
MR4-21	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*rbcL*	DQ916745
MR4-C5	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916776
MR4-C6	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916775
MR5-1	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916781
MR5-10	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916790
MR5-2	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916783
MR5-3	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916784
MR5-4	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916785

Table S2 Cont.

MR5-5	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916786
MR5-6	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916787
MR5-7	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916788
MR5-8	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916782
MR5-9	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916789
MR5-C17	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*rbcL*	DQ916746
MR5-C2	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916777
MR5-C3	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916778
MR5-C5	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916779
MR5-C6	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916780
MR6-1	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916791
MR6-2	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916792
MR6-3	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916793
MR6-4	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916794
MR6-5	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916795
MR6-6	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916796
MR6-7	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916805
MR6-8	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916804
MR6-9	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916803
MR6-C2R	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916801
MR6-C36	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*rbcL*	DQ916747
MR6-C3F	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916798
MR6-C4F	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916799
MR6-C5F	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916797
MR6-C6R	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Monte Rotondo	Italy	*CaM*	DQ916800
PB1	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916816
PB10	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916824
PB11	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916813

Table S2 Cont.

PB12	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916815
PB13	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916822
PB15	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916825
PB16	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916814
PB2	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916821
PB3	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916818
PB4	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916817
PB5	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916819
PB6	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916826
PB7	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916827
PB8	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916820
PB9	*Galdieria sulphuraria*	***Galdieria phlegrea***	Pisciarelli	Italy	*CaM*	DQ916823
SAG 108.79	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	AY119767
SFFL-5	*Cyanidiales* sp.	***Galdieria sulphuraria***	Yellowstone National Park	USA	*rbcL*	JQ269630
SP1-10	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*rbcL*	DQ916748
SP3-1	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916761
SP3-2	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916769
SP3-3	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916771
SP3-4	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916762
SP3-5	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916774
SP3-6	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916766
SP3-7	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916760
SP3-8	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916763
SP3-9	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916755
SP3-C1F	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916757
SP3-C2	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*rbcL*	DQ916749
SP3-C2F	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916767
SP3-C3F	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916772

Table S2 Cont.

SP3-C4F	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916756
SP3-C52	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916770
SP3-C56	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916765
SP3-C57	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916759
SP3-C58	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916764
SP3-C5R	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916758
SP3-C63	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916768
SP3-C6F	*Galdieria sulphuraria*	***Galdieria sulphuraria***	Sasso Pissano	Italy	*CaM*	DQ916773
UTEX 2393	*Galdieria sulphuraria*	***Galdieria sulphuraria***	unknown	USA	*rbcL*	AF233069
UTEX 2393	*Galdieria sulphuraria*	***Galdieria sulphuraria***	-	-	*CaM*	DQ916754

Fig. S1. Map of Europe and Czech Republic showing localities where *Galdieria* was found: Soos Nature Reserve and Heřmanice (a part of the city of Ostrava).

Paper IV

BARCYTĖ D, ELSTER J & NEDBALOVÁ L (2018) Plastid-encoded *rbc*L phylogeny suggests widespread distribution of *Galdieria phlegrea* (Cyanidiophyceae, Rhodophyta). *Nordic Journal of Botany* 36: e01794

Authors' contributions:

DB obtained and analyzed the data, and wrote the paper, JE took the field sample, LN read the draft of the paper

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Research

Plastid-encoded *rbc*L phylogeny suggests widespread distribution of *Galdieria phlegrea* (Cyanidiophyceae, Rhodophyta)

Dovilė Barcytė, Josef Elster and Linda Nedbalová

D. Barcytė (https://orcid.org/0000-0002-3542-1177) (dovile.barcyte@gmail.com) and L. Nedbalová, Dept of Ecology, Faculty of Science, Charles Univ., Prague, Czechia. DB also at: Dept of Zoology, National Museum, Prague, Czechia. – LN and J. Elster, The Czech Academy of Sciences, Inst. of Botany, Třeboň, Czechia. JE also at: Centre for Polar Ecology, Faculty of Science, Univ. of South Bohemia, České Budějovice, Czechia.

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The rare microscopic red alga *Galdieria phlegrea* (Cyanidiohyceae, Rhodophyta) has been discovered in the extremely acid Tinto River in Spain and this occurrence is here related to previous knowledge about the distribution and ecology of this enigmatic alga. The taxonomic affiliation of the new isolate of *G. phlegrea* was revealed by reconstructing the phylogeny of plastid-encoded *rbc*L. According to this phylogeny, the Tinto River alga is closely related to other *G. phlegrea* strains originating from extreme habitats in Czechia, Italy and Turkey, suggesting a wider distribution and higher ecological versatility than previously thought. The results suggest that *G. phlegrea*, and then possibly also other cyanidiophycean algae, are not as restricted to strongly acidic and hot microhabitats as previously believed, which, in turn, may indicate that they may commonly have been overlooked and possibly are much more widespread than is currently believed.

Keywords: Cyanidiophyceae, distribution, ecology

Introduction

Galdieria phlegrea Pinto, Ciniglia, Cascone et Pollio (Cyanidiophyceae, Rhodophyta) is a small unicellular autosporic coccoid alga originally found in extremely acidic habitats (pH 0.5–1.5) with moderately elevated temperatures (Ciniglia et al. 2004, Pinto et al. 2007). The alga was previously exclusively known from dry endolithic habitats in volcanically active areas in Italy, but was recently confirmed to also have grown in Czechia (Barcytė et al. 2018), implying a possibly wider distribution than what has previously been assumed. Its closest relative, *G. sulphuraria* (Galdieri) Melora, has a cosmopolitan distribution (Ciniglia et al. 2014) and is restricted to low pH (0.5–4.0) and high temperature (up to 56°C) environments. The recently sequenced genomes of the two species provided evidence for that it has aquired stress-adaptations through horizontal gene transfer (HGT) from prokaryotic sources

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(Schönknecht et al. 2013, Qiu et al. 2013). In addition, *G. phlegrea* underwent lineage-specific HGTs (Qiu et al. 2013). *Galdieria sulphuraria* and *G. phlegrea* seem to be morphologically indistinguishable from each other (Pinto et al. 2007, Barcytė et al. 2018) and molecular phylogenetic analyses are crucial for correct species determination. Due to the specific ecophysiological requirements not commonly encountered in prokaryotes or eukaryotes, *G. phlegrea* is an excellent potential model species for studying microbial biogeography.

Material and methods

Sampling and culturing

The strain of *Galdieria* investigated in this study was isolated from a brown microbial mat (Fig. 1) found in a spring in Anabel's Garden, Peña del Hierro (37°43′30.97″N, 6°33′32.23″W) situated in the upper part of the acidic (pH ~ 2.5) and metal rich Tinto River, Huelva, southwestern Spain. The microbial mat was taken into a sterile plastic tube in August 2005 and frozen in liquid nitrogen. In 2017, aliquots of the defrosted sample were transferred to a six-well microplate containing modified Allen's *Cyanidium* medium (Allen 1959, Watanabe et al. 2000) with pH adjusted to 2.5 by H_2SO_4. Soon all wells became dominated by microalgae with blue-green colour typical for Cyanidiophyceae. We further transferred the aliquots of these algae to new microplates using serial dilution as the isolation mode of the organism. The temperature of the Q-Cell incubator where the cultivation took place was 20°C with light provided continuously by a cool white fluorescent tube with an intensity of 20–30 µmol m^{-2} s^{-1}. The strain DB01 was further used for algal identification. To check the thermotolerant nature of the organism, we also cultivated the same strain at 35°C as described in Barcytė at al. (2018).

Figure 1. Brown mat dominated by the rhodophyte *Galdieria phlegrea* found in Tinto River, Spain, close to the origin of the river.

DNA extraction, PCR and sequencing

The total genomic DNA of the strain DB01 was extracted using a Geneaid Plant Genomic DNA Mini Kit. A partial fragment of a ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) was amplified and sequenced with the primers rC475F and rCR, including the additional sequencing primer rC910r (Ciniglia et al. 2004). The amplification reaction was performed using the following cycle parameters: 2 min at 94°C, followed by 35 cycles of 1 min at 92°C, 1 min at 50°C and 1 min at 72°C and final elongation of 10 min at 72°C. The PCR product was purified with ethanol and sent to Macrogen (Amsterdam, the Netherlands) for sequencing. The sequence has been deposited in GenBank under accession number MG661270.

Phylogenetic analyses

For phylogenetic analyses we used the same dataset as published by Barcytė et al. (2018), including the new sequence and 24 additional sequences revealed by the BLAST algorithm (Altschul et al. 1997) as close matches to the isolate DB01. In addition, we used the outgroup taxa *Cyanidioschyzon merolae* (AY119765) and *Cyanidium caldarium* (AY541298). The alignment was done using MAFFT 7 (Katoh and Standley 2013) and afterwards refined manually. The best-fit nucleotide substitution model was estimated with Modeltest (Posada and Crandall 1998) in a conjunction with the program PAUP* (Swofford 2002). Based on the Akaike information criterion (AIC), model K81 was chosen as the best-fit for the dataset and we used this model for Bayesian inference (BI) calculated by MrBayes (Huelsenbeck and Ronquist 2001). One million generations with trees sampled every 100 generations were run until the average standard deviation of split frequencies dropped below 0.006. Hierarchical likelihood ratio tests (hLRTs) selected the TrN model as best fitting the dataset and we applied this model in a maximum likelihood (ML) analysis done in MEGA (Kumar et al. 2016) with 1000 bootstrap replicates and the nearest-neighbor-interchange (NNI) heuristic search method. Additionally, we conducted a maximum parsimony (MP) analysis using the same software with 1000 bootstraps and the subtree-pruning-regrafting (SPR) search method. All aligned 964 positions (including the missing data) were used for the phylogenetic analyses. Phylogenetic trees were visualised with FigTree (Rambaut 2014) and postprocessed with Inkscape from Free Software Foundation.

p-distance

The evolutionary divergence between the sequences of *G. phlegrea* was estimated using the uncorrected pairwise distance (*p*-distance) in MEGA ver. 7. The analysis included nine nucleotide sequences. All positions with less than 95%

coverage were eliminated resulting in a total of 735 positions in the analysed dataset.

Microscopy

The alga was examined with an Olympus BX43 light microscope and microphotographs were taken with an Olympus DP27 digital camera. The Olympus micro imaging software cellSens was used to obtain morphometric measurements.

Results

Morphology and growth

The Tinto River isolate DB01 had typical cyanidiophycean characteristics: the cells were spherical, 4.2 to 9.6 µm in diameter, with thick smooth cell walls. The average size of mature cells was 6.4 µm (n = 120; SD ± 1.0). The cells contained a blue-green chloroplast without a pyrenoid (Fig. 2A). The alga reproduced asexually by autospores produced within the parental cells which eventually became autosporangia containing 2–6 daughter cells (Fig. 2B). The released young cells usually had a single empty vacuole while senescent cells had several of them (Fig. 2A). Sometimes the cells were distorted from their usual spherical shape becoming slightly pointed at one or both ends.

The isolate DB01 showed a very slow growth in the incubator at 20°C and did not grow at 35°C, revealing that the alga is not an obligate thermophile. However, the optimal cultivation conditions for the isolate remain unclear.

Phylogenetic analyses

The bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) phylogenies were largely congruent and the main *Galdieria* clades were well supported in all the analyses (Fig. 3). The new isolate from Tinto River fell within the *G. phlegrea* clade and it was identical (924 bps) to the strain CCALA 965 isolated from a mud pool discharging CO_2 in the Soos Nature Reserve in Czechia (Barcytė et al. 2018). The same clade also accommodated sequences from Italy and Turkey (Fig. 3). The analysed strains did not group according to geography, while all methods recovered biogeographical clustering of *G. sulphuraria* as previously reported (Barcytė et al. 2018).

p-distance

The *p*-distances among analysed *G. phlegrea* strains (DB01 from Spain; DBV 002 (Pisciarelli), DBV 009 (Viterbo) and DBV 012 (Benevento) from Italy; along with ACUF642 (Diyarbakir) and ACUF664 (Bitlis) from Turkey) ranged from 0.1 to 1.2% (Table 1). Two other Turkish representatives, ACUF785 and 'clone T09', showed much higher *p*-distances (from 1.8 to 3.0%) when compared with their relatives within the *G. phlegrea* clade and 3.9% when compared to each other. The maximum difference between *G. phlegrea* and *G. sulphuraria* SAG 108.79 was 12.8% (Table 1).

Discussion

The occurrence of coccoid red algae (Cyanidophyceae) in the extremely acid Tinto River (Spain) has been reported previously (Moreira et al. 1994, Gross and Gross 2001, Gómez et al. 2011). For example, Moreira et al. (1994) studied three *Galdieria* strains isolated from two different water drainages of leaching piles (pH 1.6 and 2.5) and from under the surface of amorphous gypsum crystals of an acid soil area (pH 3) near the origin of the river. The authors demonstrated that the isolates might constitute two new *Galdieria* species other than *G. sulphuraria*. One of these potential new species was found living in both acidic water and in the endolithic habitat (Moreira et al. 1994). We speculated that one of the unknown species could have been *G. phlegrea* because our strain was also isolated from a sample taken near the river source. Gross et al. (2002) also found a new *Galdieria* species from an extremely acidic area without geothermal activity in

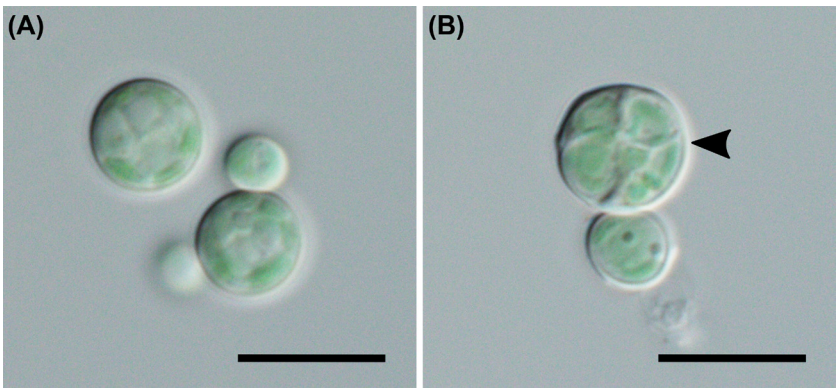

Figure 2. Light micrographs of *G. phlegrea* isolated from Tinto River. (A) vegetative cells containing several vacuoles, (B) arrowhead points to autosporangium. Scale bars represent 10 µm in both images.

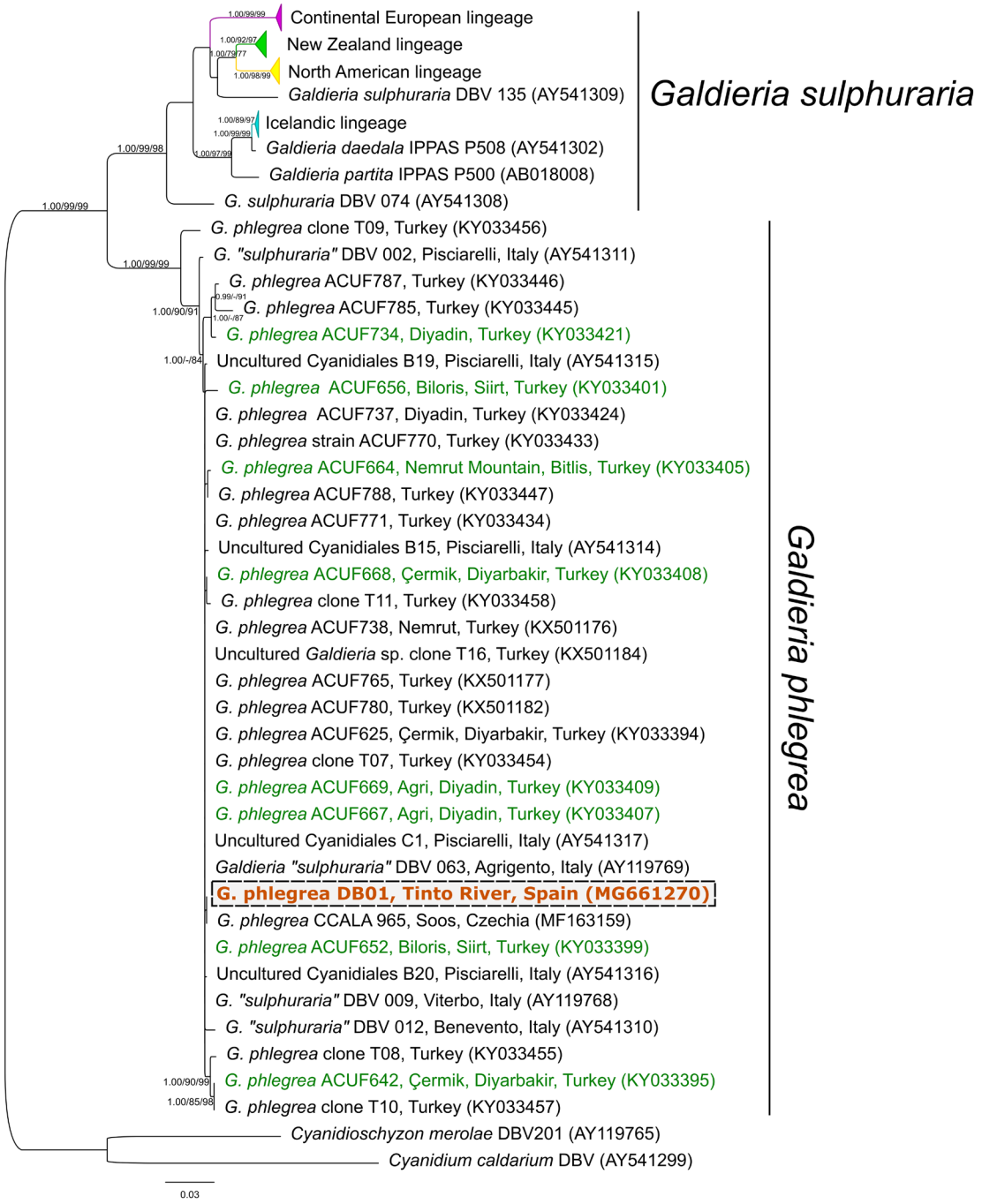

Figure 3. Phylogenetic tree inferred from maximum likelihood (ML) analysis of *rbc*L gene sequences. Only Bayesian posterior probabilities ≥ 0.99 and bootstrap values (ML/MP) ≥ 75% are shown. New sequence is in orange bold. The *G. phlegrea* dataset includes sequences from Turkey, Italy and Czechia. Sequences from neutral pH habitats are in green (data from ACUF).

Czechia, a species which was later shown to be *G. phlegrea* (Barcytė et al. 2018).

Our Tinto River isolate was identical to the Czech *G. phlegrea* CCALA 965, while some of the new sequences from Turkey were identical to the Italian ones (Fig. 1). This suggests that *G. phlegrea* is capable of long distance dispersal as was also recently suggested for the closely related species *G. sulphuraria* (Barcytė et al. 2018). On the other hand, the isolates of *G. phlegrea* may represent historical relics.

The estimated *p*-distances based on the *rbc*L gene (Table 1) showed that molecular variability within the *G. phlegrea* clade is generally low. It was by one order of magnitude less than the average distance existing among *G. sulphuraria* strains (Cozzolino et al. 2000). Even strains from different

Table 1. Pairwise genetic distances based on *p*-distances of the *rbc*L gene of selected *G. phlegrea* sequences included in this study.

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]
[1] *G. phlegrea* DB01, Tinto River, Spain								
[2] *G. "sulphuraria"* DBV 009, Viterbo, Italy	0.001							
[3] *G. "sulphuraria"* DBV 012, Benevento, Italy	0.003	0.001						
[4] *G. phlegrea* ACUF664, Bitlis, Turkey	0.003	0.001	0.003					
[5] *G. "sulphuraria"* DBV 002, Pisciarelli, Italy	0.007	0.005	0.007	0.007				
[6] *G. phlegrea* ACUF642, Diyarbakir, Turkey	0.008	0.007	0.008	0.008	0.012			
[7] *G. phlegrea* ACUF785, Turkey	0.019	0.018	0.019	0.019	0.022	0.024		
[8] *G. phlegrea* clone T09, Turkey	0.026	0.024	0.026	0.026	0.024	0.030	0.039	
[9] *G. sulphuraria* SAG 108.79, USA	0.116	0.114	0.116	0.116	0.114	0.120	0.128	0.112

countries had intraspecific variation below 1%, except the Turkish strain ACUF785 and a sequence designated as 'clone T09'. Since *p*-distances between sister species of red algae vary, for example, from 1 to 2% (Boo et al. 2014), especially sequence 'clone T09' could represent members originating from other undiscovered and undescribed populations or it could even represent a new taxon. The topology of our phylogenetic tree (Fig. 2) shows a rather separate and basal position of this sequence relative to other sequences designated *G. phlegrea*. This suggests that *G. phlegrea* may harbour hidden biodiversity and that extended sampling could uncover more cryptic species. The divergence between *G. phlegrea* and *G. sulphuraria* was more than 11% which is considerably higher than the interspecific divergence in other rhodophyte species (Boo et al. 2014). In relation to this, it is worth mentioning that Moreira et al. (1994) found two of the unknown species to be more related to each other than to *G. sulphuraria*.

Galdieria phlegrea was described by Pinto et al. (2007) and for a decade known from Italy only. However, the recent report from Czechia (Barcytė et al. 2018), along with newly available sequences from thermal springs or caves in Turkey and the acid river in Spain (this study) demonstrate that *G. phlegrea* may have a much wider distribution than anticipated. Since *G. sulphuraria* and another cyanidiohycean alga, *G. maxima* Sentsova, are globally distributed in thermoacidic habitats where their ecophysiological requirements are met, the same could also be expected for *G. phlegrea*. In addition, *G. phlegrea* is not restricted to extremely acidic and dry endolithic habitats as previously reported and discussed (Ciniglia et al. 2004, Barcytė et al. 2018). The algal collection at the Dept of Biology of Federico II University in Naples, Italy (ACUF; <www.acuf.net>) contains strains of *G. phlegrea* isolated from habitats with neutral pH and moderate or elevated temperatures (25–45°C) in Turkey. Our analyses demonstrated that these isolates fall within the same clade as the strains from the acidic environments. This shows that *G. phlegrea* is ecologically very plastic and suggests that the alga may be found in different acid or neutral habitats, especially in small springs or pools which could get relatively warm. Due to limited sampling efforts and the general assumption that cyanidiophycean algae all need acidic and hot conditions for survival, *G. phlegrea* may have been overlooked. Therefore, every record of the species is important to consider from both ecological and biogeographical points of view.

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Paper V

BARCYTĖ D, HODAČ L, NEDBALOVÁ L & ELSTER J (2018) ***Chloromonas arctica*** **sp. nov., a psychrotolerant alga from snow in the High Arctic (Chlamydomonadales, Chlorophyta).** ***International Journal of Systematic and Evolutionary Microbiology*** **68: 851–859**

Authors' contributions:

DB obtained the data and wrote the paper, LH analyzed the molecular data and wrote the corresponding parts, LN read the draft of the paper, JE funded the sampling trip

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MICROBIOLOGY SOCIETY

Chloromonas arctica sp. nov., a psychrotolerant alga from snow in the High Arctic (Chlamydomonadales, Chlorophyta)

Dovilė Barcytė,[1,*] Ladislav Hodač,[2] Linda Nedbalová[1,3] and Josef Elster[3,4]

Abstract

With the advent of molecular phylogenetic methods, it has become possible to assess the bioversity of snow algae more accurately. In this study, we focused on a morphological, ultrastructural and taxonomic description of a new *Chloromonas*-like alga isolated from snow in the High Arctic (Svalbard). Light and transmission electron microscopy revealed broad ellipsoidal or ellipsoidal–cylindrical, occasionally spherical cells with a chloroplast without a pyrenoid, an inconspicuous eyespot and a papilla. The size difference and the aforementioned morphological traits clearly distinguished the alga from its closest counterparts within the genus *Chloromonas*. Moreover, we were able to cultivate the alga at both 5 and 20 °C, revealing the psychrotolerant nature of the strain. Phylogenetic analyses of the plastid *rbc*L and nuclear 18S rRNA gene showed that the alga is nested within a clade containing a number of psychrotolerant strains within the *Chloromonadinia* phylogroup (Chlorophyceae). In the *rbc*L phylogeny, the alga formed an independent lineage, sister to the freshwater species *Chloromonas paraserbinowii*. Comparisons of secondary structure models of a highly variable ITS2 rDNA marker showed support for a distinct species identity for the new strain. The ITS2 secondary structure of the new isolate differed from the closest matches '*Chlamydomonas*' *gerloffii* and *Choloromonas reticulata* by three and five compensatory base changes, respectively. Considering the morphological and molecular differences from its closest relatives, a new psychrotolerant species from the Arctic, *Choromonas arctica* sp. nov., is proposed.

INTRODUCTION

Knowledge of Arctic snow microflora

Only a handful of psychrotolerant and psychrophilic strains of *Chlamydomonas*-like algae have been isolated from snow in polar and alpine regions compared to algae from other habitats. Consequently, there is no comprehensive modern taxonomy-based list of snow-thriving species, especially for polar regions. Although the number of Arctic isolates greatly exceedes that of Antarctic isolates (e.g. Culture Collection of Cryophilic Algae – CCCryo), detailed phylogenetic studies have mainly focused on the latter due to their unique cold adaptations [1, 2]. However, the referred Antarctic organisms were isolated from cold habitats other than snow. Therefore, the snow flora of both poles remains poorly investigated and understood. For example, no Arctic-specific *Chlamydomonas*-related taxa were recently circumscribed from the High Arctic, implying a cosmopolitan snow algae distribution in the far North [3]. However, because restricted geographical distribution is a known phenomenon in snow algae [4, 5], separate lineages might be expected in the High Arctic as well.

Chloromonads from cold habitats

The majority of psychrotolerant and psychrophilic strains are members of the *Chloromonadinia* clade within the Chlamydomonadales (or Volvocales) superclade of the class Chlorophyceae [6], whereas some of the best studied psychrophiles fall within the *Moewusinia* or *Monadinia* clades [1, 2]. Hoham *et al.* [7] studied cold-tolerant *Chloromonas* (*Cr.*) species and demonstrated that snow species are spread within at least two subclades of the

Author affiliations: [1]Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2, 128 44, Czech Republic; [2]Department of Systematics, Biodiversity and Evolution of Plants (with Herbarium), Georg-August-University of Göttingen, Untere Karspüle 2, Göttingen, 37073, Germany; [3]The Czech Academy of Sciences, Institute of Botany, Dukelská 135, Třeboň, 379 82, Czech Republic; [4]Centre for Polar Ecology, University of South Bohemia, Na Zlaté stoce 3, České Budějovice, 370 05, Czech Republic.
***Correspondence:** Dovilė Barcytė, dovile.barcyte@gmail.com
Keywords: *Chloromonas arctica*; morphology; phylogeny; snow algae; taxonomy.
Abbreviations: AIC, Akaike information criterion; BI, Bayesian inference; CCALA, Culture Collection of Autotrophic Organisms; CCAP, Culture Collection of Algae and Protozoa; CCCryo, Culture Collection of Cryophilic Algae; ITS, internal transcribed spacer; MCMC, Markov chain Monte Carlo; ML, maximum-likelihood; PRSF, potential scale reduction factor; *rbc*L, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; SAG, Culture Collection of Algae at Göttingen University; TEM, transmission electron microscopy; UTEX, Culture Collection of Algae at the University of Texas at Austin.
The GenBank/EMBL/DDBJ accession numbers for the *rbc*L gene sequence and 18S-ITS1-5.8S-ITS2 rDNA region sequence of strain CCALA 10278^T are MG189706 and MG189707, respectively.
One supplementary figure is available with the online version of this article.

Chloromonas clade *sensu* Pröschold *et al.* [8] or *Chloromonadinia* clade *sensu* Nakada *et al.* [6]. Further taxonomic investigations of the genus *Chloromonas* not only confirmed the existence of two main clades [9] encompassing psychrotolerant species, such as *Cr. reticulata* (Goroschankin) Gobi or *Cr. augustae* (Skuja) Pröschold, Marin, Schlösser et Melkonian [10], and psychrophilic species, such as the assumed cosmopolitan *Cr. nivalis* (Chodat) Hoham and Mullet and *Cr. brevispina* (Fritsch) Hoham, Roemer et Mullet, but also revealed a number of novel snow lineages. Recently described *Chloromonas* snow species include *Cr. chenangoensis* and *Cr. tughillensis* Hoham, Berman, Rogers, Felio, Ryba et Miller found in the USA [11], *Cr. miwae* (Fukushima) Muramoto, Nakada, Shitara, Hara et Nozaki described from green snow in Japan [12], *Cr. fukushimae* along with *Cr. tenuis* Matsuzaki and Nozaki recognized, respectively, from snow in Japan and USA [13], and *Cr. krienitzii* Matsuzaki and Nozaki, also from Japan [4]. The increasing *Chloromonas* diversity shows evidence that molecular variability within the genus has not been fully assessed and that extended sampling could change the way we understand this genus, especially for snow-dwelling lineages. For example, *Cr. nivalis* and *Cr. brevispina* may in fact veil multiple cryptic species as they both were identified using just aplanozygote (resting spore) morphology [4].

Aim of the study

Because taxonomic studies of the genus *Chloromonas* were traditionally based solely on light microscopic observations [14, 15], the combination of electron microscopy and molecular phylogenies have enabled re-examination and elucidation of many cryptic taxa within the genus with new taxonomic proposals, as discussed above [4, 11–13]. Due to difficulties in cultivating snow algae leading to a limited number of existing cultures, strain-based studies are highly valuable. The aim of this study was to identify and ascertain the phylogenetic position of a new *Chloromonas* strain isolated from snow in the High Arctic.

METHODS

Sampling and culturing

The snow sample was taken from the surface of the Svenbreen glacier (78° 43′ 645″ N 16° 17′ 037″ E), Svalbard, High Arctic, in mid-August 2016. No prominent algal bloom was noticed on the glacier. The snow was dug (~5 cm deep) with a pickaxe up to the ice surface and placed in a 500 ml sterile plastic bag. An aliquot of the sample was transported to Prague, Czech Republic, in a liquid state in a 50 ml sterile plastic tube. The alga was isolated from the original sample using a glass micro-pipette and cultivated in Bold's basal medium [16] in a refrigerator (Polar 370) at 5 °C and a Q-Cell 200 incubator (PolLab) at 20 °C. Light was continuous and provided by a cool white 8 W fluorescent tube with intensity of photosynthetically activate radiation of 20–30 μmol m^{-2} s^{-1}. The living culture of the isolate has been deposited and is available at the Culture Collection of Autotrophic Organisms (CCALA) under strain number 10278 (referenced as such hereafter).

Light and transmission electron microscopy

Isolate CCALA 10278 was studied under Olympus BX43 and Nikon Eclipse E400 light microscopes. Photomicrographs were taken using Olympus DP27 and DP71 digital cameras and processed using the Quick Photo Camera 2.3 software (Promicra). For transmission electron microscopy (TEM) the sample was fixed for 24 h in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and postfixed in 2 % OsO_4 in the same buffer. Fixed cells were dehydrated in a graded ethanol series (35, 50, 70, 80, 96, 100 % for 15 min), transferred to acetone (3×100 % for 15 min) and finally embedded in Araldite–Poly/Bed 812 mixture (Polysciences). Ultrathin sections were cut on a Reichert-Jung Ultracut E ultramicrotome and stained using uranyl acetate and lead citrate. Sections were examined using a JEOL JEM-1011 electron microscope. Photomicrographs were obtained using a Veleta CCD camera (EMSIS) equipped with the image analysis software Olympus Soft Imaging Solution. Pictures were postprocessed with Inkscape 0.91 (Free Software Foundation).

DNA extraction, PCR and sequencing

Total genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen). PCRs were done using PPP Master Mix (Top-Bio) in a total volume of 25 μl. A small subunit (18S) ribosomal RNA gene was amplified using universal eukaryotic primers NS1 [17] and 18L [18] under the following programme: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 3 min and final extension at 72 °C for 10 min. The region was sequenced with primers 34F, 370R, 1122F [19], 895R, 1422F [20] and 891F, 1122R and 1422R (T. Friedl, unpublished). The entire internal transcribed spacer (ITS) region was amplified using an ITS1 and ITS4 primer combination [17] using the following cycle parameters: initial denaturation at 95 °C for 10 min with a subsequent 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1.5 min and final extension at 72 °C for 10 min. The segment was sequenced with forward primers 1800F [21] and 5.8SbF [22] and reverse primers ITS2 and ITS4 [17]. A part of the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) gene was amplified and sequenced with primers rbcL1F and rbcL23R with given PCR cycle parameters [7]. The PCR products were purified using ethanol and sent to Macrogen for sequencing. New sequences are available in GenBank under accession numbers MG189706 and MG189707.

Phylogenetic analyses

The closest related *rbc*L, 18S rRNA and ITS sequences to strain CCALA 10278 and other representatives of the Chlamydomonadales were acquired from GenBank by using the BLAST algorithm [23]. The sequence alignments were computed using MAFFTv.6 [24]. The aligned sequences were

checked for possible misaligned positions in BioEdit 7.0.9.0 [25]. The *rbc*L alignment comprised 41 sequences/966 positions (362 variable, 294 parsimony-informative). Based on Akaike's information criterion (AIC) in jModelTest 0.1.1 [26], the GTR+Γ+I nucleotide substitution model was selected as the best fitting for the dataset. A maximum-likelihood phylogeny was computed in RAxML 7.0.4 [27] under the proposed model and statistical support values were derived from rapid bootstrapping (1000 replicates) in the same program. For additional statistical support, Bayesian posterior probabilities were computed in MrBayes 3.2.1×64 [28] with sequence dataset partitioned by codon positions. We carried out two Markov chain Monte Carlo (MCMC) runs for one million generations each with one cold and three heated chains under the GTR+Γ+I evolutionary model (parameters were estimated from the data) and trees were sampled every 100 generations. After 10^6 generations the average standard deviation of split frequencies dropped below 0.008 and the potential scale reduction factor (PSRF) approached 1.000–1.001 for convergence diagnostic parameters. The 18S rRNA alignment comprised 83 sequences/1705 positions (362 variable, 262 parsimony-informative). Computation of the best substitution model and the maximum-likelihood tree were conducted in the same way as described above. Bayesian posterior probabilities were computed based on the non-partitioned dataset and after 10^6 generations the average standard deviation of split frequencies dropped below 0.006 while the PSRF approached 1.000–1.001 for convergence diagnostic parameters. The final trees were displayed using FigTree [29].

ITS2 rDNA secondary structure analysis

Annotation of ITS2 including the 5.8 and 28S flanking regions was accomplished by the ITS2 online database [30–34]. A minimum energy secondary structure model of ITS2 was computed with RNAstructure 5.3 [35] and displayed by Varna 3.8 [36]. A sequence and structure alignment including four sequences that were the most similar to strain CCALA 10278 ['*Chlamydomonas*' *gerloffii* CCAP 11/72 (FR865610), *Chloromonas* sp. CCAP 11/110 (FR865527), *Cr. reticulata* CCCryo 213–05 (HQ404885), *Cr. reticulata* CCCryo 338–08 (HQ404900)] was built employing the ClustalW algorithm implemented in 4SALE 1.7. [37, 38]. The same software computed compensatory base changes (CBCs) [39] among the sequences.

RESULTS

Chloromonas arctica Barcytė and Hodač, sp. nov.

Diagnosis: solitary vegetative cells, 10–20 µm long and 6–16 µm wide, broad ellipsoidal or ellipsoidal–cylindrical in shape; or spherical. Two flagella of equal length; 1.0× cell length or longer. Chloroplast single, parietal, cup- or urn-shaped and lobed with number of lobes ranging from two to eight. Lobes are never disconnected. Eyespot pale red, small and elliptical in the lateral anterior part of the cell; in older cells usually not visible. Two apical contractile vacuoles. No pyrenoid. Papilla non-distinct, hemispherical. Nucleus central. Old cells globular with cytoplasmic oil droplets occupying most of the cell volume. Asexual reproduction via production of two, four or eight zoospores. Zoospores may lose their flagella, become spherical and act as aplanospores. Formation of cell aggregates is a common phenomenon in culture. Sexual reproduction unclear. The species differs from other species of the genus in the nuclear 18S rDNA, ITS rDNA and plastid *rbc*L gene sequences.

Holotype: the alga is preserved permanently at the Herbarium of University of South Bohemia in České Budějovice (CBFS) under number A-90–1. The authentic strain has also been deposited at the Culture Collection of Autotrophic Organisms (CCALA, http://ccala.butbn.cas.cz/) in Třeboň, Czech Republic, as an active culture under strain number 10278. Figs 1, 2 and 3 show the morphology of the holotype.

Type locality: snow on the Svenbreen glacier, Svalbard, Norway (78° 43′ 645″ N 16° 17′ 037″ E; altitude 391 m above sea level).

Etymology: arc'ti.ca. L. fem. adj. *arctica* northern, from the Arctic.

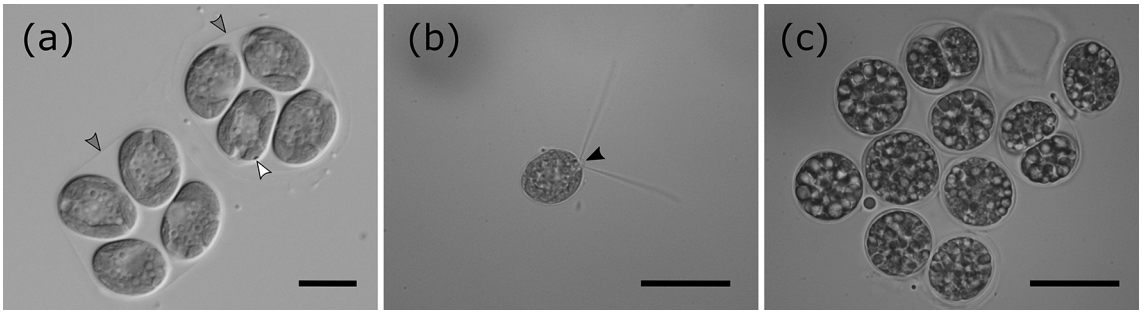

Fig. 1. Morphology of *Cr. arctica* CCALA 10278: (a) vegetative cells; (b) zoospore with two equal flagella; (c) old cells full of lipid droplets. White arrow points to the eyespot, grey arrows to the mother cell walls and black arrow to the papilla. Bars, 10 µm (a); 20 µm (b and c).

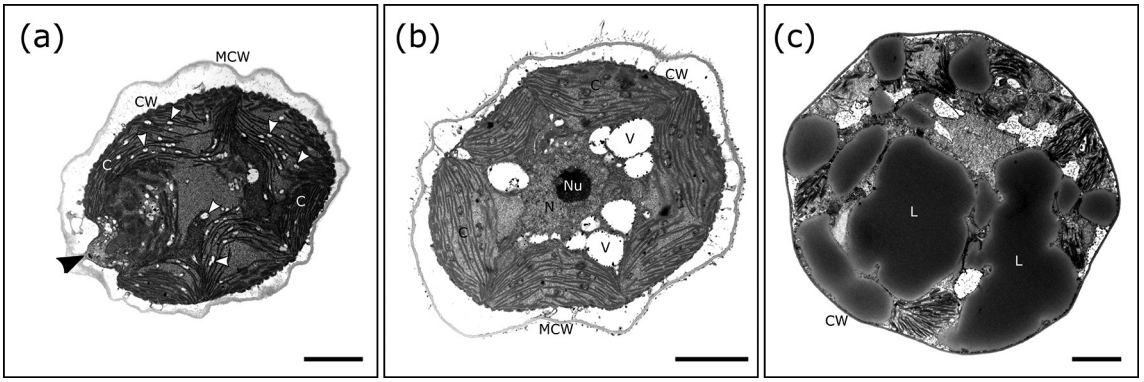

Fig. 2. Ultrastructure (TEM) of *Cr. arctica* CCALA 10278: (a) zoospore; (b) mature vegetative cell; (c) old cell. C, chloroplast; CW, cell wall; MCW, mother cell wall; N, nucleus; Nu, nucleolus; V, vacuole; L, lipid droplet. Black arrow shows the papilla, while white arrows point to the starch grains. Bars, 2 µm.

Morphology and ultrastructure

The alga investigated in this study grew at both 5 and 20 °C revealing the psychrotrophic nature of the organism rather than it being an obligate psychrophile. The isolate had general morphological and ultrastructural characteristics common to *Chloromonas*-like microalgae. It was unicellular, with broad ellipsoidal or ellipsoidal-cylindrical cells (Fig. 1a, b). Spherical cells were observed in culture that was more than 1 month old (Fig. 1c). Single cup- or urn-shaped multilobed parietal plastid occupied most of the cell volume and surrounded the central nucleus; no pyrenoid was observed (Figs 1 and 2). Small starch grains were spread in the interthylakoidal spaces over the entire chloroplast (Fig. 2a). It reproduced asexually by two to eight zoospores which soon became immotile. Motile cells (Fig. 1b) were only observed when aliquots of the culture were transferred to fresh medium, in which case motility lasted for a very short time (noticed only at 20 °C). Zoospores then quickly lost their flagella and became aplanospores. These cells were always surrounded by a parental cell wall (Fig. 1a). The young cells had eyespots and inconspicuous papillae (Figs 1a, b and 2a), while in mature older cells stigmata and papillae were not visible. Repeated divisions of daughter cells in parental cell walls resulted in cell aggregates covered by mother cell walls (Fig. 1a), especially under lower temperature conditions (5 °C compared to 20 °C). Sexual reproduction was not observed. Large oil droplets were noticed in the senescent cells (Figs 1c and 2c).

Phylogenetic analyses

Cr. arctica CCALA 10278 was placed within the *Chloromonadinia* phylogroup [6] of the class Chlorophyceae. In the *rbc*L phylogeny, *Cr. arctica* CCALA 10278 clustered within clade 1 (Fig. 3) as a part of a supported subclade [maximum-likelihood support (ML)/Bayesian inference (BI): 90/1.00] containing the *Chloromonas* type species *Cr. reticulata* with the epitype strain UTEX 1970 (=SAG 29.83) [8]. The most similar *rbc*L gene sequence in GenBank was *Cr. paraserbinowii* SAG 71.72, which differed by 28 nucleotides from *Cr. arctica* CCALA 10278. 18S rRNA gene sequence analysis supported the same phylogenetic placement (Fig. 4). *Cr. arctica* CCALA 10278 differed by two nucleotides from '*Chlamydomonas*' *gerloffii* CCAP 11/72, its closest relative available in GenBank. Both sequences were nested within a moderately supported (ML/BI: 65/0.99) subclade of clade 1 (upper part of Fig. 4) along with *Cr. reticulata*. Analysis of the ITS2 secondary structures revealed the highest similarity between *Cr. arctica* 10278 and '*Chlamydomonas*' *gerloffii* CCAP 11/72, although both sequences still differed by three compensatory base changes within helix III (Fig. 5). Three other strains of *Cr. reticulata* with similar ITS2 sequences (CCAP 11/110, CCCryo 213-05, CCCryo 338-08) differed by five compensatory base changes from *Cr. arctica* CCALA 10278.

DISCUSSION

Biogeography of snow algae

Limited research has been undertaken to explore the biodiversity of eukaryotic snow microalgae using modern methods, particularly in the polar regions [3]. On the other hand, polyphasic taxonomic approaches have revealed a number of new green algal taxa from snowfields of the USA and Japan [4, 11–13]. Interestingly, the newly described species appeared to have a local rather than cosmopolitan distribution, implying the existence of both large- and small-scale biogeographical patterns [4]. Thus, the discovery here of the novel species *Cr. arctica* in the High Arctic is not surprising. In contrast, Lutz *et al.* [3] revealed a distribution of cosmopolitan snow algae throughout the Arctic. For example, *Cr. polyptera* (Fritsch) Hoham, Mullet et Roemer, known only from Antarctica, was shown to be the second most abundant snow alga in the Arctic [3]. However, such results should be interpreted with great care because the authors used the evolutionary highly conserved 18S rRNA gene which has been shown to be poorly discriminating

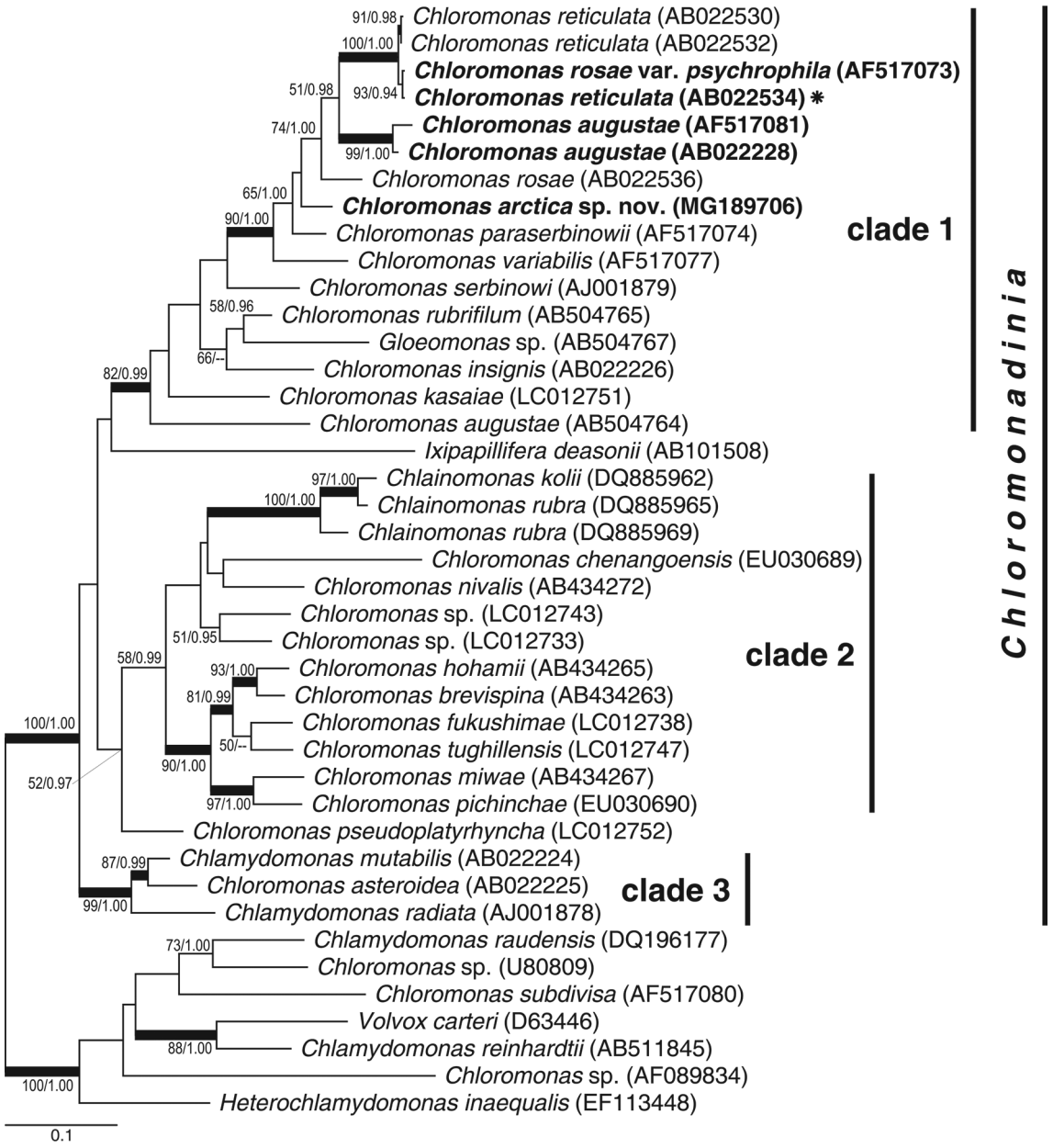

Fig. 3. Phylogenetic position of *Cr. arctica* CCALA 10278 within the *Chloromonadinia* phylogroup (Chlorophyceae) based on maximum-likelihood tree of *rbc*L gene sequences. *Reinhardtinia* lineages were used as an outgroup. Numbers next to branches indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities). Thick lines indicate branches with high statistical support. *Chloromonas* clades 1, 2 and 3 were delimited according to Hoham *et al.* [7]. Snow strains in clade 1 are marked in bold. Asterisk shows the type species of the genus *Chloromonas*. Bar, 0.1 changes per nucleotide position.

between *Chloromonas* species. In addition, *Cr. polyptera* is found only in ecosystems with high animal nutrient input [9]. Our study also showed that the 18S rRNA gene would not have been adequate in defining *Cr. arctica*. Therefore, the conclusion that distinct snow algal species are cosmopolitan based on this single marker [3] should be reconsidered because only the application of a multigene approach along with modern microscopy can accurately distinguish taxa of the genus *Chloromonas* [40]. The study by Lutz *et al.* [3] serves as a good example of how different methods and approaches can affect the assessment of Arctic (and not only) snow algae distribution.

Observations at the type locality

The best-investigated site for Arctic snow algae is probably north-western Spitsbergen of the Svalbard archipelago, where many psychrotolerant and psychrophilic strains were isolated from persistent snowfields and glaciers at the coast

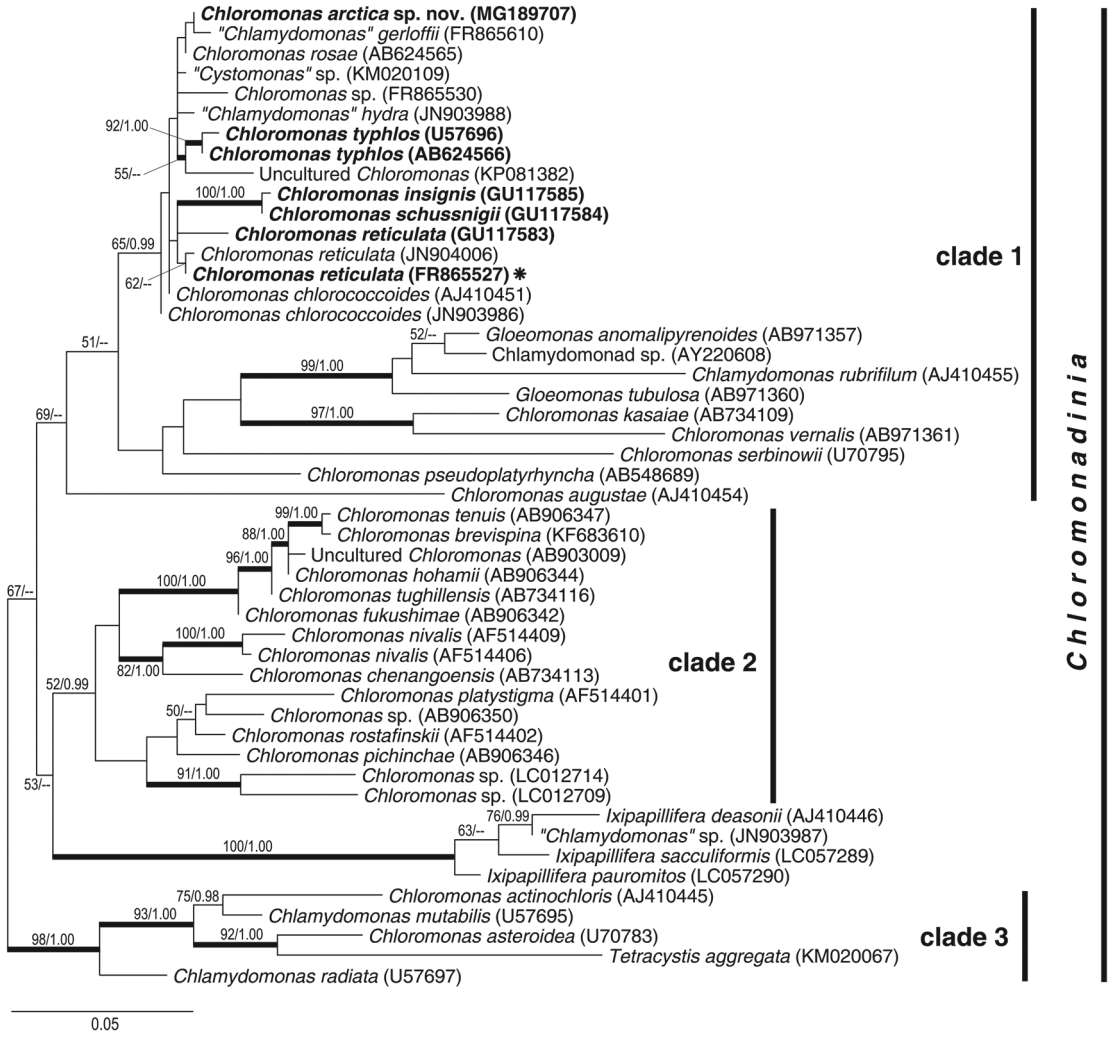

Fig. 4. Maximum-likelihood tree of 18S rRNA gene sequences of *Cr. arctica* CCALA 10278 and other members of the *Chloromonadinia* phylogroup. Numbers next to branches indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities). Thick lines indicate branches with high statistical support. *Chloromonas* clades 1, 2 and 3 were delimitated according to Hoham *et al.* [7]. Snow strains in clade 1 are marked in bold. Asterisk shows the type species of the genus *Chloromonas*. Bar, 0.05 changes per nucleotide position.

and further inland thanks to a 3-year extensive sampling done by the CCCryo group (http://cccryo.fraunhofer.de) [41]. Species of the genera *Chloromonas* and *Chlamydomonas* were the most frequently occurring representatives of the High Arctic cryoflora [41]. The Svenbreen glacier where *Cr. arctica* CCALA 10278 was found is located in Petuniabukta, Central Svalbard, where no comprehensive study of the cryoflora has been performed. The first attempt to briefly describe the community of snow algae there was done by Kvíderová [42]. However, that study encompassed the cryoflora of temporary snowfields only and was based just on microscopic observations. The samples were dominated by coloured cysts of *Chlamydomonas* cf. *nivalis* [42]. *Cr. arctica* CCALA 10278 was isolated from a permanent supraglacial habitat exposed to the sun and meltwater during the boreal summer. The alga was found along with the common ice species zygnematophytes *Ancylonema nordenskioeldii* Berggern and *Cylindrocystis brebissonii* Ralfs (De Bary) and spherical red spores resembling *Chlamydomonas* cf. *nivalis* (see Fig. S1, available in the online version of this article). The ice species probably appeared due to snow and ice mixing in the sample taken. The reason why *Cr. arctica* was not previously discovered may not be only the lack of sampling of snow habitats but also the possibility that the alga is not a dominant or bloom-forming species of snow algal communities, such as *Cr. nivalis* or *Cr. brevispina* [14]. Therefore, it could have been overlooked among other conspicuous and abundant snow species and their different life stages. For example, strain CCALA 970 was isolated from a red snow (it belongs to the species *Cr. reticulata;* pers.

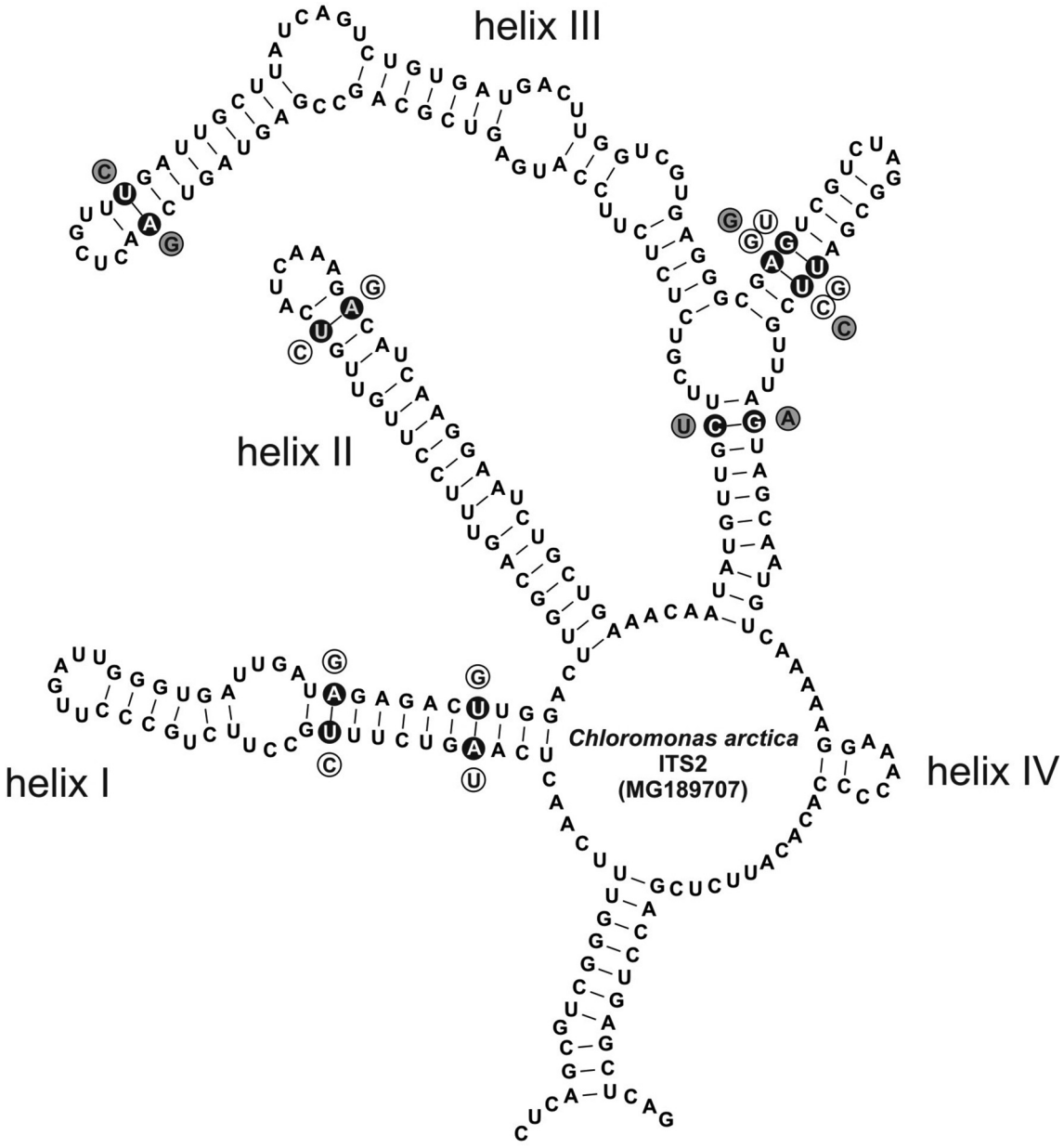

Fig. 5. Secondary structure of the ribosomal DNA internal transcribed spacer 2 (ITS2 rDNA) of *Cr. arctica* CCALA 10278. Nucleotides highlighted in black circles mark CBCs between *Cr. arctica* CCALA 10278 and *Cr. reticulata* CCAP 11/110 (white circles) and between *Cr. arctica* CCALA 10278 and '*Chlamydomonas*' *gerloffii* CCAP 11/72 (grey circles).

commun. with Nedbalová) but it did not form the initial algal bloom. In addition, the distribution of *Cr. arctica* could also be influenced by habitat type and its physico-chemical conditions [11]. On the other hand, the alga may be an endemic species to the Arctic where it commonly occurs on the permanent snowfields. For example, *Cr. polyptera* is acknowledged to be an endemic species to Antarctica [9], or the recently described *Cr. nivalis* subsp. *tatrae* Procházková, Remias, Řezenka et Nedbalová was revealed to be a likely endemic taxon to the High Tatra Mountains (Slovakia) [5]. However, the dispersal capabilities of snow algal species probably differ. Based on extensive sampling in the states of Colorado and Washington (USA), Brown *et al.* [43] demonstrated that *Coenochloris* species populations from snow were strongly geographically structured, whereas *Chlamydomonas* species were not.

Growth and morphology of *Cr. arctica*

The new isolate is a psychrotolerant alga because it grew at both tested temperatures (5 and 20 °C). This is not

surprising considering that only few species isolated from snow are true psychrophiles that form blooms [10] and the majority of algae are usually mesophiles that have some degree of cold tolerance [44]. With ongoing climate change, the unique polar or alpine ecosystems could soon be lost by accommodating more psychrotolerant or cosmopolitan organisms [45]. Thus, the identification of organisms and description of their distribution could not only help to better understand the biotic interactions within cold ecosystems but also allow us to predict how they could change in the future [46].

Cells of *Cr. arctica* were observed mostly as non-motile vegetative stages. Stibal [47] also reported the reproduction of *Cr. nivalis* solely by non-motile stages in the culture. It is not clear what exact growth conditions could induce the motility of the zoospores or loss of flagella. Sensitivity to changes of environmental/growth conditions is well known in snow flagellates [48]. The observed morphological features were consistent with those reported for *Chloromonas*-like algae [49, 50], including the standard lack of a pyrenoid. The two sister lineages of *Cr. arctica*, *Cr. rosae* Ettl and *Cr. serbinowii* Wille, also do not have pyrenoids. *Cr. gerloffii* (='*Chlamydomonas*' *gerloffii*) Ettl the closest relative of *Cr. arctica* (revealed by 18S rRNA gene and ITS2 rDNA analyses), has stretched, egg-like cells without papillae [50]. Moreover, the alga has a large protruding eyespot. In contrast, the eyespot of *Cr. arctica* is inconspicuous and usually not present (or not visible) at all, as also noted, for example, in its other sister lineage *Cr. variabilis* (Dangeard) Wille [50]. In addition, cells of *Cr. gerloffii* are almost twice as narrow (4–8 µm) as those we report for *Cr. arctica* (6–16 µm), although the length of the cells is the same (10–16 µm for *Cr. gerloffii* and 10–20 µm for *Cr. arctica*). Another closely related taxon, *Cr. paraserbinowii* (Skuja) Gerloff and Ettl (revealed by *rbc*L analysis), has bigger cells (20–38 µm long and 13–29 µm wide) that are ovate or ellipsoid-ovate in shape. In contrast to the aforementioned *Chloromonas* species, *Cr. paraserbinowii* has an exceptionally large papilla. Like *Cr. arctica*, it has a large chloroplast composed of many closely connected lobes, although the eyespot is big and located in the lateral middle part of the cell [50]. The presence of the parental cell wall (=primary cell wall) is a common phenomenon in *Chloromonas* snow species observed both in the field and under culture conditions [9, 51].

Conclusions

We predict that Arctic *Chlamydomonas*/*Chloromonas*-like snow flora may hide a number of novel, possibly endemic, species yet to be discovered. Here we showed that the isolation and cultivation of unialgal strains is a valuable and vital tool for better understanding of actual snow microalgae biodiversity. Therefore, this study serves as a good starting point for looking more deeply into Arctic cryoflora using a combination of both culture-based and modern molecular techniques.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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International Journal of Systematic and Evolutionary Microbiology

Chloromonas arctica sp. nov., a new psychrotolerant alga from snow in the High Arctic (Chlamydomonadales, Chlorophyta).

Dovilė Barcytė[1], Ladislav Hodač[2], Linda Nedbalová[1,3], Josef Elster[3,4]

[1]Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2, 128 44, Czechia

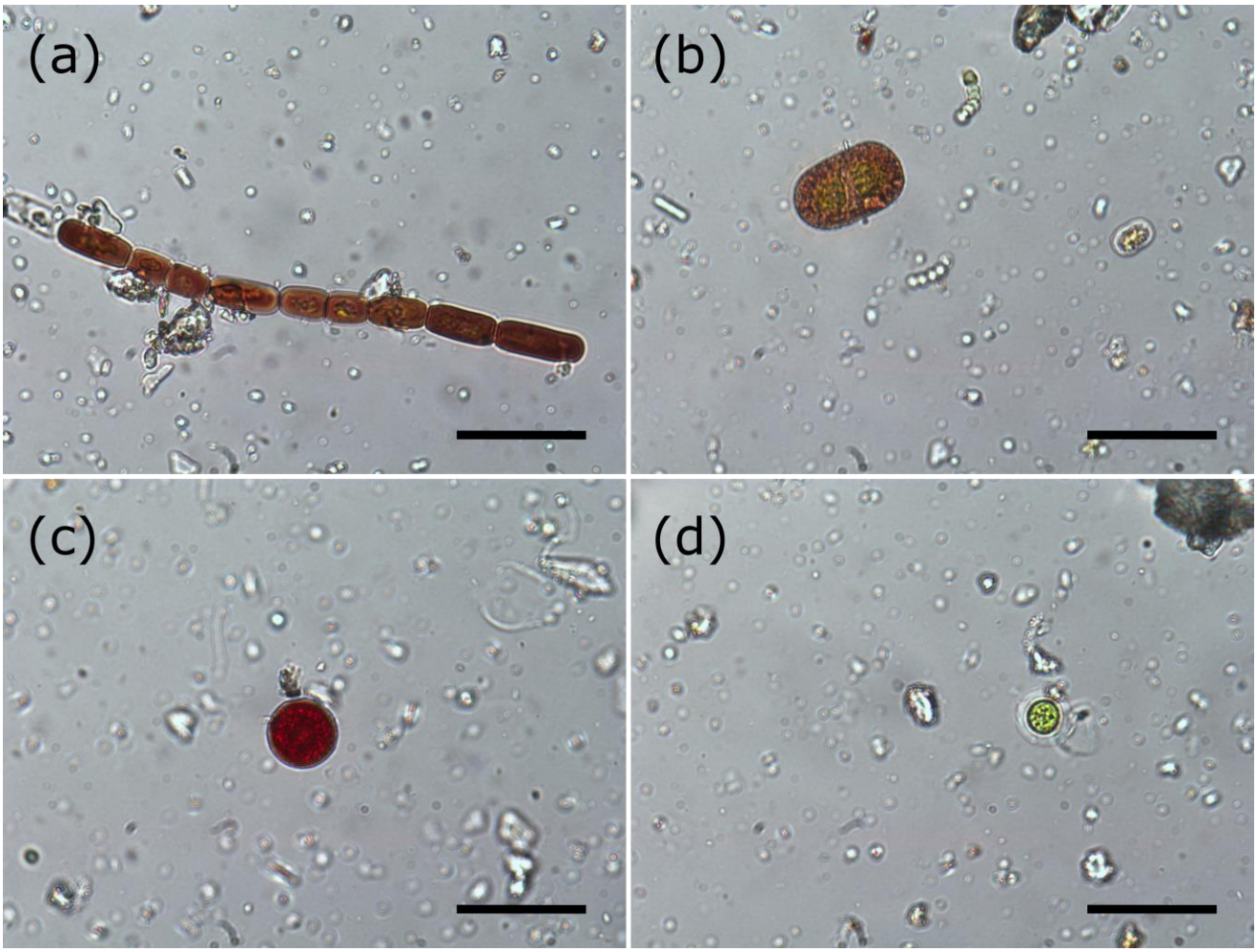
[2]Department of Systematics, Biodiversity and Evolution of Plants (with Herbarium), Georg-August-University of Göttingen, Untere Karspüle 2, 37073, Göttingen, Germany

[3]The Czech Academy of Sciences, Institute of Botany, Dukelská 135, 379 82, Třeboň, Czechia

[4]Centre for Polar Ecology, University of South Bohemia, Na Zlaté stoce 3, 370 05, České Budějovice, Czechia

Correspondence: Dovilė Barcytė, dovile.barcyte@gmail.com

Fig. S1. Algae observed in the field sample. (a) *Ancylonema nordenskioeldii*; (b) *Cylindrocystis brebissonii*; (c) spore of *Chlamydomonas* cf. *nivalis*; (d) unidentified green alga. Bars, 20 µm.



Paper VI

BARCYTĖ D, HODAČ L, NEDBALOVÁ L & ELSTER J (2018) ***Chloromonas svalbardensis*** **n. sp. with insights into the phylogroup** ***Chloromonadinia*** **(Chlorophyceae).** ***Journal of Eukaryotic Microbiology*** **65: 882–892**

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DB obtained the data and wrote the paper, LH analyzed the molecular data and wrote the corresponding parts, LN read the draft of the paper, JE funded the sampling trip

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ORIGINAL ARTICLE

Chloromonas svalbardensis n. sp. with Insights into the Phylogroup *Chloromonadinia* (Chlorophyceae)

Dovilė Barcytė[a], Ladislav Hodač[b], Linda Nedbalová[a,c] & Josef Elster[c,d]

a Department of Ecology, Faculty of Science, Charles University, Viničná 7, 2 128 44 Prague, Czechia
b Department of Systematics, Biodiversity and Evolution of Plants (with Herbarium), Georg-August-University of Göttingen, Untere Karspüle 2, 37073 Göttingen, Germany
c Institute of Botany, The Czech Academy of Sciences, Dukelská 135, 379 82 Třeboň, Czechia
d Centre for Polar Ecology, University of South Bohemia, Na Zlaté stoce 3, 370 05 České Budějovice, Czechia

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Correspondence
D. Barcytė, Department of Ecology, Faculty of Science, Charles University, Viničná 7, 2 128 44 Prague, Czechia
Telephone number: +420-221-951-809;
FAX number: +420-221-951-673;
e-mail: dovile.barcyte@gmail.com

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ABSTRACT

The traditional green algal genus *Chloromonas* accommodates mesophilic, cold-tolerant and cold-adapted microorganisms. In this paper, we studied a new strain isolated from a wet hummock meadow in the High Arctic. We used morphological, ultrastructural and molecular data to assess the taxonomic position and phylogenetic relationships of the new isolate. The observed morphological features generally corresponded to the cold-tolerant *Chloromonas* characteristics. However, ellipsoidal or wide ellipsoidal vegetative cells, a massive parietal cup-shaped chloroplast with a number of continuously connected lobes, a thick cell wall, a prominent hemispherical papilla and the anterior position of an oblong or round eyespot distinguished the alga from all previously described *Chloromonas* species. Analyses of *rbc*L and 18S rRNA genes showed that the new strain formed an independent lineage within a clade containing mesophilic and psychrotolerant *Chloromonas* species. Comparisons of secondary structure models of a highly variable ITS2 rDNA marker supported a separate species identity of the new isolate. Considering the morphological and molecular differences from its relatives, a new psychrotolerant species, *Chloromonas svalbardensis*, is proposed. Further, our results demonstrated the paraphyletic origin of *Chloromonas* within *Chloromonadinia* with genetically, morphologically and ecologically well-defined clades. We discuss a scenario of a possible *Chloromonas* split and revision.

VOLVOCALES (Chlorophyceae) is the largest and the most phylogenetically diverse green algal group encompassing 21 strongly supported monophyletic groups (Nakada et al. 2008). Some of them have already been reevaluated with new taxonomic proposals and combinations, for example, *Moewusinia* phylogroup (Watanabe and Lewis 2017), while others are still awaiting a critical reassessment.

Phylogroup *Chloromonadinia* (Nakada et al. 2008) was established based on its representative freshwater genus *Chloromonas* Gobi (1899–1900). Traditionally, *Chloromonas* was understood as *Chlamydomonas* Ehrenberg without a pyrenoid (Ettl 1970, 1983) and this straightforward separation prevailed until the advent of molecular phylogenetics. Buchheim et al. (1997) showed that the traditional concept of *Chloromonas* is polyphyletic and circumscribed the monophyletic group encompassing three *Chloromonas* species and a few "*Chlamydomonas*" species. Later, Pröschold et al. (2001) resolved *Chloromonas* as a monophyletic genus with *Cr. reticulata* (Goroschankin) Gobi as the type species corresponding to *Chloromonas* lineage sensu Buchheim et al. (1997). Matsuzaki et al. (2012) revised the *Cr. reticulata* sensu Pröschold et al. (2001) strains and subdivided them into four separate taxa. The set of these species constitute a clade containing mesophilic and psychrotolerant organisms (Hoham et al. 2002).

A number of previous phylogenetic analyses (Novis et al. 2008; Nozaki et al. 2010; Remias et al. 2016) demonstrated that another freshwater genus *Gloeomonas* Klebs (1886) and a prominent snow alga *Chlainomonas* Christen (1959) fall within *Chloromonas* as well. These

two genera were described based on their unique morphological features. In contrast to *Chloromonas*, *Gloeomonas* has two separated flagellar bases (Klebs 1886), while *Chlainomonas* has four flagella and a swollen or gelatinous cell wall separated from the protoplast (Christen 1959). In addition, a new genus *Ixipapillifera* Nakada was recently proposed within *Chloromonadinia* based on a combination of molecular and morphological data (Nakada et al. 2016). Therefore, the current phylogenetic status of the phylogroup is not clear, especially the monophyly of the genus *Chloromonas*.

The problems in classifying organisms within *Chloromonadinia* have been addressed before with contrasting solutions proposed. Novis et al. (2008) suggested that *Chlainomonas* from snow could represent a derived form of two fused ancestral biflagellate cells based on its molecular phylogenetic position, ultrastructure of flagellar apparatus, and peculiar cell division. On the other hand, snow-inhabiting *Chlainomonas* could also represent a certain stage of a life cycle of a biflagellate organism since they have never been maintained in culture (Novis et al. 2008). Meanwhile, Nozaki et al. (2010) proposed that all species of *Chloromonas* should be transferred to *Gloeomonas* based on the priority principle (McNeill et al. 2012). However, the lack of strains of *Gloeomonas* type species complicated the matter because the authors did not know how to interpret the genus once established by Klebs (1886). Finally, doubts in monophyly of *Chloromonas* itself have been raised and the necessity of a taxonomic revision within *Chloromonadinia* was addressed (Matsuzaki et al. 2012; Nakada et al. 2015).

In this paper, we studied a new cold-tolerant *Chloromonas* strain isolated from a wet hummock meadow in the High Arctic. We used morphological and molecular attributes to accurately place this organism within *Chloromonadinia*. This allowed us to critically look at the genus *Chloromonas* and highlight the existing problems in classifying cold-adapted and cold-tolerant species within the framework of *Chloromonadinia*.

MATERIALS AND METHODS

Sampling, isolation and culturing

Chloromonas sp. was isolated from a water sample taken in August 2016 from a wet hummock moss-dominating meadow at Petuniabukta, Billefjorden, central Svalbard, Norway (78°43′01,8″N 16°26′39,3″E). The detailed description of the meadow is given in Elster et al. (2012). The aliquots of the sample were transferred to a microplate filled with Bold's Basal Medium (Bischoff and Bold 1963) and kept in a Polar 370 refrigerator (Polar Refrigeration, Bristol, U.K.) at 5°C. We used a serial dilution as the isolation mode of the alga. Finally, the isolate was transferred to an Erlenmeyer flask and cultivated in a Q-Cell 200 incubator (PolLab, Wilkowice, Poland) at 20°C. The light was continuous and provided by a cool white Ostram 8 W fluorescent tube (Munich, Germany) with 20–30 μmol/m^2/s intensity.

Light and transmission electron microscopy

The isolate was studied under an Olympus BX43 light microscope (Tokyo, Japan). Microphotographs were taken using an Olympus DP27 digital camera (Tokyo, Japan). The Olympus micro imaging software cellSens v1.15 (Tokyo, Japan) was used to obtain morphometric measurements of the alga. For the transmission electron microscope the sample was fixed for 24 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and postfixed in 2% OsO_4 in the same buffer. Fixed cells were dehydrated in a graded ethanol series (35%, 50%, 70%, 80%, 96%, 100% for 15 min), transferred to acetone (3 × 100% for 15 min) and finally embedded in Araldite-Poly/Bed® 812 mixture (Polysciences, Warrington, PA). Ultrathin sections were cut on a Reichert-Jung Ultracut E ultramicrotome (Wien, Austria) and stained using uranyl acetate and lead citrate. Sections were examined using a JEOL JEM-1011 electron microscope (Tokyo, Japan). Photomicrographs were obtained using a Veleta CCD camera (EMSIS GmbH, Muenster, Germany) equipped with the image analysis software Olympus Soft Imaging Solution (EMSIS GmbH). The pictures were post-processed with Inkscape 0.91 (Free Software Foundation, Boston, MA).

DNA extraction, PCR and sequencing

The total genomic DNA was extracted using the Geneaid Plant Genomic DNA Mini Kit (New Taipei City, Taiwan). The cells were disrupted using the bead-beating method: the harvested biomass was transferred to a bead-beating tube containing 2.8 mm zirconium oxide beads and 100 μl of lysis buffer. The sample was beaten two times for 20 s at the maximum speed in a bead-based Precellys®24 homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). Polymerase chain reactions (PCRs) were done using PPP Master Mix (Top-Bio, Prague, Czechia) in a total volume of 25 μm (12.5 μl of the mix, 9.7 μl of sterile PCR Ultra water (Top-Bio), 0.65 μl of primers (10 pmol/ml), and 1.5 μl of DNA). A partial fragment of a ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbc*L) was amplified and sequenced with primers rbcL1F and rbcL23R (Hoham et al. 2002). The 18S rRNA gene was amplified and sequenced with primers NS1 (White et al. 1990) and 18L (Hamby et al. 1988) and additional sequencing primers 895R (Remias et al. 2012) and 1122F (5′-GGC TGA AAC TTA AAG GAA TTG-3′; T. Friedl, unpubl. observ.). Internal transcribed spacer 2 (ITS2) was amplified with primers AL1500af (Helms et al. 2001) and LR3 (Vilgalys and Hester 1990) and sequenced with primers ITS1 and ITS4 (White et al. 1990). The PCR amplification of the 18S rDNA region began with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturating at 94°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 3 min, with a final extension at 72°C for 10 min. The ITS2 region was amplified as follows: initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for

1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. The PCR cycle parameters for *rbc*L gene amplification in given in Hoham et al. (2002). All PCR were performed in a GenePro thermal cycler (BIOER, Hangzhou, China). PCR products were purified with ethanol and sent to Macrogen (Amsterdam, the Netherlands) for sequencing.

Phylogenetic analyses

The BLAST algorithm (Altschul et al. 1997) was employed to search the GenBank database for sequences closely related to our new strain. For phylogenetic comparisons we acquired 42 chloroplast *rbc*L sequences, 49 nuclear 18S rDNA sequences and five ITS2 rDNA sequences. Multiple sequence alignments of *rbc*L and 18S rDNA were computed using the MAFFT algorithm (Katoh and Toh 2008) with two different methods: FFT-NS-1 for *rbc*L sequences and Q-INS-i for 18S rDNA sequences. The multiple sequence alignments were checked for misaligned positions in BioEdit 7.0.9.0 (Hall 1999). The *rbc*L alignment comprised 43 sequences/936 positions (350 variable, 286 parsimony-informative) and the 18S rDNA alignment comprised 50 sequences/1682 positions (241 variable, 196 parsimony-informative). Based on the AIC criterion in jModelTest 0.1.1 (Posada 2008), the GTR+Γ+I nucleotide substitution model was selected as best fitting both sequence alignments (including all three plastid gene partitions). Maximum-likelihood phylogenies for both markers were computed in RAxML 7.0.4 (Stamatakis et al. 2008), using the proposed model and statistical support values were acquired from rapid bootstrapping (1,000 replicates) in the same program. Additional statistical support based on Bayesian posterior probabilities was computed in MrBayes 3.2.1 x64 (Ronquist et al. 2012) with sequence dataset partitioned by codon positions in the case of the *rbc*L marker and nonpartitioned in the case of the 18S rDNA marker. We carried out two Markov chain Monte Carlo runs for one million generations each with one cold and three heated chains under the GTR+Γ+I evolutionary model (parameters were estimated from the data); trees were sampled every 100 generations. After 10^6 generations the average standard deviation of split frequencies dropped below 0.006 and the potential scale reduction factor approached 1.000–1.001 for convergence diagnostic parameters. The final trees from RAxML and MrBayes were displayed using FigTree program 1.4.2 (Rambaut 2007). Additional sequence comparisons based on numbers of different nucleotides were computed in MEGA6 (Tamura et al. 2013).

ITS2 rDNA secondary structure analysis

The BLAST search revealed only a few sequences of the ITS2 corresponding to strains closely related to the new strain (clade 1; Hoham et al. 2002). For ITS2 secondary structure comparisons we acquired multiple sequences of *Cr. reticulata*, including the authentic strain (FR865527, HQ404900, MF033356), "*Chlamydomonas*" *gerloffii* H. Ettl (FR865610) and *Cr. arctica* Barcytė and Hodač (MG189707) within the *Chloromonadinia* phylogroup. Annotation of the ITS2 region bordering on 5.8S and 28S flanking regions was accomplished by the ITS2 database (Keller et al. 2009; Koetschan et al. 2010, 2012; Schultz et al. 2006; Selig et al. 2008). Secondary structures of the annotated ITS2 sequences were folded based on the minimum energy model in the program RNAstructure 5.3 (Reuter and Mathews 2010) and visualized by VARNA 3.8 (Darty et al. 2009). The ITS2 sequences with secondary structures were aligned employing the ClustalW algorithm implemented in the program 4SALE 1.7. (Seibel et al. 2006, 2008) and the resulting alignment of six sequences had 340 positions (103 variable, 42 parsimony-informative). Compensatory base changes (CBCs) among the sequences were computed with the same software.

RESULTS

Taxonomic treatment

Chloromonas svalbardensis Barcytė and Hodač n. sp.

Description. Vegetative cells unicellular, biflagellate, ellipsoidal or wide ellipsoidal, 12–20 μm long and 7–16 μm wide, or spherical. Anterior papilla prominent and hemispherical. Eyespot oblong or round in the lateral anterior end of the chloroplast, in the anterior third of the cell. Chloroplast parietal, cup- or urn-shaped and multilobed; lobes never disconnected. No pyrenoid. Nucleus central and two apical contractile vacuoles. Asexual reproduction by forming two or four zoospores. Sexual reproduction unknown and cell aggregates not observed.

Holotype. The strain is permanently cryopreserved at the Culture Collection of Algae of Charles University (CAUP; https://botany.natur.cuni.cz/algo/caup.html), Prague, Czechia under strain number CAUP G 1301-CRYO. Figure 1 shows the morphology of the authentic strain.

DNA sequences available: nuclear 18S rDNA (MH161373), ITS2 rDNA (MH161374), and plastid *rbc*L (MH161375) from the strain CAUP G 1301-CRYO.

Type locality. Wet hummock meadow at Petuniabukta, Billefjorden, central Svalbard, Norway (78°43′01,8″N 16°26′39,3″E).

Etymology. The species name was chosen to emphasize the original area of the holotype; from Svalbard.

Growth, morphology and ultrastructure

The growth of *Cr. svalbardensis* CAUP G 1301-CRYO was observed at 5 and 20°C though cells reproduced much faster at higher temperature and they were motile. The alga had ellipsoidal, broad ellipsoidal or cylindrical cells (Fig. 1), 12–20 μm long and 7–16 μm wide. The cell wall was thick (Fig. 2) with a prominent hemispherical papilla in the front (Fig. 1A, 2A). Two apically localized flagella of equal length were emerging from under the papilla (Fig. 2). The flagella were shorter than or of similar length as the cells. The cells possessed cup- or urn-shaped single chloroplasts surrounding the periphery of the cell and

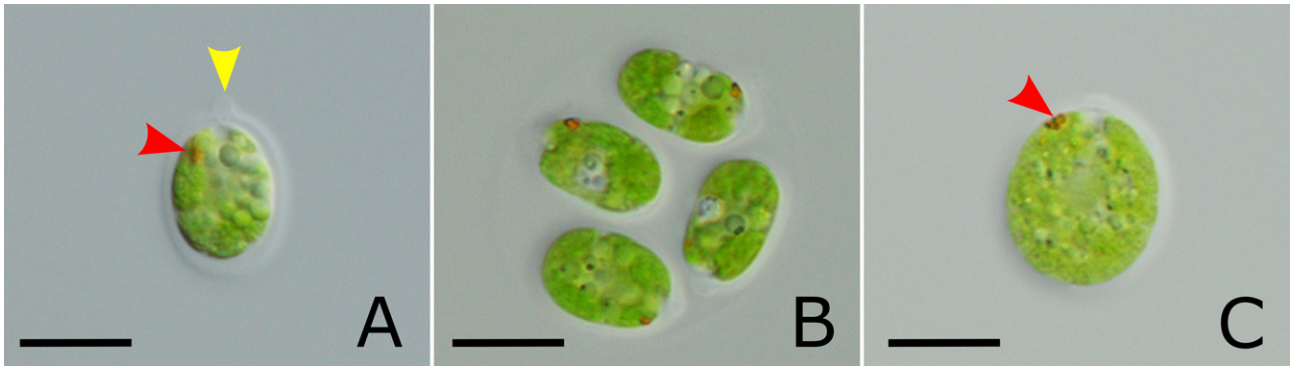

Figure 1 Light microscopic images of *Chloromonas svalbardensis* CAUP G 1301-CRYO. (**A**) Vegetative cell with a distinct eyespot and a papilla. (**B**) Zoospores within mother cell wall. (**C**) Rounded mature vegetative cell. Red arrowheads point to eyespots, yellow—to a papilla. Scale bars = 10 μm.

filling most of its volume (Fig. 1, 2). The plastids were divided into multiple lobes, each connected with the adjacent one. No pyrenoids were observed but there were numerous small starch grains scattered within the chloroplast interthylakoidal spaces (Fig. 2). An oblong or round eyespot was also present in the lateral anterior end of the plastid, in the anterior third of the cell (Fig. 1). A number of cytoplasmic globular vacuoles either empty or containing nonmembranous electron-dense deposits were present in the vegetative cells (Fig. 2). Nucleus was centrally located with several mitochondria and the Golgi apparatus situated in the middle part of the cell (Fig. 2). In addition, mitochondia were usually squeezed in between the lobes of the chloroplast. The alga reproduced by zoospores formed in doublets or tetrads (Fig. 1B). Sexual reproduction was not observed. Non-motile cells were a common phenomenon in the senescent cultures but they did not form cell aggregates resulting from repeated divisions of daugter cells retained within a parental cell wall.

Molecular analyses

Chloromonas svalbardensis CAUP G 1301-CRYO fell within the *Chloromonadinia* phylogroup (Nakada et al. 2008) of the green algal class Chlorophyceae. In the *rbc*L gene phylogeny, *Cr. svalbardensis* CAUP G 1301-CRYO was placed within clade 1 (Fig. 3) as part of a highly supported subclade (ML/BI: 85/1.00) containing the *Chloromonas* type species, *Cr. reticulata*. The most similar *rbc*L sequence was *Cr. paraserbinowii* (Skuja) Gerloff and H. Ettl SAG 71.72 (AF517074), which differed by 42 nucleotides from *Cr. svalbardensis* CAUP G 1301-CRYO. Other relatives, for example, *Cr. arctica* CCALA 10278 (MG189706) or *Cr. reticulata* UTEX 1970 (ex-epitype strain; Pröschold et al. 2001 [AB022534]), showed 46 and 48 nucleotide differences, respectively. The 18S rRNA gene phylogeny supported the same placement of the new isolate (Fig. 4). The nearest revealed relatives were *Cr. rosae* (H. and O. Ettl) H. Ettl SAG 51.72 (AB624565) and *Cr. chlorococcoides* (H. Ettl and K. Schwarz) Matsuzaki, Y. Hara and Nozaki SAG 12.96 (AJ410451) with three nucleotide differences. Based on ITS2 rDNA, the closest relative of *Cr. svalbardensis* CAUP G 1301-CRYO was the *Cr. reticulata* strain SYKOA Ch-054-11 (MF033356) isolated from pink snow in the subpolar Ural with a total of 56 nucleotide differences, including four CBCs. The second closest cultured match with 58 different nucleotides, including four CBCs, was the *Cr. reticulata* strain CCCryo 338-08 (HQ404900) isolated from snow in France. The other near relatives isolated from snow, *Cr. arctica* CCALA 10278 (MG189707) and *Cr. reticulata* CCAP 11/110 (= UTEX 1970; FR865527), differed from *Cr. svalbardensis* CAUP G 1301-CRYO by 62 and 60 nucleotides and four and five CBCs, respectively (Fig. 5).

DISCUSSION

Polar wetlands are particularly characterized by seasonal water fluctuations with meltwater as the main source of liquid water. Nevertheless, polar wetlands contain the most diverse and abundant algal communities compared to other polar biotopes. Both prokaryotic cyanobacteria and eukaryotic algae are common inhabitants of polar wetland ecosystems though such algal communities are still little studied and understood (Elster et al. 1994). As far as we know, no *Chloromonas* species has yet been isolated and described from the polar wetlands. Since *Chloromonas* is generally known to occur on temporary and permanent snowfields, we cannot exclude the fact that *Cr. svalbardensis* CAUP G 1301-CRYO could have been brought to the meadow from the snowfield by the melting water. Thus, polar wetlands could serve as important secondary habitats for snow dwelling microorganisms.

The morphological features observed in *Cr. svalbardensis* CAUP G 1301-CRYO are generally common in its sister species revealed by *rbc*L and 18S rDNA phylogenies (Fig. 3, 4). In particular, the similar shape and size of the cells, the chloroplast morphology and the presence of an eyespot and a papilla verify the affiliation to the same genus. However, a combination of morphological features such as an ellipsoidal to wide ellipsoidal cell shape with a

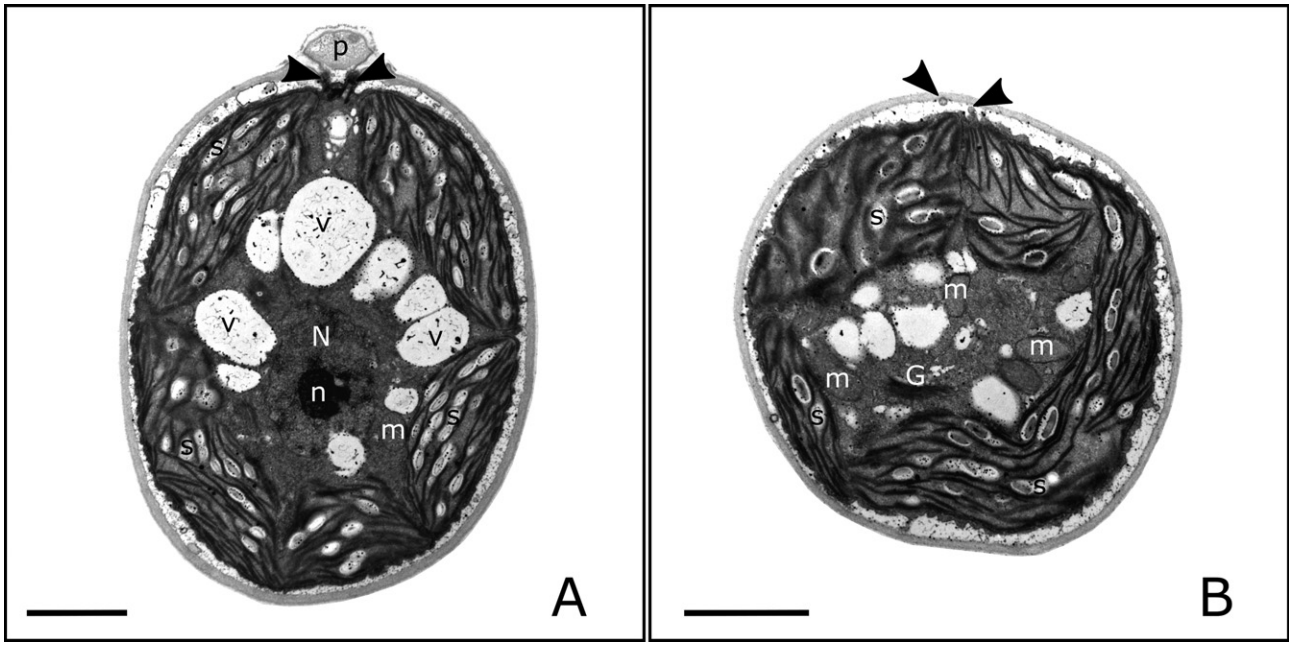

Figure 2 Ultrastructure of *Chloromonas svalbardensis* CAUP G 1301-CRYO. (**A**) TEM image of a longitudinal section of a vegetative cell with a prominent papilla. (**B**) TEM image of a transverse section of a vegetative cell. N, nucleus; n, nucleolus; p, papilla; v, vacuole; s, starch; m, mitochondrion; G, Goldi apparatus. Arrowheads point to the flagellar bases. Scale bars = 2 μm.

thick cell wall and a prominent hemispherical anterior papilla, a cup-shaped chloroplast with connected lobes and without pyrenoids and an oblong or round eyespot positioned in the anterior third part of the cell is unique just for *Cr. svalbardensis* CAUP G 1301-CRYO within the core *Chloromonas* clade (Fig. 3, 4) or other described *Chloromonas* species sensu lato (Ettl 1983). For example, the sister species of *Cr. svalbardensis*, *Cr. paraserbinowii* (revealed by *rbc*L analysis) has ovate to ellipsoid-ovate or sometimes obovate cells, an exceptionally huge papilla and an elliptic or round eyespot located in the lateral middle part of the cell (Ettl 1983). In contrast, another sister species *Cr. arctica* has both a small eyespot and a papilla, and a thin cell wall (Barcytė et al. 2018). Besides, motile vegetative cells of *Cr. svalbardenisis* were observed in older than 1 mo cultures unlike in the cultures of *Cr. arctica* (both algae were cultivated under identical conditions). This probably explains why no cell aggregates were present in *Cr. svalbardensis* cultures as the cells could move away from each other. The formation of cell aggregates resulting from the repeated divisions of daughter cells within a parental cell wall is currently considered as a diagnostic feature in delineating cold-adapted *Chloromonas* species (e.g. Matsuzaki et al. 2018). *Chloromonas svalbardensis* exhibits similar characteristics as the *Chloromonas* type species, *Cr. reticulata*, however, a round eyespot was not reported for the latter alga (Matsuzaki et al. 2012). Other close relatives, *Cr. insignis* (Anachin) Gerloff and H. Ettl or *Cr. schussnigii* H. Ettl (revealed by 18S rDNA phylogeny) have wide ovate or spherical cells, respectively, with low arched papillae and eyespot positioned in the anterior half of the cell in *Cr. insignis* and in the middle part of the cell in the case of *Cr. schussnigii*. *Cr. insignis* possesses a relatively thick cell wall while *Cr. schussnigii* has a delicate one. The morphology of additional closely related species, *Cr. rosae*, generally matches the descripion of *Cr. svalbardensis*. However, the species bears an obtuse and cone-shaped papilla and the cell wall is not as thick as in *Cr. svalbardensis*. *Cr. serbinowii* Wille also highly resembles *Cr. svalbardenis* in the cell and papilla shape and size along with the chloroplast morphology and the position of the eyespot. However, both asexual and sexual reproduction was reported for the alga along with the existence of oval aplanospores (Ettl 1983). In addition, *Cr. svalbardensis* CAUP G 1301-CRYO is phylogenetically separated from the authentic strain of *Cr. serbinowii*, UTEX 492 (Fig. 3, 4). One more morphologically similar species is *Cr. alaskensis* (Skuja) Ettl described from freshwaters in Alaska (no strain available), the main differences being that the alga was depicted as having a small hemispherical papilla and a cell wall with many distinct pores (Ettl 1983). To conclude, *Cr. svalbardensis* exhibits its own specific set of features and phylogenetically closely related *Chloromonas* species could be distinguished by their appearance although morphological identification is not straightforward.

In addition to the morphological data, a new species identity of *Cr. svalbardensis* CAUP G 1301-CRYO was also supported by the molecular data. The species differed from other members of the *Chloromonadinia* phylogroup by the *rbc*L, 18S rDNA and ITS2 rDNA sequences. The topology of the phylogenetic trees along with the ITS2

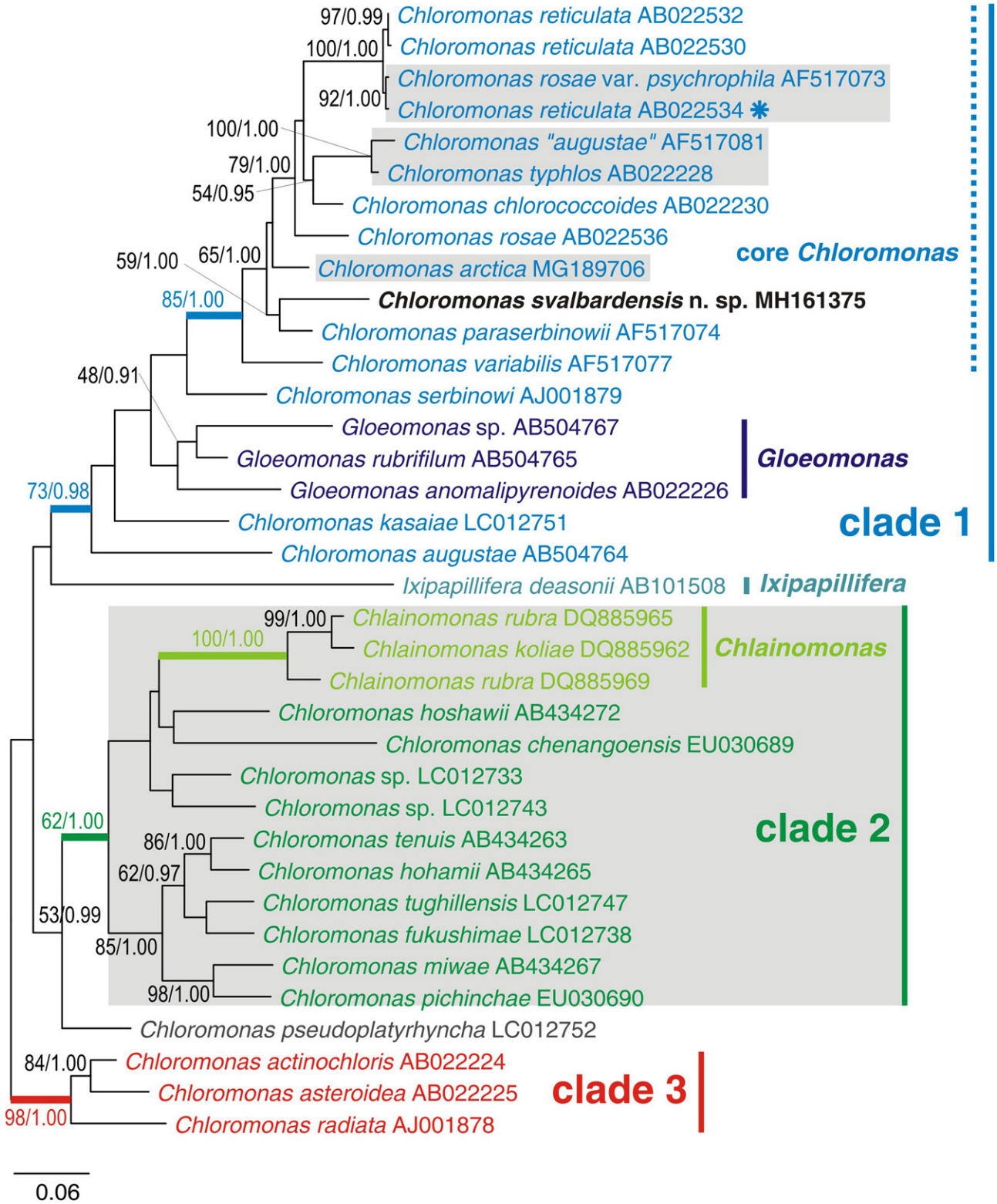

Figure 3 Maximum-likelihood tree of the *Chloromonadinia* phylogroup based on *rbc*L sequences. Numbers next to branches indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities). Thick lines indicate main clades within *Chloromonadinia*. *Chloromonas* clades 1, 2 and 3 were delimited according to Hoham et al. (2002). Snow strains are gray underlaid. Asterisk shows the authentic strain of *Cr. reticulata*, the type species of the genus *Chloromonas*.

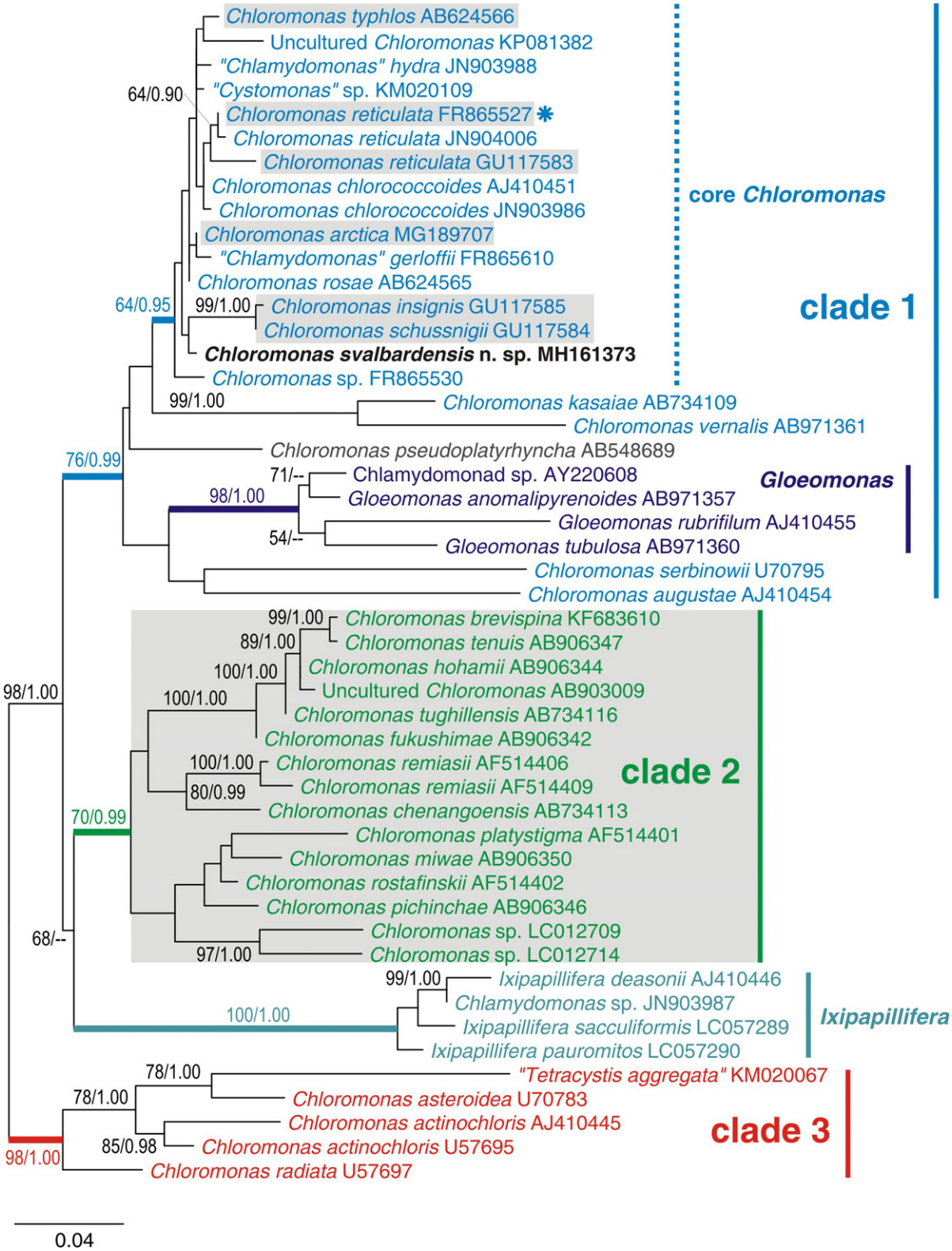

Figure 4 Maximum-likelihood tree of the *Chloromonadinia* phylogroup based on 18S rDNA sequences. Numbers next to branches indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities). Thick lines indicate main clades within *Chloromonadinia*. *Chloromonas* clades 1, 2 and 3 were delimitated according to Hoham et al. (2002). Snow strains are gray underlaid. Asterisk shows the authentic strain of *Cr. reticulata*, the type species of the genus *Chloromonas*.

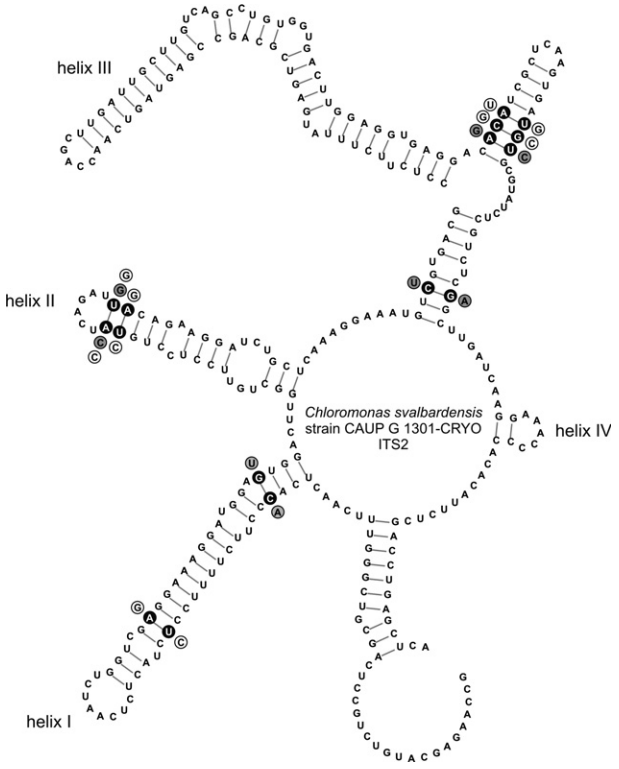

Figure 5 Secondary structure model of the ITS2 of *Chloromonas svalbardensis*. Nucleotides highlighted in circles mark compensatory base changes between *Cr. svalbardensis* (black circles) vs. *Cr. arctica* (gray circles) and between *Cr. svalbardensis* vs. *Cr. reticulata* authentic strain (white circles).

CBC species concept (Wolf et al. 2013) suggested the recognition of *Cr. svalbardensis* as a new species as well.

Chloromonas svalbardensis CAUP G 1301-CRYO is a psychrotolerant organism rather than psychrophile since it can grow at 20°C and majority of its closest relatives were also isolated from cold habitats. For example, *Cr. arctica*, *Cr. insignis* and *Cr. schussnigii* were isolated from snow in Svalbard. *Cr. arctica* like *Cr. svalbardensis* was growing at both 5 and 20°C (Barcytė et al. 2018) while the latter two species are facultative psychrophiles (T_{max} and T_{opt} below 20°C; Leya 2004). Aside from snow, other members of the *Chloromonas* clade (marked by a dash line in Fig. 3, 4) were isolated from a lake/pond water or a soil revealing ecological versatility of the genus rather than restriction to certain type of habitats. However, cold environments may be more prefered. For example, *Cr. reticulata* was isolated from a lake in Norway, snow in the USA, and a moss in Antarctica. The ecological plasticity of the core *Chloromonas* could probably explain its survival in various environments and the worldwide dispersal.

Our phylogenetic trees along with a number of previous studies (Nakada et al. 2015, 2016; Remias et al. 2013) demonstrate that *Chloromonas* is paraphyletic and that the genus should be split into a number of smaller entities. Moreover, deep branching suggests independent evolution of the main clades within *Chloromonadinia* (Fig. 3, 4), which are also ecologically well-defined. For example, clade 1 (containing true *Chloromonas*) encompasses mesophilic and psychrotolerant organisms that likely do not form snow-blooms though are commonly found there and clade 2 encompass psychrophilic organisms forming blooms, whereas clade 3 includes mesophilic algae only. Psychrophilic species within clade 2 are physiologically adapted to low temperatures (optimum for growth below 15°C) enabling them to dominate snow algal communities (Hoham et al. 2008). In addition, their dominance in extreme snow habitats is also determined by a number of other adaptations, for example, accumulation of carotenoids providing photoprotection against UV and visible light, or complex life cycles, including the formation of resting stages (zygospores and cysts). In contrast, species of clade 1 do not require low temperatures for survival and strains isolated from snow can grow in elevated temperatures (Barcytė et al. 2018; Leya 2004; Lukeš et al. 2014). Furthermore, no prominent sexually produced dormant cysts have been described within clade 1 (though asexual resting spores were reported for *Cr. rosae* var. *psychrophila*; Hoham et al. 2002). The topology of our phylogenetic trees suggests that psychrophilic life mode within *Chloromonadinia* was derived from the mesophilic counterparts, not the opposite. Moreover, the psychrophilic character within *Chloromonadinia* has evolved probably just once in the life history of the phylogroup.

Chloroplast morphology also supports a scenario of a possible *Chloromonas* split and revision. For example, species within *Chloromonas* clade 1 have a massive parietal cup- or urn-shaped chloroplast with a number of continuously connected lobes (the "*reticulata*" type as already discussed above). Clade 2 bears chloroplast mostly composed of parietal bands/platelets or disks which may or may not be connected to each other (Hoham et al. 2006; Matsuzaki et al. 2014, 2018) while clade 3 exhibit asteroid chloroplast morphology (Pröschold et al. 2001). Furthermore, majority of *Chloromonas* species within clade 1 have eyespot (with an exception of *Cr. typhlos* (Gerloff) Matsuzaki, Y. Hara and Nozaki) and several species also bear pyrenoids (e.g. *Cr. chlorococcoides*, *Cr. typhlos*). Meanwhile, *Chloromonas* taxa within clade 2 generally lack both eyespots and pyrenoids. Interestingly, *Cr. hohamii* H. U. Ling and Seppelt was originally described as usually possessing an eyespot. However, strain UTEX SNO67 designated as such does not have one (Matsuzaki et al. 2014). On the other hand, the currently described species *Cr. remiasii* Matsuzaki, Nozaki and Kawachi possesses an ellipsoidal to elongated D-shaped eyespot (Matsuzaki et al. 2018). For comparison, members of clade 3 have both an eyespot and a central pyrenoid (Pröschold et al. 2001). This way, morphological criteria rather accurately reflect phylogenetic differentiation.

Clade 2 encompasses a number of snow species reported just from their type localities in the USA and Japan and a cosmopolitan-assumed *Cr. nivalis* (Chodat) Hoham and Mullet. It is determined by its specific zygospore shape and undulated flanges on the cell surface

(Remias et al. 2010). However, *Cr. nivalis* veils multiple species and extended molecular phylogenetic investigations could considerably reshape its taxonomic status and biogeography (Matsuzaki et al. 2015, 2018). In addition, here we suggest that the genus *Chloromonas* would be understood just as a part of clade 1 (= core *Chloromonas*; Fig. 3, 4), a subclade containing the type species, and corresponding only to *Cr. reticulata* clade sensu Pröschold et al. (2001). Other clades or lineages should be carefully revised and new generic names should be proposed. For example, strain SAG 46.72 (formerly classified as *Cr. carrizoensis*; Pröschold et al. (2001)) was recently transferred to *Ixipapillifera* based on the fact that the strain does not fall to the same clade as *Chloromonas* type species (Nakada et al. 2016). The phylogenetic placement of *Ixipapillifera* within *Chloromonadinia* is unclear. For example, the topology of our *rbc*L tree suggests *Ixipapillifera* as a sister lineage to *Chloromonas* clade 1 though no statistical support was obtained. However, while 18S rRNA gene sequence analyses place *Ixipapillifera* as a sister lineage to *Chloromonas* clade 2 (Nakada et al. 2016), our study does not show statistical support. In contrast, the multigene phylogenetic analyses of *Chloromonadinia* placed *Ixipapillifera* as a sister lineage to clade 3 (Matsuzaki et al. 2010; Nozaki et al. 2010). Thus, the phylogenetic placement of the genus within *Chloromonadinia* remains unclear. Since members of *Ixipapillifera* are mesophiles, the genus is more likely to be closer related to clade 1 or 3 rather than clade 2.

In conclusion, here we summarized the current knowledge of the *Chloromonadinia* phylogroup and discussed the phylogenetic and taxonomic problems in the genus *Chloromonas*. Our study, along with the data provided by a number of other authors (references herein), suggests that the genus is paraphyletic and needs a proper taxonomic revision. It could help to better organize the existing biodiversity within *Chloromonadinia* and would significantly increase species richness in cold ecosystems where *Chloromonas* sensu lato is commonly found. Additional discoveries of new lineages, such as *Cr. svalbardensis*, could also provide a more robust estimation of the relationships between *Chloromonas*, *Gloeomonas*, *Chlainomonas*, and *Ixipapillifera*.

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Paper VII

BARCYTĖ D, HODAČ L & NEDBALOVÁ L (unpublished manuscript) Overlooked diversity with terrestrial lifestyle within the predominantly freshwater and snow phylogroup *Chloromonadinia*. *European Journal of Phycology*, under review

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DB obtained the data, LH analyzed the molecular data; DB and LH jointly wrote the paper; LN read the draft of the paper

Overlooked diversity with terrestrial lifestyle within the predominantly freshwater and snow phylogroup *Chloromonadinia* (Volvocales, Chlorophyceae)

Dovilė Barcytė[a,b], Ladislav Hodač[c], Linda Nedbalová[a]

[a]Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2, 128 44, Czechia

[b]Department of Zoology, National Museum, Cirkusová 1740, Prague 9, 193 00, Czechia

[c]Department of Systematics, Biodiversity and Evolution of Plants (with Herbarium), Georg-August-University of Göttingen, Untere Karspüle 2, 37073, Göttingen, Germany

Correspondence to Dovilė Barcytė:

Department of Ecology, Faculty of Science, Charles University in Prague, Viničná 7, 128 43, Prague, Czechia

dovile.barcyte@gmail.com

Abstract

The phylogroup *Chloromonadinia* (Volvocales, Chlorophyceae) encompasses an ecologically versatile array of green microalgae species with a predominantly freshwater and snow lifestyle. A few species have been detected in soils, however, the diversity of volvocalean flagellates with a terrestrial lifestyle is generally underestimated, since these habitats have been less sampled. We have isolated a new strain of volvocalean flagellate from tree bark (CAUP G 1401/Czechia). This alga shares similar morphological features with the terrestrial species *Chlamydomonas* (*Cd.*) *chlorostellata* discovered in New Zealand (SAG 12.72/soil) and *Cd. chlorostellata* var. *gracillima* (SAG 25.87/soil) discovered in Czechia. Since these latter two algae have been assigned to the freshwater species *Cd. meslinii*, we have also investigated the authentic *Cd. meslinii* strain (SAG 75.81/France). For comparison, a new freshwater strain (CAUP G 1302/Svalbard) of a *Chloromonas* (*Cr.*) species (*Cr. reticulata*) was also included in the study. Because the terrestrial strains differed from the freshwater strains in morphology and lifestyle, we expected them to represent separate evolutionary lineages at the species level or higher. We examined their identity based on cell ultrastructure (by TEM) and molecular phylogenetic analysis (nuclear 18S+ITS2 rDNA and plastid *rbc*L). We found that the new isolate, CAUP G 1401, and the two authentic "*Chlamydomonas*" strains, SAG 12.72 and SAG 75.81, belong to a distinct phylogenetic lineage within the *Chloromonadinia* phylogroup, denominated *Ostravamonas* (*Os.*) gen. nov. The terrestrial strains (CAUP G 1401 and SAG 12.72) were assigned to the species *Os. chlorostellata*, while the freshwater strain (SAG 75.81) was reassigned to the species *Os. meslinii*. The core *Chloromonas* clade accommodated the terrestrial strain SAG 25.87 (*Cr. gracillima*). The species *Os. chlorostellata* includes the first known terrestrial examples of *Chloromonadinia* microalgae originating in acidic environments. In addition, our data suggest that terrestrial vs aquatic lifestyle could have significantly influenced evolutionary diversification of the *Chloromonadinia* phylogroup.

Keywords: *Chloromonas*; cryptic diversity; ecology; freshwater; polyphasic approach; terrestrial

Introduction

The phylogroup *Chloromonadinia* is a part of the order Volvocales, within the green algae class Chlorophyceae. It encompasses four genera of unicellular flagellates isolated mainly from freshwater and snow. The most widespread and species rich genus is *Chloromonas* Gobi accomodating morphologically, ecologically, and physiologically diverse organisms. Barcytė *et al*. (2018a) demonstrated that the current concept of *Chloromonas* is paraphyletic, and suggested that the genus should be split into a number of smaller taxonomic entities for better systematic organization of *Chloromonadinia* microalgae. The genus *Chloromonas* was proposed to be the monophyletic clade containing the type species *Cr. reticulata*, and corresponding only to the *Cr. reticulata* clade *sensu* Pröschold *et al*. (2001) (Barcytė *et al*., 2018a). Both pyrenoid-lacking and pyrenoid-bearing species fall into this circumscription as previously demonstrated (Matsuzaki *et al*., 2012). Having been subdivided into three main clades, *Chloromonas* now awaits an integrative taxonomic revision with the establishment of new genera. Since both morphology and phylogeny have certain limitations alone, polyphasic taxonomic approaches are currently the most popular way to classify and reclassify microorganisms. For example, by combining morphological and molecular analyses, Matsuzaki *et al*. (2012, 2014, 2018, 2019) identified and re-identified multiple *Chloromonas* strains and proposed new species combinantions in some cases, and new descriptions in others. However, attempts to reclassify *Chloromonadinia* microalgae at the genus level based on current systematics methods has not yet begun. The taxonomic history of *Chloromonas*-like flagellates is not simple, especially for snow-dwelling species (many of which have

resting stages) that were placed in the genus *Scotiella* F.E. Fritsch (Hoham & Mullet, 1978; Hoham *et al*., 1983) and *Cryocystis* Kol (Hoham *et al*., 1979).

In contrast to freshwater taxa, the majority of which are psychrotolerant and psychrophilic *Chloromonas* species (Hoham *et al*., 2002; Barcytė *et al*., 2018a, b; Matsuzaki *et al*., 2014, 2018, 2019), much less attention has been paid to the terrestrial species of *Chloromonadinia* microalgae. Consequently, the uncharacterized strains limit our understanding of *Chloromonas*-like morphotypes and phylotypes thriving in (aero)-terrestrial environments. For example, our current knowledge is limited to several sequenced strains isolated from soils in different parts of the world. However, since terrestrial lifestyles are commonly encountered among Volvocalean green algae, more such organisms probably lay within the *Chloromonadinia* as well, which have yet to be discovered and described.

Apart from *Chloromonas*, other genera of the *Chloromonadinia* phylogroup include freshwater *Gloeomonas* G.A. Klebs, which is a sister lineage to the psychrotolerant *Chloromonas*; snow alga *Chlainomonas* Christen, a close relative of the psychrophilic *Chloromonas*; and terrestrial and freshwater *Ixipapillifera* Nakada whose phylogenetic placement within *Chloromonadinia* continues to be debated (Barcytė *et al*., 2018a). The phylogenetic position of freshwater *Cr. pseudoplatyrhyncha* (Pascher) P.C. Silva is also unclear, since it does not cluster within any of the mentioned *Chloromonas* clades (Barcytė *et al*., 2018a).

In this paper, we have focused on terrestrial members of the *Chloromonadinia* phylogroup. We have studied the new volvocalean flagellate strain CAUP G 1401, isolated from a highly unusual terrestrial habitat – a burning coal spoil heap. We have compared it with morphologically similar authentic terrestrial strains of the species *Cd. chlorostellata* E.A. Flint & H. Ettl SAG 12.72 and *Cd. chlorostellata* var. *gracillima* H. Ettl SAG 25.87, both assigned to the freshwater species *Cd*. *meslinii* Bourrelly (authentic strain SAG 75.81). For comparison and taxonomic implications, we have also studied a new isolate of *Cr*. *reticulata*

CAUP G 1302. We aimed to evaluate or re-evaluate the taxonomic status of the new and authentic strains, respectively, and test if adaptation to terrestrial vs aquatic environments plays a significant role in their diversification. Based on the results, we discuss relationships between species circumscribed within the *Chloromonadinia* phylogroup. For the purpose of accommodating the investigated strains within *Chloromonadinia*, we propose here a new genera-level taxonomy.

Materials & Methods

Strain origins, cultivation, and microscopy

Two strains used in this study were newly isolated (Table 1) using multiple serial dilutions and an inverted phase contrast Nikon Diaphot 200 microscope (Nikon Corp., Tokyo, Japan). These strains have been deposited in the Culture Collection of Algae of Charles University in Prague, Czechia (CAUP; https://botany.natur.cuni.cz/algo/caup.html). Three strains were obtained from the Culture Collection of Algae at the University of Göttingen, Germany (SAG; http://sagdb.uni-goettingen.de/) (Table 1).

Cultures of each strain were maintained in Bold's Basal Medium (BBM; Bischoff & Bold, 1963) in a Q-Cell 200 incubator (PolLab, Wilkowice, Poland) at 20°C. Continuous light was provided by cool white 8W fluorescent tubes (Osram, Munich, Germany) at approximately 30 μmol photons m^{-2} s^{-1}.

Morphological investigations of cultures were conducted using an Olympus BX43 light microscope (Tokyo, Japan). Microphotographs were taken with an Olympus DP27 digital camera (Tokyo, Japan). The Olympus micro imaging software cellSens v1.15 (Tokyo, Japan) was used for morphometric measurements of the algae. Vegetative cells were observed and documented using one month old cultures. One hundred cells of each strain were measured for size comparisons. The ultrastrusture of cells was studied using transmission electron microscopy (TEM) as previously described in Barcytė *et al*. (2017).

Table 1. List of studied strains. New isolates are in bold.

Species	Strain	Habitat	Locality
Chloromonas reticulata	**CAUP G 1302**	**snowmelt stream**	**Bjørndalen, Svalbard, Norway**
Chloromonas gracillima	SAG 25.87	soil from spruce forest	Hrubý Jeseník mountains, Czechia
Ostravamonas chlorostellata	**CAUP G 1401**	**tree bark**	**Heřmanice coal spoil heap, Ostrava, Czechia**
Ostravamonas chlorostellata	SAG 12.72 as	acidic soil	Southern Alps at Bealey, New Zealand
Ostravamonas meslinii	SAG 75.81	ditch	Jardin Alpin du Museum, Paris, France

DNA extraction, fragment amplification, and sequencing

Genomic DNA from each investigated strain was extracted using a Geneaid Plant Genomic DNA Mini Kit (New Taipei City, Taiwan). Nucleotide sequences of 18S-ITS1-5.8-ITS2 rDNA regions were amplified using the following primer pairs: 18SF/18SR (Katana *et al*., 2002), AL1500af (Helms *et al*., 2001)/ITS2 and ITS1/ITS4 (White *et al*., 1990). A partial fragment of a ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbc*L) was amplified with primers rbcL1F and rbcL23R (Hoham *et al*., 2002). Polymerase chain reactions (PCRs) were performed using PPP Master Mix (Top-Bio, Prague, Czechia) in a total volume of 25 µl, or a Qiagen Multiplex PCR Plus Kit (Hilden, Germany) in a total volume of 10 µl. Cycling conditions have been previously described in Barcytė *et al*. (2018a) and Hoham *et al*. (2002). The PCR products were cleaned using ethanol or ExoSAP-IT (Affymetrix, Cleveland, USA). Sequencing was performed by Macrogen (Amsterdam, the Netherlands) in both directions using the PCR primers described above, and the internal primers 985R, 1422F (Remias *et al*., 2012) and 1422R (5′-CTA AGG GCA TCA CAG ACC TG-3′; T. Friedl, unpubl.). Sequences were assembled using SeqAssem ver. 07/2008 (Hepperle, 2004). These have been deposited in GenBank under accession numbers MK908411-MK908414, MK912124-MK912127 and MK912141-MK912145.

Phylogenetic analyses

Employing the BLAST search algorithm (Altschul *et al*., 1997), we have obtained sequences from GenBank that are closely related to our newly sequenced strains. Phylogenetic analyses relied upon 75 nuclear 18S rDNA sequences, and 57 plastid *rbc*L sequences. Multiple sequence alignments were computed using the MAFFT algorithm (Katoh & Toh, 2008) employing Q-INS-i (18S rDNA) and FFT-NS-1 (*rbc*L) strategies. We checked the alignments for misaligned positions using the program BioEdit 7.0.9.0 (Hall, 1999). The 18S rDNA alignment covered 1687 positions (336 variable, 230 parsimony-informative), while the *rbc*L

alignment covered 936 positions (353 variable, 297 parsimony-informative). The Akaike information criterion (AIC) implemented in the software jModelTest 0.1.1 (Posada, 2008) selected the best fitting nucleotide substitution model for the 18S rDNA dataset (GTR+Γ+I) and the *rbc*L dataset (GTR+Γ+I for the *rbc*L 1st codon position, JC+I for the *rbc*L 2nd codon position, and GTR+Γ+I for the *rbc*L 3rd codon position). The 18S-based maximum-likelihood phylogeny was computed in RAxML 7.0.4 (Stamatakis *et al*., 2008) under the proposed model. Statistical support values were acquired by rapid bootstrapping (1000 replicates) in the same program. The *rbc*L-based maximum-likelihood phylogeny was computed with the program Garli 2.01 (Zwickl, 2006) in accord with the above mentioned nucleotide substitution models, with 100 bootstrap replicates. Bayesian posterior probabilities were computed in MrBayes 3.2.1 x86 (Ronquist *et al*., 2012) using the non-partitioned 18S rDNA alignment, and the *rbc*L alignment partitioned into each of the three codon positions. In both cases, we performed two Markov chain Monte Carlo (MCMC) runs for 10^6 generations with one cold and three heated chains under the proposed evolutionary models, with trees sampled every 100 generations and a burn-in of 25%. After 10^6 generations, the average standard deviation of split frequencies dropped below 0.008, and the potential scale reduction factor (PSRF) approached 1.000 – 1.003 for convergence diagnostic parameters. For visualization of the phylogenetic trees, we used the program FigTree v1.4.2 (Rambaut, 2007). Sequence comparisons based on numbers of different nucleotides were conducted using the software MEGA6 (Tamura *et al*., 2013).

ITS2 rDNA secondary structure analysis

The ITS2 region between 5.8S and 28S rDNA flanking regions was annotated using the ITS2 database (Koetschan *et al*., 2012). Secondary structure models of the annotated ITS2 regions were folded based on the minimum energy criterion in the program RNAstructure 5.3 (Reuter & Mathews, 2010) and visualized by Varna 3.8 (Darty *et al*., 2009). The primary ITS2

sequences along with their secondary structures were aligned employing the ClustalW algorithm implemented in the program 4SALE 1.7. (Seibel *et al*., 2008). The sequence+structure alignment had 320 positions and was analyzed for the presence of compensatory base changes (CBCs; Wolf *et al*., 2013) among sequences in 4SALE 1.7.

Results

Morphology/Cytology

Cells of the new unidentified terrestrial Czech strain CAUP G 1401 have broad ellipsoidal or broad cylindrical, sometimes almost spherical cells, with a radially lobed chloroplast containing a central pyrenoid (Figs 1–3). Two equal flagella emerge from a low but prominent papilla, which is hemispherical, or cone-shaped with a round face (Fig. 3). The flagella are about 1.5 times as long as the cell. Two prominent contractile vacuoles are present below the papilla. The eyespot is elliptical lateral, located in the second third of the anterior end of the cell (Figs 2, 3). The eyespot is often camouflaged by the massive chloroplast (Fig. 1). Cell walls are smooth and robust. They are often somewhat remote from the protoplast, especially towards the posterior end of cells. Cell size of the strain CAUP G 1401 ranged from 16–27 μm in length and 11–22 μm in width. The alga reproduced asexually, forming two or four zoospores.

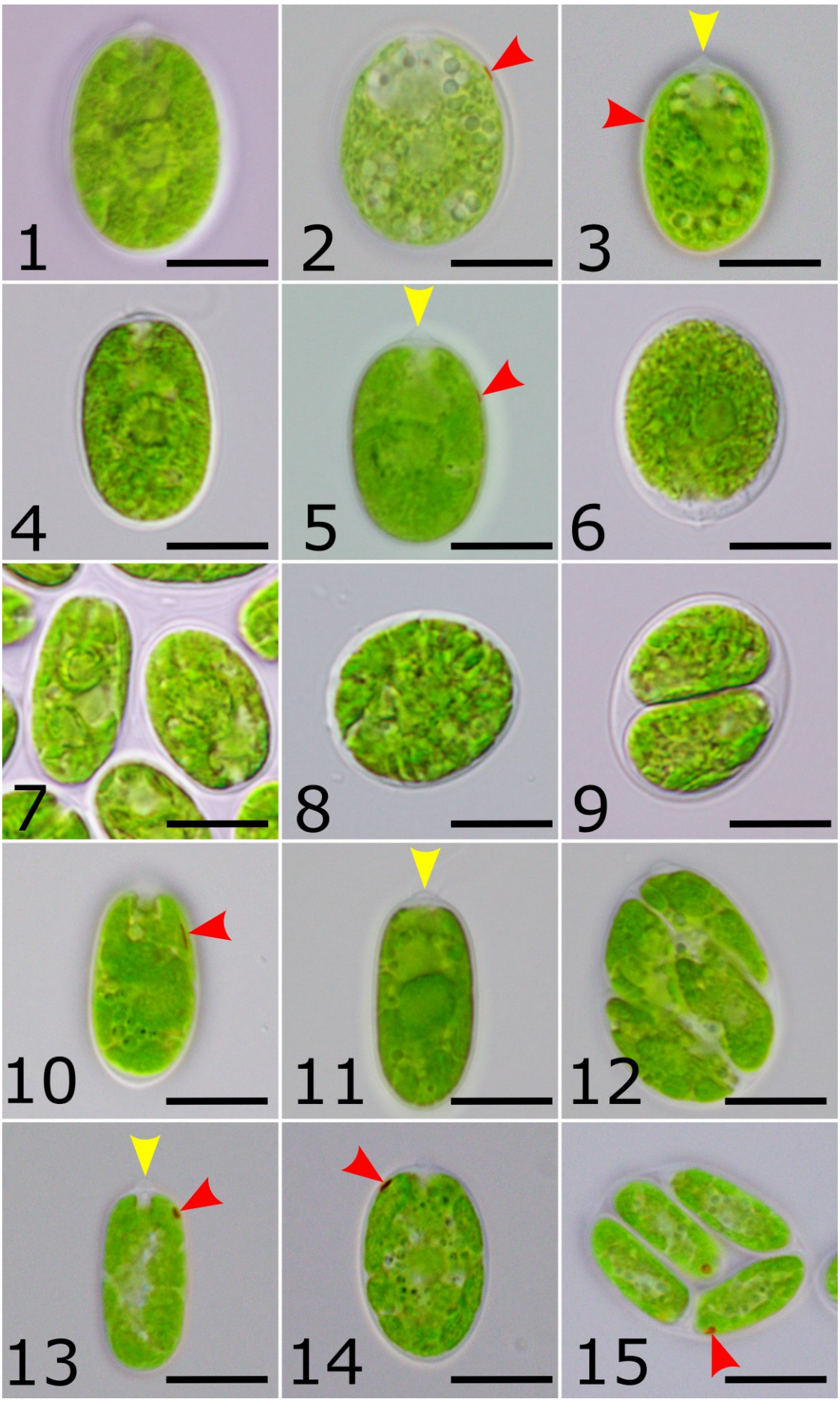
The curated strain of the terrestrial *Cd. chlorostellata* SAG 12.72 had broadly ellipsoidal or broadly cylindrical cells with a broadly rounded posterior end (Figs 4–6). Sometimes the cells were almost spherical. The cells had a rather thick outline and a low hemispherical or cone-shaped papilla with a round face. An extra layer of mother cell walls often coated the cells. Its chloroplast was radially lobed, and had a prominent pyrenoid in the middle (Figs 4–6). The eyespot was elliptical, located in the lateral position of the chloroplast, in the second or the last third of the cell's anterior end (Fig. 5). In mature cells it was often hidden (Figs 4, 6). The posterior end of the cell was sometimes not fully filled by the chloroplast and appeared empty

(Fig. 6). Two equal-length flagella, longer than the cell, were present. Cells of *Cd. chlorostellata* SAG 12.72 were 15–27 μm long, and 9–22 μm wide. The strain reproduced asexually, forming two to four zoospores.

The second terrestrial Czech isolate, the authentic strain of the species *Cd. chlorostellata var. gracillima* SAG 25.87 has broadly ellipsoidal or cylindrical (Fig. 7), sometimes almost spherical cells (Fig. 8). Its cell walls are thick, with a distinct hemispherical papilla in the front. Cells have 2 flagella, which are approximately as long as the cell. The chloroplast is composed of large parietal lobes, some of which extend towards the cell's center, and surround the pyrenoid (Fig. 7). In mature cells, the chloroplast was highly divided and asteroid-shaped (Fig. 8). Occasionally, cells were observed to contain two pyrenoids (Fig. 7). An ellipsoidal or slightly roundish eyespot was present in the lateral anterior end of the chloroplast, in the first third of the anterior end of the cell. The eyespot was often hidden. Cells were 12–22 μm in length, and 7–18 μm in width. The alga reproduced by forming two, four, or eight zoospores (Fig. 9).

The authentic strain of freshwater species *Cd. meslinii* SAG 75.81 has ellipsoidal or cylindrical cells with rounded ends (Figs 10, 11). Sometimes the posterior end of the cell is pointed. A small distinct hemispherical papilla is present in the front (Fig. 11). Cells possessed 2 equal flagella of variable length: shorter than, equal to, or longer than the cell. A contractile vacuole was located close to each flagellar base. The cell walls are approximately as thick as the membranes. The chloroplast filled most of the cell's volume and was composed of radially emerging lobes, along with additional parietal or central ellipsoidal discs (Figs 10, 11). A large pyrenoid was located approximately in the middle of the cell. A long, thin, string-like eyespot is present at the lateral margins of the chloroplast, in the second third of the cell's anterior (Fig. 10). Cells are 16-27 μm long, and 7–20 μm wide. Asexual reproduction forming two or four zoospores was observed (Fig. 12).

By contrast, cells of the freshwater Arctic strain CAUP G 1302 are ellipsoidal or ellipsoidal-cylindrical, occasionally ovoid (Figs 13, 14). They have two equal flagella about as long as the cell, and a thin cell wall with a hemispherical papilla (Fig. 13). Sometimes the top of the papilla is flat. The chloroplast is parietal and cup-shaped, composed of multiple parietal lobes, which are always connected to adjacent lobes (Figs 13, 14). The eyespot is located in the lateral anterior end of the chloroplast, in the first third of the anterior end of the cell, and is D-shaped or ellipsoidal (Figs 13–15). The pyrenoid is absent. Numerous vacuoles occupy as much as half of the cell by volume (Figs 13, 14). Cells are 10–20 μm long, and 5–13 μm wide. Asexual reproduction yields four or eight zoospores (Fig. 15).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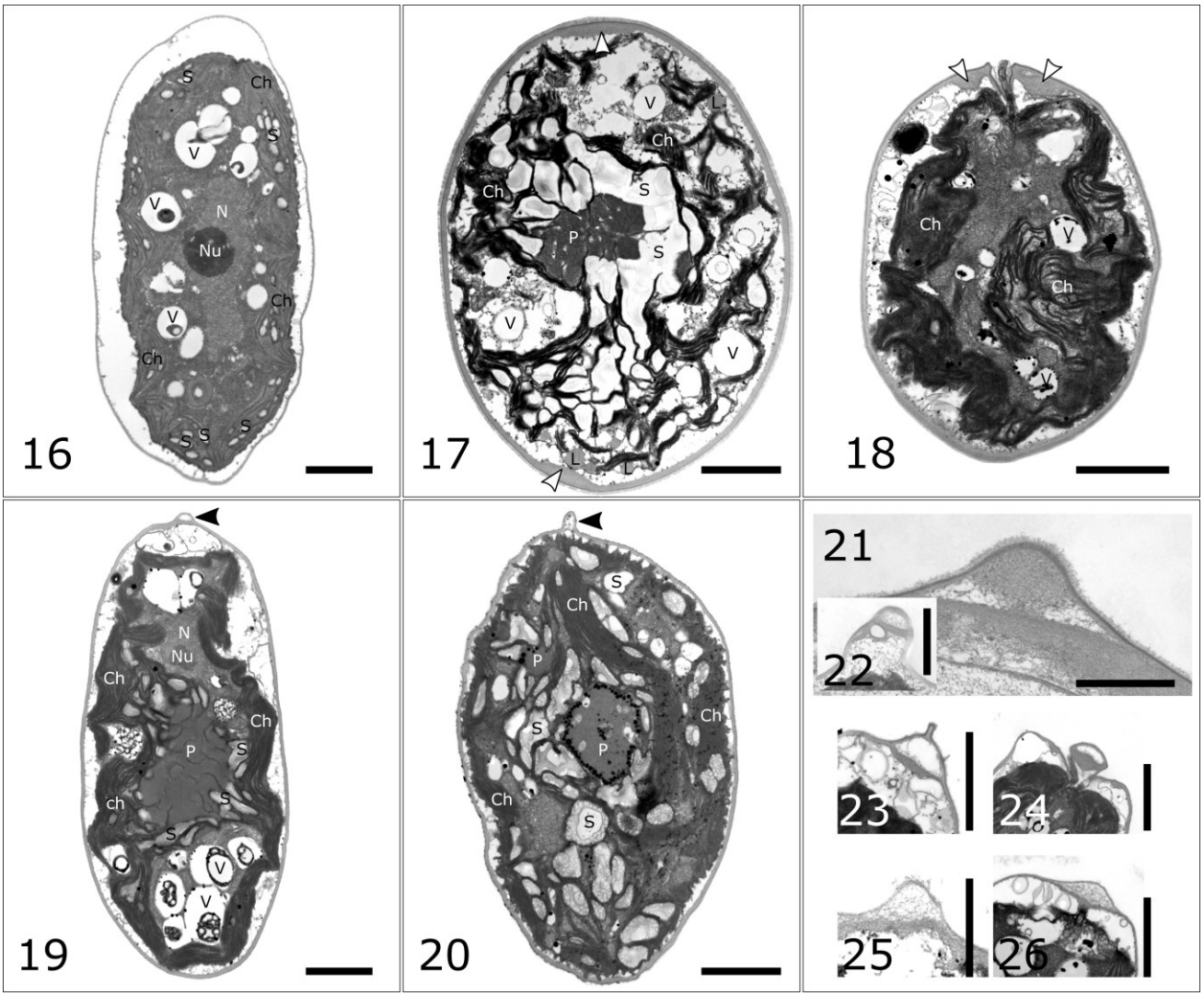
Figs 1–15. Morphology and reproduction of studied strains. **Figs 1–3.** *Os. chlorostellata* CAUP G 1401. **Figs 4–6**. *Os. chlorostellata* SAG 12.72. **Figs 7–9.** *Cr. gracillima* SAG 25.87. **Figs 10–12**. *Os*. *meslinii* SAG 75.81. **Figs 13–15.** *Cr. reticulata* CAUP G 1302. Yellow arrowheads indicate papilla, red arrowheads indicate eyespot. Scale bars = 10 μm.

Ultrastructure

TEM confirmed observed differences in chloroplast exteriors in the studied strains (Figs 16–20). For example, only the Arctic strain CAUP G 1302 had a cup-shaped chloroplast with parietal lobes that did not enter the cell center, and lacked a pyrenoid (Fig. 16). The other examined strains possessed a chloroplast radiating from the cell periphery towards its center, where a pyrenoid was located (Figs 17–20). In the new Czech isolate CAUP G 1401, and in *Cd. chlorostellata* SAG 12.72 chloroplast thylakoids undulated along the periphery of the chloroplast envelope, and extended radially where they surrounded the pyrenoid, which was spherical or subspherical in shape (Fig. 17). The chloroplast of *Cd. meslinii* SAG 75.81 had rather distinct peripheral lobes, some of which extended radially and encircled the pyrenoid (Fig. 19). By contast, *Cd. chlorostellata* var. *gracillima* SAG 25.87 had both radially and upright stretching chloroplast thylakoids, forming a net-like structure in which the pyrenoid was entangled (Fig. 20). For a detailed pyrenoid description see below.

The cell walls of the investigated strains were smooth and without ornamentation (Figs 16–20). Presence of cells with thick walls was confirmed in the strains CAUP G 1401, *Cd. chlorostellata* SAG 12.72 and *Cd. chlorostellata* var. *gracillima* SAG 25.87. The cell walls of CAUP G 1401 and *Cd. chlorostellata* SAG 12.72 sometimes had prominent anterior and posterior thickenings (Figs 17, 18). TEM confirmed the presence of hemispherical papillae in all examined strains (Figs 17, 19–26). However, the shape and expression of the papilla was variable within the same strain (Figs 21, 22). A nucleus with a prominent nucleolus was located in the cell center in the Arctic strain CAUP G 1302 (Fig. 16), while in the other strains

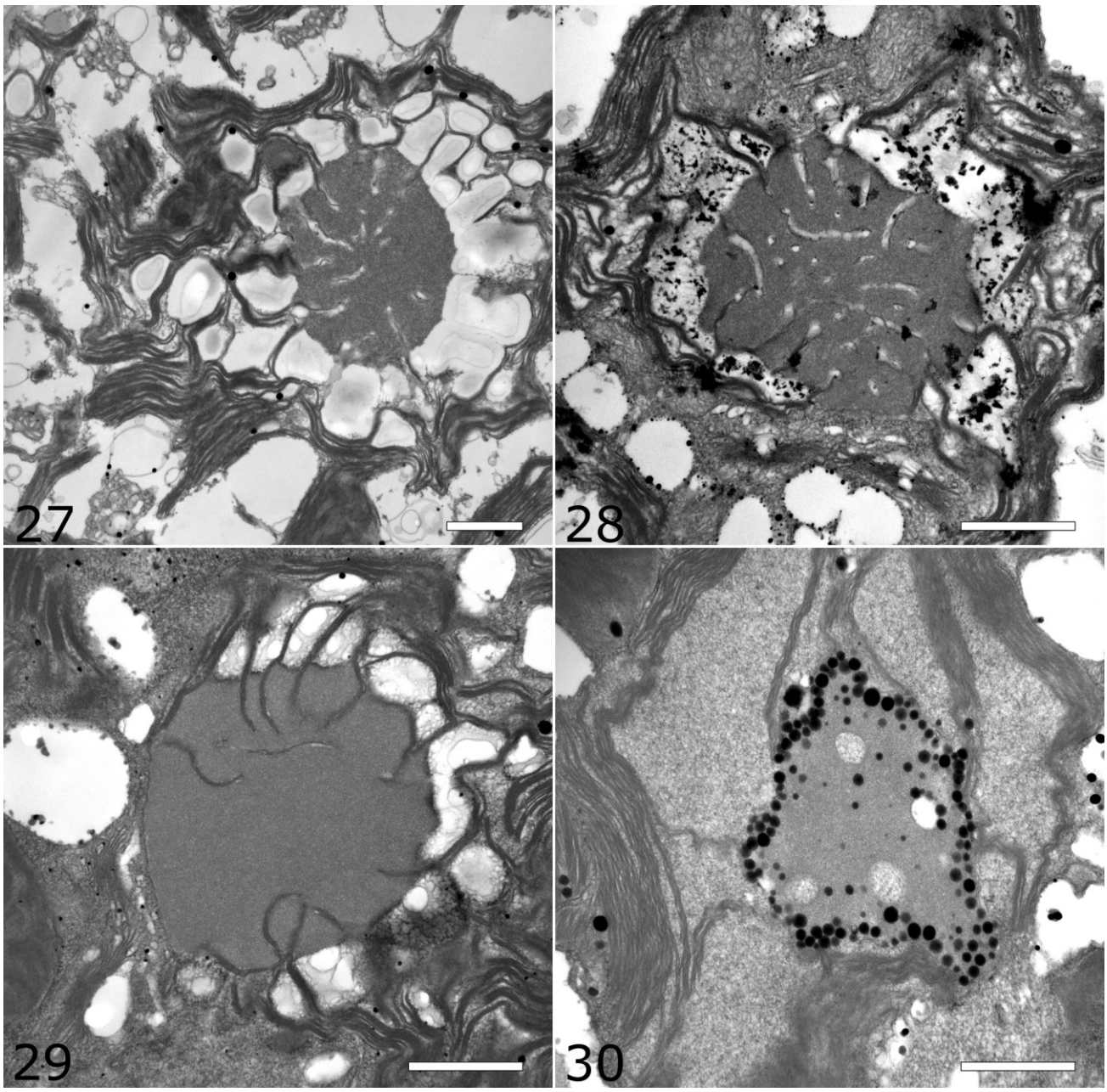
it was placed above the pyrenoid (Fig. 19). All investigated strains had numerous cytoplasmic vacuoles, some of which contained electron-dense structures (Figs 16–19). Lipid globules were detected in the cytosol only in strains CAUP G 1401 and *Cd. chlorostellata* SAG 12.72 (Fig. 17).



Figs 16–26. Transmission electron micrographs of studied strains. **Fig. 16.** *Cr. reticulata* CAUP G 1302 **Fig. 17.** *Os. chlorostellata* CAUP G 1401 **Fig. 18.** *Os. chlorostellata* SAG 12.72 **Fig. 19.** *Os. meslinii* SAG 75.81 **Fig. 20.** *Cr. gracillima* SAG 25.87. Papillae of strains: **Figs 21–22.** CAUP G 1401 **Fig. 23.** SAG 12.72 **Fig. 24** SAG 75.81 **Fig. 25.** SAG 25.87. **Fig. 26.** CAUP G 1302. White arrowheads indicate cell wall thickenings, black arrowheads indicate papillae. Abbreviations: Ch, chloroplast; L, lipid droplet; N, nucleus, Nu, nucleolus;

P, pyrenoid; S, starch; V, vacuole. Scale bars **Figs 21, 25** = 1 μm **Figs 16–20, 22–24**, **26** = 2 μm.

The pyrenoids of strains CAUP G 1401 and *Cd. chlorostellata* SAG 12.72 were surrounded by numerous starch plates and granules, and penetrated by both thylakoids and fine starch grains (Figs 27, 28). The pyrenoid of strain SAG 75.81 was aslo surrounded by starch, and thylakoids penetrated the pyrenoid's matrix, but starch grains usually did not (Fig. 29). The pyrenoid of strain *Cd. chlorostellata* var. *gracillima* SAG 25.87 was either surrounded by starch plates and grains (Fig. 20) or naked (Fig. 30). In addition, numerous plastoglobuli surrounded the pyrenoid, which was not observed in any other strain investigated in this study. Several starch grains were present in the pyrenoid matrix in *Cd. chlorostellata* var. *gracillima* SAG 25.87 as well (Fig. 30). Sometimes two or three pyrenoids were observed within a single cell in each of the four pyrenoid-containing species. Of note, the pyrenoid-less Arctic strain CAUP G 1302 never contained starch granules as large as those in other strains, just fine grains scattered within the interthylakoidal spaces (Fig. 16).



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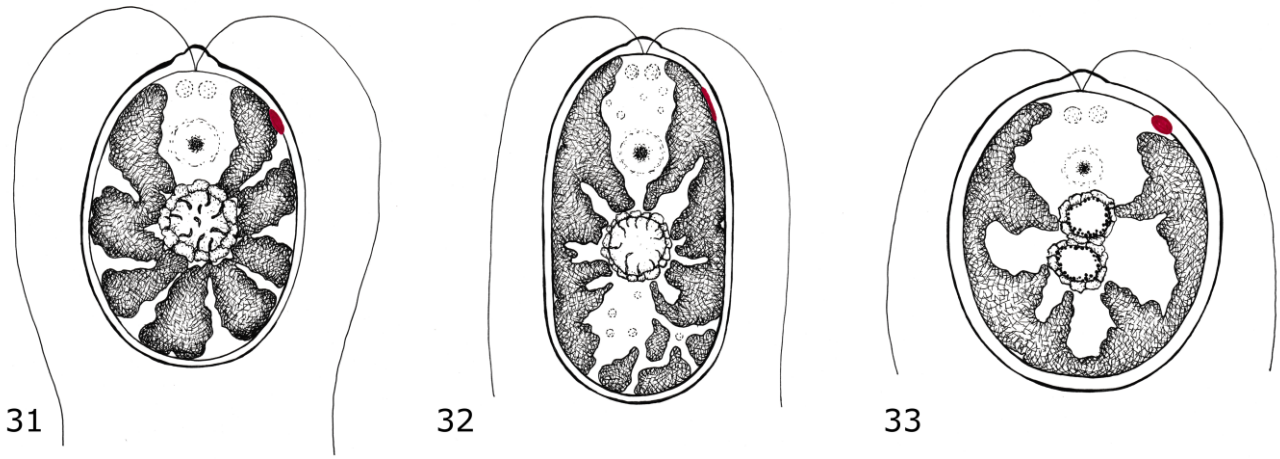
Figs 27–30. Pyrenoid ultrastructure. **Fig. 27.** *Os. chlorostellata* CAUP G 1401 **Fig. 28.** *Os. chlorostellata* SAG 12.72 **Fig. 29.** *Os. meslinii* SAG 75.81 **Fig. 30.** *Cr. gracillima* SAG 25.87. Scale bars = 1 μm.

Line drawings of the species re-examined by light and transmission electron microscopy are shown in Figs 31–33.



Figs 31–33. Line drawings of re-examined species. **Fig. 31.** *Os. chlorostellata* **Fig. 32.** *Os. meslinii* **Fig. 33.** *Cr. gracillima.*

Molecular phylogeny based on 18S rRNA and* rbc*L genes

Phylogenetic analysis of 18S rDNA revealed that the five strains investigated in this study clustered into two main phylogenetic lineages within the *Chloromonadinia* phylogroup (Fig. 34). In the 18S rDNA phylogenetic tree, all strains were nested within a highly diversified clade 1 (*sensu* Hoham *et al*., 2002), which was unlikely to be monophyletic. The new Arctic strain CAUP G 1302 of the species *Cr. reticulata*, and *Cd. chlorostellata* var. *gracillima* (hereafter *Cr. gracillima*) SAG 25.87 clustered within the core *Chloromonas* clade, with moderate support for being monophyletic. The strains of the newly proposed genus *Ostravamonas* (CAUP G 1401 and SAG 12.72 as *Os. chlorostellata* and SAG 75.81 as *Os. meslinii*) were placed as sister lineages to *"Cd." moewusii* var. *microstigmata* CCAP 11/108 and *Cr. pseudoplatyrhyncha* NIES-2563 (Fig. 34). Comparison of 1687 nucleotide positions within the 18S rDNA between the newly isolated *Cr. reticulata* strain CAUP G 1302 and the authentic strain CCAP 11/110 revealed just one nucleotide difference. *Chloromonas gracillima* SAG 25.87 differed from its closest revealed relative *"Cystomonas"* sp. SAG 35.97 by one nucleotide substitution as well. The two strains of *Os. chlorostellata* SAG 12.72 and

CAUP G 1401 differed from *"Cd." moewusii* var. *microstigmata* CCAP 11/108 by 16 or 17 nucleotide substitutions, respectively, and by one nucleotide from each other. The closest known relative of *Os. chlorostellata* strains SAG 12.72 and CAUP G 1401 was the newly sequenced *Os. meslinii* SAG 75.81, which differed from them by 24 or 25 nucleotide substitutions, respectively. *Ostravamonas meslinii* SAG 75.81 differed by a considerable number of nucleotide substitutions (18 nucleotides) from *"Cd." moewusii* var. *microstigmata* CCAP 11/108. Assignment of the whole *Ostravamonas* clade and *"Cd." moewusii* var. *microstigmata* CCAP 11/108 was strongly statistically supported. Assignment of *Cr. pseudoplatyrhyncha* NIES-2563 as its sister lineage has moderate statistical support (Fig. 34).

By contrast to the 18S rDNA phylogenetic tree topology, the *rbc*L-based phylogeny statistically supported monophyly of both clade 1 and the core *Chloromonas* clade (Fig. 35). In addition, the three *Ostravamonas* strains (i.e. CAUP G 1401, SAG 12.72, SAG 75.81) clustered out of clade 1 (Fig. 35). Similar to the 18S rDNA inference, *Cr. pseudoplatyrhyncha* NIES-2563 appeared to be closely related to the three strains mentioned above, but failed to achieve statistical support. *Ostravamonas chlorostellata* CAUP G 1401 and SAG 12.72 were identical in their *rbc*L sequence (936 aligned nucleotides) and differed from *Os. meslinii* SAG 75.81 by 52 nucleotide substitutions. Meanwhile, *Cr. gracillima* SAG 25.87 differed from the most similar sequence of *Cr. chlorococcoides* SAG 72.81 by 19 nucleotide substitutions, but the 2 sequences do not supportedly form a monophyletic clade (Fig. 35). Despite multiple efforts to amplify the *rbc*L sequence of *Cr. reticulata* CAUP G 1302, we failed to obtain any analyzable *rbc*L fragment. However, as we will show in the following ITS2 comparisons, the strain is phylogenetically closely related to *Cr. reticulata* CCAP 11/110 (Fig. 36), as was also demonstrated by 18S rDNA phylogeny (Fig. 34).

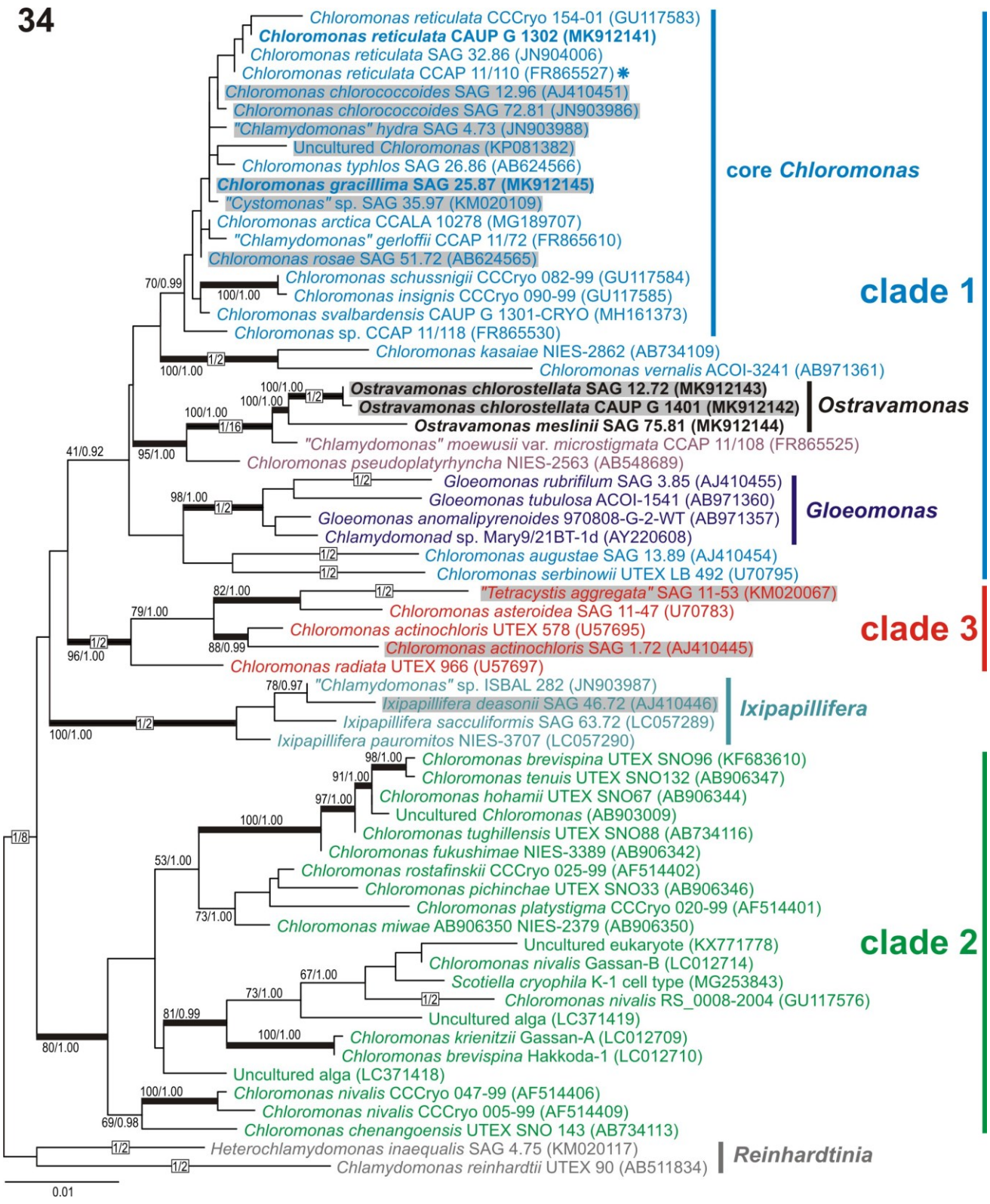


Fig. 34. Maximum-likelihood tree of the *Chloromonadinia* phylogroup based on 18S rDNA sequences. Numbers next to branches indicate statistical support values: ML bootstraps and BI posterior probabilities. Thick lines indicate branches with statistical support: ML ≥ 80 / BI ≥ 0.95. *Chloromonas* clades 1, 2, and 3 were delimited according to Hoham *et al*. (2002) and adopted in the previous studies of Barcytė *et al*. (2018a, b). New sequences are in bold. Grey

underlined strains were isolated from terrestrial habitats. The asterisk indicates the authentic strain of *Cr. reticulata*, which is the type species of the genus *Chloromonas*.

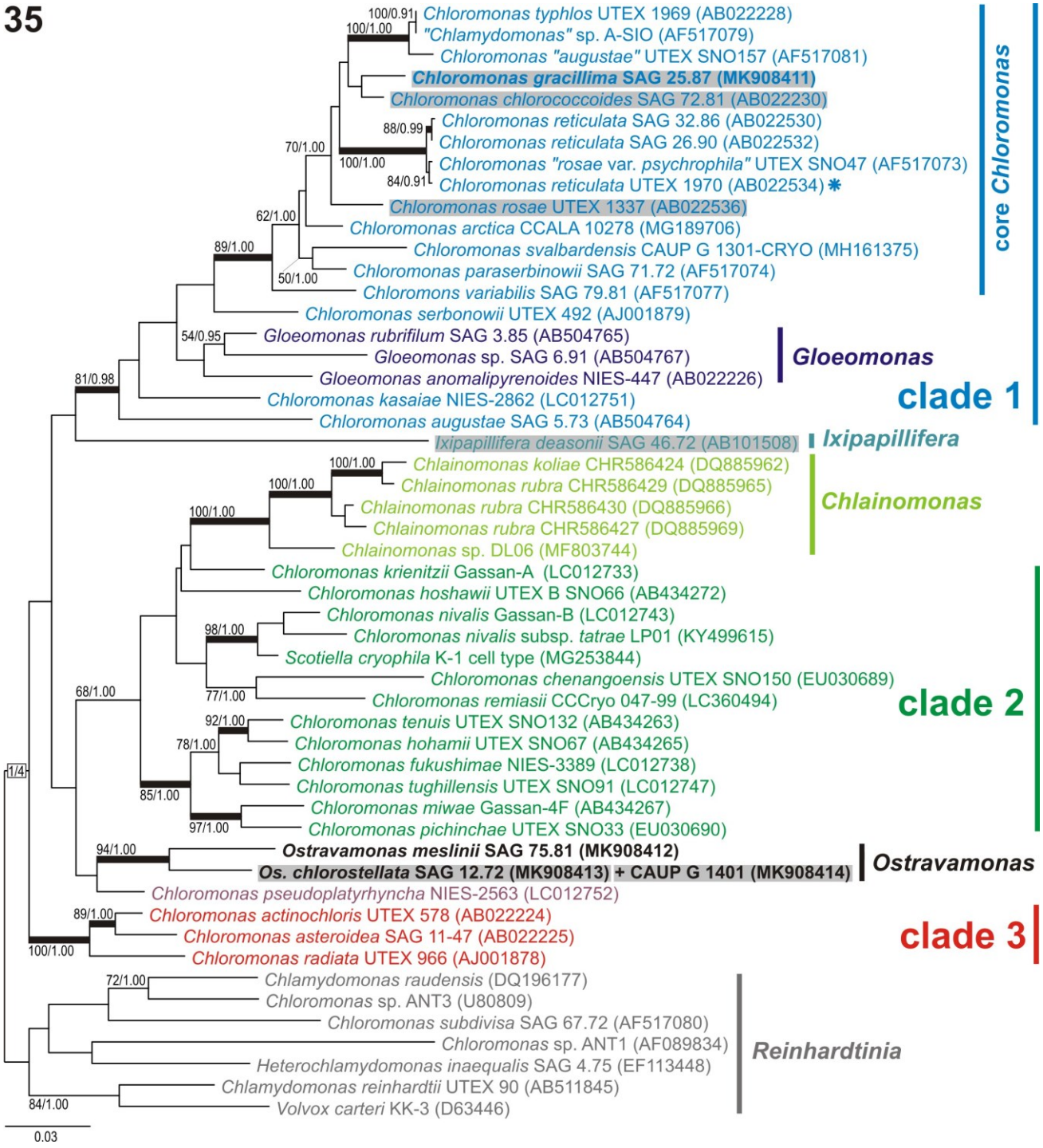


Fig. 35. Maximum-likelihood tree of the *Chloromonadinia* phylogroup based on *rbc*L sequences. Numbers next to branches indicate statistical support values: ML bootstraps and BI posterior probabilities. Thick lines indicate branches with statistical support: ML ≥ 80 / BI ≥ 0.95. *Chloromonas* clades 1, 2, and 3 were delimited according to Hoham *et al*. (2002) and

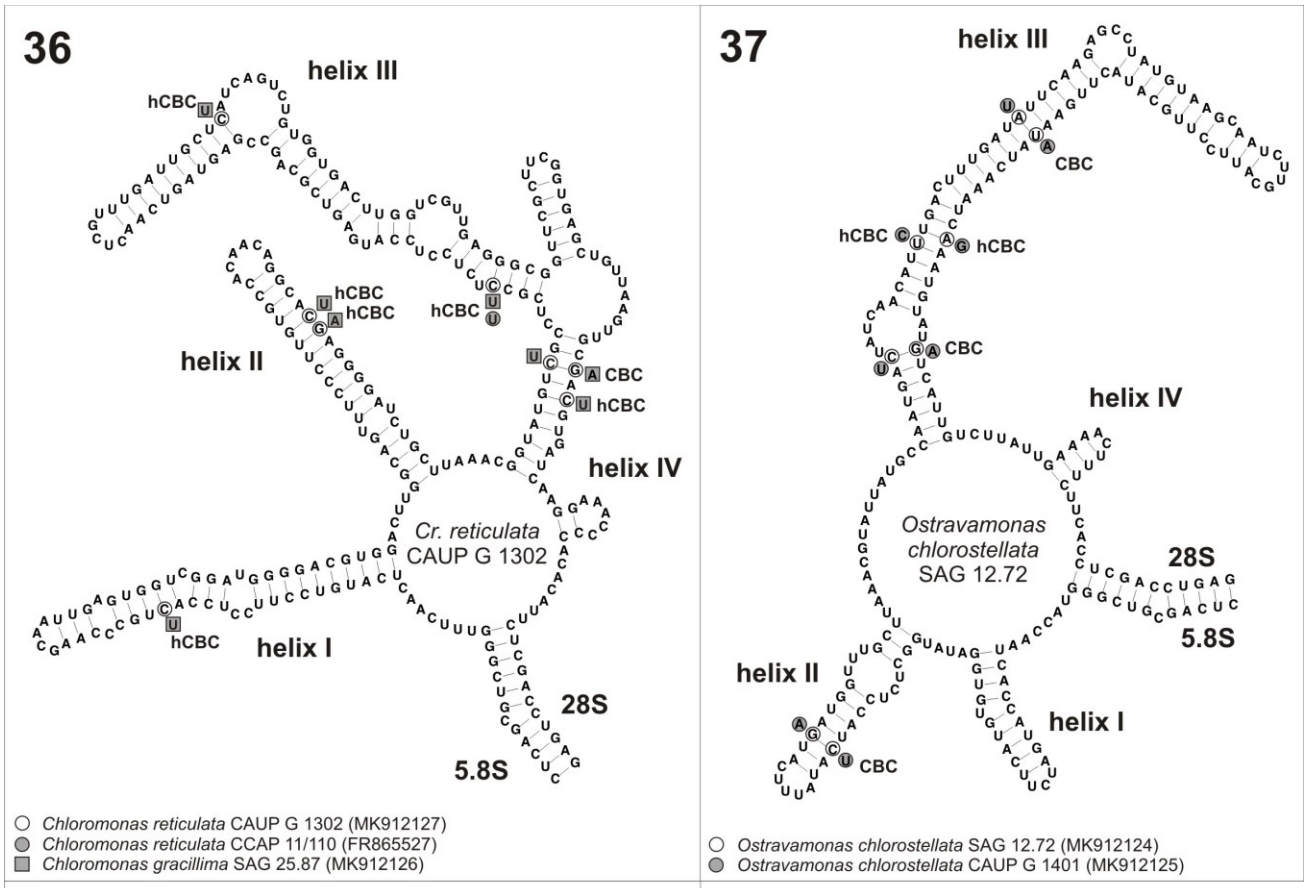
adopted in the previous studies of Barcytė *et al*. (2018a, b). New sequences are in bold. Grey underlined strains were isolated from terrestrial habitats. The asterisk shows the authentic strain of *Cr. reticulata*, which is the type species of the genus *Chloromonas*.

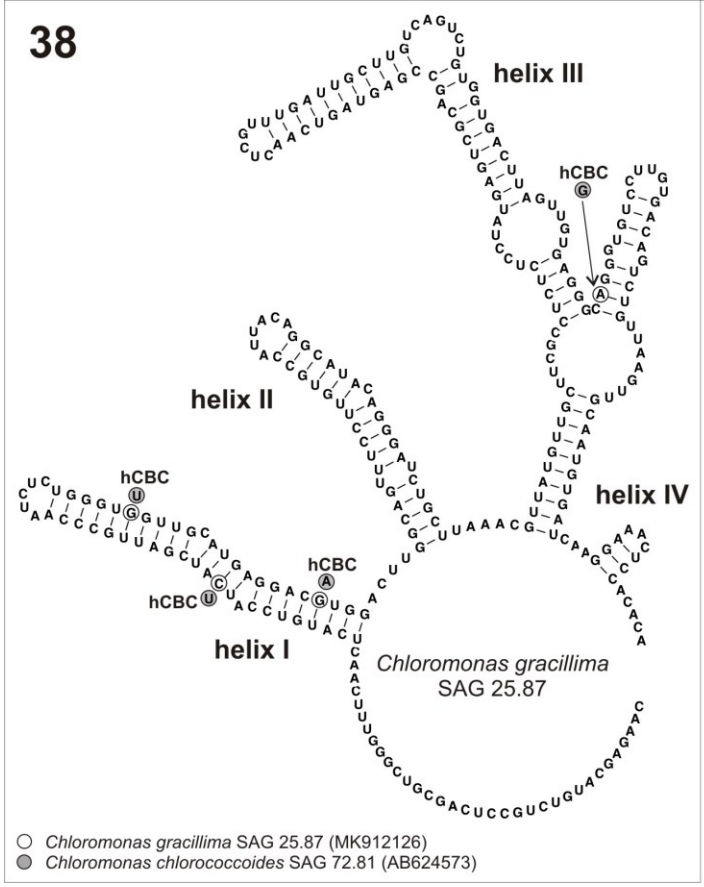
Comparison of ITS2 rDNA secondary structures

Comparing 275 aligned nucleotides of the core *Chloromonas* strains, *Cr. reticulata* CAUP G 1302 and *Cr. gracillima* SAG 25.87, identified 38 nucleotide substitutions in the primary sequences of the ITS2 region. The two sequences exhibited one compensatory base change (CBC; helix III; Fig. 36) and six one-sided mutations (hCBC) when their secondary structure models were compared. The strain CAUP G 1302 differed by three nucleotide substitutions (including one hCBC) from the authentic strain of *Cr. reticulata* CCAP 11/110 and also from *Cr. rosae* var. *psychrophila* UTEX SNO47. Meanwhile, *Cr. gracillima* SAG 25.87 was most similar (with one nucleotide difference) to an uncultured Chlamydomonadales clone 750 (GenBank accession number MF481961) isolated from soil.

Ostravamonas chlorostellata SAG 12.72 and CAUP G 1401, which had identical *rbc*L sequences and almost identical 18S sequences, differed by 13 nucleotides within the ITS2 regions (231 alignable positions). The nucleotide substitutions between SAG 12.72 and CAUP G 1401 comprised also three compensatory base changes (CBC; 1x helix II; 2x helix III; Figure 37) and two hCBC (helix III). In the case of *Os. meslinii* SAG 75.81, even repeated amplifications of the ITS region failed to reveal the analyzable full-length ITS2 sequence. We succeeded in sequencing a 63 base pair fragment, which could be annotated as 27 bp of the 5.8S, and 36 bp of the very beginning of the ITS2 region. This short sequence was most similar to *"Chlamydomonas"* sp. CCAP 11/122 (six nucleotide differences) and *"Cd." moewusii* var. *microstigmata* CCAP 11/108 (eight nucleotide differences), in agreement with the above described phylogenetic placement based on the 18S rDNA molecular marker.

Chloromonas gracillima SAG 25.87, which had *rbc*L sequence most similar to *Cr. chlorococcoides* SAG 72.81 (Fig. 35), differed from this strain by 21 nucleotides within the ITS2 region (251 alignable positions), comprising four hCBCs (Fig. 38).





Figs 36–38. Secondary structure models of the nuclear ITS2. **Fig. 36.** An ITS2 secondary structure model of the strain *Cr. reticulata* CAUP G 1302 isolated in the Arctic compared

with two closely related strains of the core *Chloromonas* lineage. The strain CCAP 11/110 represents the authentic strain of *Cr. reticulata*, and the strain SAG 25.87 represents *Cr. gracillima*. Differences in double-strand compensatory base changes (CBC) and single strand compensatory base changes (hCBC) are marked as grey circles (CCAP 11/110) or squares (SAG 25.87) **Fig. 37.** ITS2 secondary structure model of *Ostravamonas chlorostellata* SAG 12.72 compared to the conspecific strain CAUP G 1401. Differences in double-strand compensatory base changes (CBC) and single strand compensatory base changes (hCBC) are marked as grey circles **Fig. 38.** ITS2 secondary structure model of *Chloromonas gracillima* SAG 25.87 compared to the closely related *Cr. chlorococcoides* strain SAG 72.81. Differences in double-strand compensatory base changes (CBC) and single strand compensatory base changes (hCBC) are marked as grey circles.

New taxonomic proposals

Ostravamonas Barcytė & Hodač, *gen. nov.*

DESCRIPTION: Vegetative cells are ellipsoidal, broadly ellipsoidal, or broadly cylindrical, and occasionally spherical. Cells possess 2 equivalent flagella of variable length, a robust cell wall with a smooth surface, a low but prominent hemispherical or cone-shaped papilla with a round face, 2 apical contractile vacuoles. Chloroplasts are single and radially lobed, or radial with additional discs. The pyrenoid is central, with thylakoids entering its matrix, and surrounded by starch plates and granules. The eyespot is elliptical or string-like, and located in the lateral anterior end of the chloroplast, in the second or last third of the anterior end of the cell. The nucleus is above the pyrenoid. The posterior end of the cell often stands away from the protoplast. Cells reproduce asexually by forming 2 or 4 biflagellate zoospores. Sexual reproduction is unknown.

IDENTIFICATION: The genus exhibits a set of unique morphological features, and is supported by molecular phylogenetic analyses using 18S-ITS1-5.8S-ITS2 rDNA, and *rbc*L sequence comparisons (including ITS2 secondary structures).

ETYMOLOGY: the name refers to the City of Ostrava where the genus was rediscovered.

TYPE SPECIES: *Ostravamonas chlorostellata* (E.A. Flint & H. Ettl) Barcytė & Hodač

***Ostravamonas chlorostellata* (E.A. Flint & H. Ettl) Barcytė & Hodač, *comb. nov.* (Figs 4–6, 18, 28)**

BASIONYM: *Chlamydomonas chlorostellata* E.A. Flint & H. Ettl, 1966, *New Zealand Journal of Botany* **4**: 420–422; fig. 2 (descr. et ic. prima, iconotypus).

EPITYPE (designated here): The authentic SAG 12.72 strain is permanently cryopreserved in a metabolically inactive state in the Culture Collection of Algae at Göttingen University (SAG) in Germany.

TYPE LOCALITY: Tekoa, Southern Alps at Bealey, New Zealand

NOTE: Additional strain CAUP G 1401 is housed in the Culture Collection of Algae of Charles University in Prague (CAUP), Czechia

***Ostravamonas meslinii* (Bourelly) Barcytė & Hodač, *comb. nov.* (Figs 10–12, 19, 29)**

BASIONYM: *Chlamydomonas meslinii* Bourrelly, 1951, *Hydrobiologia* **3**: 258; pl. 3 fig. 52 (descr. et ic. prima, iconotypus).

EPITYPE (designated here): The authentic strain SAG 75.81 is permanently cryopreserved in a metabolically inactive state in the Culture Collection of Algae at Göttingen University (SAG) in Germany.

TYPE LOCALITY: Alpine garden of the National Museum in Paris, France

***Chloromonas gracillima* (H. Ettl) Barcytė & Hodač, *comb. nov.* (Figs 7–9, 20, 30)**

BASIONYM: *Chlamydomonas chlorostellata* var. *gracillima* H. Ett, 1976, *Nowa Hedwigia* **49**: 401–402; pl. 61, fig. 1 (descr. et ic. prima, iconotypus).

EPITYPE (designated here): The authentic SAG 25.87 strain is permanently cryopreserved in a metabolically inactive state in the Culture Collection of Algae at Göttingen University (SAG) in Germany.

TYPE LOCALITY: Hrubý Jeseník mountains, Czechia

Discussion

Morphological comparison and search for distinctive features

The newly isolated volvocalean flagellate strain CAUP G 1401, falling within the *Chloromonadinia* phylogroup and constituting the novel genus *Ostravamonas*, exhibited several morphological differences from the monophyletic genus *Chloromonas* (=core *Chloromonas* clade; Figs 34, 35), who's members usually possess a cup- or urn-shaped chloroplast with parietal lobes, and generally lack pyrenoids (e.g., Barcytė *et al*., 2018a, b). For example, the morphology of the new Arctic isolate CAUP G 1302 fit well within such circumscription. In addition, the shape of papilla along with the position and shape of the eyespot hinted at its affiliation with the *Chloromonas* type species, *Cr. reticulata* (Matsuzaki *et al*., 2012). Their conspecifity was also supported by phylogenetic analysis of 18S rRNA gene sequences (Fig. 34), and confirmed by ITS2 secondary structure analysis (Fig. 36). A few exceptions do exist (Matsuzaki *et al*., 2012). For example, the re-examined *Cr. gracillima* SAG 25.87 had a parietal and radially lobed chloroplast, and one to two pyrenoids (Fig. 33).

When compared with other taxa, *Os. chlorostellata* CAUP G 1401 morphologically resembled numerous species of *Chlamydomonas* Ehrenberg *sensu lato* (Ettl 1976, 1983). For example, it was similar to *Cd. radiata* Deason & Bold, *Cd. actinochloris* Deason & Bold, *Cd. augustae* var. *eupapillata* H. Ettl, *Cd. macrostellata* J.W.G. Lund, *Cd. gerloffii* H. Ettl or *Cd. spinosa* H. Ettl in bearing a massive, radially lobed chloroplast with a centrally located

pyrenoid. However, *Cd. radiata* and *Cd. actinochloris* do not bear a papilla, and the nucleus is located below the pyrenoid in the former. *Cd. augustae* var. *eupapillata* and *Cd. spinosa* have clearly-expressed papillae as large as that of the newly investigated strain CAUP G 1401 of the species *Os. chlorostellata*. However, the papilla of *Cd. augustae* var. *eupapillata* has a flat keel-shaped face, while *Cd. spinosa* has a cone-shaped papilla with an acute face. *Cd. macrostellata* and *Cd. gerloffii* have much smaller hemispherical papillae, while the papilla of *Cd. hydra* is keel-shaped. Other morphological comparisons, including shape of the chloroplast, shape and position of the eyespot, position of the pyrenoid and nucleus, and cell size, are summarized in Table 2.

The most morphologically similar species to the newly studied strain CAUP G 1401 was *Cd. chlorostellata*. The authentic strain (SAG 12.72) of this species was available for comparison (see results section). Apart from the similar chloroplast outline (Figs 1–6), the two isolates had the same shape of papilla and eyespot and the same type of pyrenoid (Figs 27, 28), and they both shared an empty posterior end of their cells, along with anterior and posterior cell wall thickenings, suggesting conspecificity of the two strains. The molecular phylogenetic results confirmed that the strain SAG 12.72 belongs among the *Chloromonadinia*, and also is a close relative of the strain CAUP G 1401 (Figs 34, 35). Since *Chlamydomonas* is polyphyletic, and species in clades other than the clade containing the type species *Cd. reinhardtii* should be transfered to other genera (Pröschold *et al*., 2001), we have classified both strains SAG 12.72 and CAUP G 1401 as *Os. chlorostellata* gen. et comb. nov. Moreover, our investigation of the morphologically similar species *Cd. meslinii* SAG 75.81 confirmed its close phylogenetic relationships to the discussed species, resulting in its accommodation within the genus *Ostravamonas* as well (Figs 34, 35).

Of note, numerous species within the genus *Chlamydomonas sensu lato*, such as the aforementioned *Cd. radiata*, *Cd. actinochloris* and *Cd. augustae* var. *eupapillata* have been transferred to the genus *Chloromonas* (Pröschold *et al*., 2001). They are divided among

Chloromonas clade 1 and clade 3 *sensu* Hoham *et al*. 2002 (Figs 34, 35). Furthermore, the previous (Barcytė *et al*., 2018a, b) and the current 18S rDNA-based phylogenetic analyses demonstrated that the authentic strains of *Cd. gerloffii* CCAP 11/72 and *Cd. hydra* var. *micropapillata* SAG 4.73 are part of the core *Chloromonas* clade, as is the currently re-examined *Cr. gracillima* SAG 25.87. This shows that morphological identification of the genus *Chloromonas* is not that simple, and that the core *Chloromonas* clade contains great morphological diversity (Table 2). The electron dense globules surrounding the pyrenoid which we detected only in *Cr. gracillima* SAG 25.87 (Fig. 30) was also reported for *Cr. chlorococcoides* (Matsuzaki *et al*., 2012), supporting the close affiliation of the two taxa (Figs 34, 35).

Several paraphyletic lineages share very similar morphological and ultrastructual characteristics (plesiomorphic features) within the *Chloromonadinia*. As a consequence, light microscope based identification of the related taxa is not always straightforward. However, the search for morphological and ecological differences is an important complement to phylogenetic species delimitations. For example, *Os. chlorostellata* differs from the species of the *Chloromonas* clade 3 and *Cr. augustae* by a set of features (Table 2) and by often having a posterior cell end that stands away from the protoplast (Fig. 6). Meanwhile, *Os. meslinii* has a radial chloroplast with additional discs, which has not been reported for any other species within the *Chloromonadinia*. In addition, the sister lineage of *Ostravamonas*, *Cr. psedoplatyrhyncha* NIES-2563, has ovoid to spherical cells with a cup-shaped chloroplast, and multiple atypical pyrenoids, including a large D- or rod-shaped eyespot positioned in the lateral central part of the cell (Matsuzaki *et al*., 2010). Moreover, *Chlainomonas* and *Gloeomonas* have a set of unique morphological features, effectively discriminating them from among other *Chloromonadinia* microalgae (Fig. 35).

Since morphology of the Volvocalean green algae can be confusing, attempts to molecularly characterize and match new isolates with historical strains, and re-examination authentic strains is of particular importance in revising algal taxonomy.

Interpreting Chloromonadinia *diversity using conserved and variable molecular markers*

The phylogenetic species concept dominates algal taxonomy, with 18S rDNA and *rbc*L being the most used molecular markers to date. Phylogenetic analyses employing the two markers support the existence of three main *Chloromonadinia* superclades (Figs 34, 35), although topologically consistent between markers, sometimes lacking statistical support. In addition, they do not contradict the delineating of smaller monophyletic clades, such as, for example, the core *Chloromonas*, *Gloeomonas* or *Ixipapillifera* (Figs 34, 35). On the other hand, the sister relationships of the clades are not robust, and might vary using additional sequences or even concatenated data (see discussion in Barcytė *et al*., 2018a).

Delineation of closely related species, especially sister species, using 18S rDNA and *rbc*L is not straightforward (Figs 34, 35). The ITS2 rDNA, especially its secondary structure analysis, is the most popular tool to help resolve ambiguous sister relationships at the molecular level (e.g., Wolf *et al*., 2013; Matsuzaki *et al*., 2012; Barcytė *et al*., 2018a, b). For instance, ITS2 rDNA secondary structure analysis strongly supports classification of morphologically different *Cr. reticulata* CAUP G 1302 and *Cr. gracillima* SAG 25.87 as separate species (Fig. 36).

According to the ITS2/CBC approach, the two *Ostravamonas* strains CAUP G 1401 and SAG 12.72 should be considered distinct species as well (Fig. 37). However, one nucleotide difference in the 18S rDNA sequences (Fig. 34) and identical *rbc*L sequences (Fig. 35), together with similar morphology, ultrastructure and ecology (acidic habitats; see discussion below) do not support such separation. While ITS2 is useful for investigating closely related species, as discussed above, use of this marker to delineate species without additional

supporting data might be tricky. For example, morphologically different *Cr. gracillima* SAG 25.87 and *Cr. chlorococcoides* SAG 72.81 are well separated by 18S phylogeny (Fig. 34), closely related by *rbc*L phylogeny (Fig. 35), and the CBC species concept does not support thei representation as different species (Fig. 38).

Table 2. Morphological comparison of similar *Chloromonadinia* species. Data taken from Ettl (1983) unless otherwise indicated.

Species	Chloroplast	Papilla	Stigma	Pyrenoid	Nucleus	Length x width (µm)
Ch. chlorostellata[a]	lobed and appears to be stellate	relatively large, clear, hemispherical	large, elliptical or slighty rounded, lies in the middle or towards the apical end of the cell	central and surrounded by a number of small starch grains	above pyrenoid	18-24 x 11-17
Ch. chlorostellata* var. *gracillima[b]*	central part not so massive, lobes much delicate and narrow; spaces between lobes much larger	distinct, large, broadly hemispheric	significantly smaller	with larger starch grains	above pyrenoid	12-20 x 5-10
Ch. meslinii	composed of radiating lobes which become wider close to cell wall and divide into small elliptical discs	hemispherical, very small but very distinct	long, string-like	axial, located below the lower second third of the cell	above pyrenoid	20-22 x 15
Ch. radiata	asteroid-shaped; divided by deep and wide incisions into several radial lobes	without	oblong, in the front third of the cell on one of the lobes	big, spherical, ± central	below pyrenoid	12-21 x 7,5-13
Ch. actinochloris	very massive, radially divided and lobed by deep cuts; lobes relatively thick, irregular, with the widest part close to cell wall	without	in the first third of the cell, small	big, spherical, central	above pyrenoid	10-20 x 4-9
Ch. augustae	in principal cup-shaped but with radiating lobes from the cell center	very low, barely noticeable, obtuse keel-shaped	Stigma in the middle of a cell height, small, elliptical	spherical, central	above pyrenoid	8-18 x 5-13
Ch. macrostellata	massive, radially lobed and asteroid-shaped with lobes unevenly distributed	low and little	small line-shaped, often difficult to see, in the first third of the cell	broadly ellipsoidal to shperical, central	above pyrenoid	14-19 x 8-12
Ch. gerloffii*	consists of a prominent central part, from which several irregular lobes emerge radially	sharp low hemispherical	in half height or more in front, small, elliptical	big, broadly ellipsoidal, central, also indistinct	above pyrenoid	10-18 x 8-18
Ch. spinosa	Chloroplast divided by deep incisions in several irregular lobes, which are wider radially and peripherally	large and sharp, pointed conical or spike-like	in the anterior cell half, oblong spot-like, sometimes pale	wide ellipsoidal to polygonal, central	above pyrenoid	9-18 x 6-14
Ch. hydra	parietal, asteroid-shaped; lobes widely separated from each other and irregularly curved	low but distinct, rounded keel-shaped	large, elliptic, in the anterior cell half	broadly ellipsoidal to spherical, in the posterior half of the cell	above pyrenoid	9,5-14 x 6-12
Ch. hydra* var. *micropapillata[b]*	parietal, asteroid-shaped with a prominent central part from which lobes radiate to all sides	kiel-shaped and smaller	very small, elliptic, in the last third of the anterior cell end	broadly ellipsoidal, central, slighly belowusually below cell centre	above pyrenoid	9,5-14 x 4,5-13,5

[a] Filnt & Ettl, 1966

[b] Ettl, 1976

*species falling within the core *Chloromonas* clade (Figs 34, 35)

Direct sequencing of the amplified ITS2 region of the strain CAUP G 1401 identified several ambiguous positions (i.e. single nucleotide polymorphisms) in independent reads, suggesting the presence of several ITS2 paralogues. For example, Stat *et al*. (2011) showed that the use of ITS2 to delineate different *Symbiodinium* (Dinophyceae) is problematic due to *Symbiodinium* carrying multiple copies of ITS2, and displaying intra-genomic variability. Divergent intragenomic ITS2 paralogues were also revealed in *Heterococcus* (Xantophyceae; Rybalka *et al*., 2013) and several Eustigmatophyceae strains (Kryvenda *et al*., 2018). On the other hand, three CBCs has never been reported for the same species as has been found in the *Os. chlorostellata* strains CAUP G 1401 and SAG 12.72. The fact that *Os. chlorostellata* represents a species covering a large distribution area, that is comprised of genetically diverse local populations may offer an explanation for this puzzle. On the other hand, it is not true for *Cr*. *reticulata*, which shows widespread distribution, occuring in cold environments including the Arctic and Antarctic. Discovery and isolation of other related strains could better characterize the ongoing diversification of the *Os. chlorostellata* lineage.

Identification of the sister lineages *Os. chlorostellata*, and *Os. meslinii* SAG 75.81 as a separate taxon is well supported by 18S rDNA and *rbc*L gene analyses (Figs 34, 35). We repeatedly sequenced both flanking regions of its annotatable 5.8S-ITS2 rDNA fragment, and the resulting high-quality sequences did not match any algae or related organism. Based on that, we conclude that the strain SAG 75.81 contains large intronic or pseudogenous indels within its ITS regions. This may constitute additional evidence supporting the unique phylogenetic placement of this microalga.

In conclusion, the value of conserved molecular markers should not be underestimated. They still provide the first crucial insights into algal taxonomy. The ITS2/CBC concept provides complementary information for species delimitation, but it should not be taken for granted because different algal groups and taxa have different evolutionary histories. More detailed exploration of both ITS1 and ITS2 multi-copy species could improve our

understanding of the evolution of *Chloromonadinia*, and all Volvocales. Moreover, morphology, and especially ecology are important criteria for circumscribing species, and should get more attention in current molecular data based taxonomy papers.

Terrestrial lifestyle within the Chloromonadinia

Members of the *Chloromonadinia* phylogroup with terrestrial lifestyles are still very little understood. Apart from the studied *Cr. gracillima* SAG 25.87, terrestrial members of the core *Chloromonas* clade may include *Cr. rosae* SAG 51.72 isolated from soil in the High Tatra Mountains (Slovakia). It is also possible that the organism originated in the snowfields on the mountain, considering its close relative, *Cr. reticulata*. Meanwhile, *Cr. chlorococcoides* strains SAG 12.96 and SAG 72.81 were obtained from soils in Germany and Australia, respectively. *"Chlamydomonas" hydra* SAG 4.73 was isolated from soil in the beech forest in Czechia, while *"Cystomonas"* sp. SAG 35.97 was isolated from a branch of a spruce tree in Germany. *Chloromonas actinochloris* SAG 1.72 and *"Tetracystis aggregata"* SAG 11-53 were found in dry soils in the USA and Mexico, respectively. Both organisms belong to the mesophilic *Chloromonas* clade (clade 3). In addition, *Ixipapillifera deasonii* SAG 46.72 was isolated from soil in the USA (Fig. 35).

Ostravamonas chlorostellata strains CAUP G 1401 and SAG 12.72 were isolated from acidic terrestrial habitats. The pH of the tree bark where the strain CAUP G 1401 was discovered is unknown. However, residues of trees on the Heřmanice spoil heap are acidic enough to accomodate acidophilic algal species (Barcytė *et al*., 2018c). The strain SAG 12.72 was obtained from acidic soil (pH = 5.3) (Flint & Ettl, 1966). Since these two strains were isolated from different hemispheres, the genus might have a worldwide distribution, and new isolates of *Ostravamonas* might be discovered in other acidic habitats. *Os*. *meslinii* is a freshwater species, but since it was isolated from a ditch, we can not reject the possibility that the alga occurrs in terrestrial habitats as well.

The low number of terrestrial species and strains is probably due to limited sampling efforts rather than rarity of such lifestyles among the *Chloromonadinia*. More detailed studies of *Chloromonas*-related microalgae could improve understanding of evolution driven by ecological speciation, and colonization of terrestrial environments by *Chloromonadinia* algae.

Disclosure statement

No potential conflicts of interest are reported by the authors.

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Author contributions

D. Barcytė: concept of the paper, isolation and cultivation of strains, light and electron microscopy, molecular lab work, drafting and editing of manuscript; L. Hodač: analyses of molecular data, drafting and editing of manuscript; L. Nedbalová: reading of the draft and financial support.

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Paper VIII

BARCYTĖ D, PILÁTOVÁ J, MOJZEŠ P & NEDBALOVÁ L (unpublished manuscript) The Arctic green alga *Cylindrocystis* (Zygnematophyceae, Streptophyta) is genetically and morphologically diverse with an effective accumulation of polyphosphate. *Journal of Phycology*, under review

Authors' contributions:

DB designed the study, isolated and cultivated the strains, did the molecular lab work and light microscopy, analyzed the molecular data, and wrote the paper, JP performed confocal laser scanning and Raman microscopy, PM wrote the Raman-related result and discussion parts, LN read the draft of the paper

THE ARCTIC GREEN ALGA *CYLINDROCYSTIS* (ZYGNEMATOPHYCEAE, STREPTOPHYTA) IS GENETICALLY AND MORPHOLOGICALLY DIVERSE WITH AN EFFECTIVE ACCUMULATION OF POLYPHOSPHATE[1]

Dovilė Barcytė[2]

Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2, 128 44, Czechia

Jana Pilátová

Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, Prague 2, 128 44, Czechia

Peter Mojzeš

Faculty of Mathematics and Physics, Institute of Physics, Charles University, Ke Karlovu 5, Prague 2, 121 16, Czechia

Linda Nedbalová

Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2, 128 44, Czechia

[2]Author for correspondence: e-mail dovile.barcyte@gmail.com.

Running title: Diversity of the Arctic *Cylindrocystis*

Abstract

The green algal genus *Cylindrocystis* is widespread in various types of environments, including extreme habitats. However, very little is known about its diversity, especialy in the polar regions. In the present study, we have isolated seven new *Cylindrocystis*-like strains from terrestrial and freshwater habitats in Svalbard (High Arctic). We aimed to compare the new isolates on molecular (*rbc*L and 18S rDNA), morphological (light and confocal laser scanning microscopy) and cytological (Raman spectroscopy) basis. Our results have demonstrated that the Arctic *Cylindrocystis* was not of monophyletic origin and that the studied strains clustered within two (tentatively named soil and freshwater/glacier) clades and four separate lineages. The morphological data (cell size, shape and chloroplast morphology) supported the presence of several distinct taxa among the new isolates. Moreover, the results have shown that the Arctic *Cylindrocystis* strains were closely related to strains originating from the temperate zone, indicating high ecological versatility and successful long-distance dispersal of the genus. A huge amount of inorganic polyphosphate (polyP) grains were detected within the chloroplasts of the cultured Arctic *Cylindrocystis* strains, suggesting an effective luxury uptake of phosphorus. This study represents the first attempt to combine molecular, morphological, ecological and biogeographical data of the Arctic *Cylindocystis*. Our novel cytological observations could also partly explain the success of *Cylindrocystis*-like microalgae in the polar regions.

Key index words: *Cylindrocystis*, diversity, ecology, morphology, phylogeny, polar habitats; Raman microscopy

Abbreviations: BI, Bayesian inference; BSC, biological soil crust; ML, maximum likelihood; P_i, inorganic phosphorus

Introduction

The streptophytic green algae or charophytes (Streptophyta, Viridiplantae) and particularly the conjugating green algae of the class Zygnematophyceae have been demonstrated to be the closest relatives of land plants (Wodniok et al. 2011, Ruhfel et al. 2014). The increased scientific attention in terms of stress-tolerance, adaptation, molecular diversity and evolution has been thus paid to this particular algal group (Pichrtová et al. 2014a, b, 2016, 2018, Herburger et al. 2015, 2016, de Vries et al. 2016, 2018, Rippin et al. 2017, Holzinger et al. 2018). The obtained such data help to better elucidate the colonization of terrestrial environments by streptophytic algae and the origin of all embryophytes. For example, desiccation tolerance is the key trait in the emergence of life on land and zygnematophytes have been shown to withstand the intense and prolonged periods of water shortage (Pichrtová et al. 2014a, b, Herburger et al. 2015, Rippin et al. 2017).

The Zygnematophyceae encompass morphologically diverse green algae, ranging from non-motile unicells or unbranched filaments to small colonial forms. They are widely distributed and found mostly in freshwaters, including limnoterrestrial habitats, such as ephemeral pools. Apart from the periodic desiccation exposure, zygnematophytes can also survive low pH and temperature, high visible light and UV radiation, and low nutrient conditions (Hall and McCourt 2017). Since the first land plants were likely exposed to such environmental stresses (Wodniok et al. 2011), diversity studies of streptophytes in extreme environments are an important research direction. For example, using the stress of low temperature and high light, de Vries et al. (2018) demonstrated that Zygnematophyceae have many of the stress-signaling factors known from land plants, i.e. embryophyte stress signaling evolved already in their algal progenitors. That said, little explored zygnematophytes found living in challenging environmental conditions could hold the key to the terrestrialization and our Earth as it is.

Polar habitats include the aforementioned physical extremes and constitute some of the harshest environments on Earth. Nevertheless, Zygnematophyceae constitute a successful group of organisms both in the Arctic and Antarctic. For example, zygnematophytes form excessive scum mats in hydro-terrestrial environments (Pichrtová et al. 2018) and thrive on bare ice of glaciers (Yallop et al. 2012, Lutz et al. 2018). However, despite their indisputable role as primary producers and influence on acceleration of ice sheet melting (Cook et al. 2019), little is known about diversity and ecology of the polar streptophytic microalgae. This is due, in part, to limited sampling efforts along with difficulties in cultivating cold-adapted microorganisms. One of such enigmatic microorganisms in the polar regions is the green alga *Cylindrocystis*.

Cylindrocystis is a genus of saccoderm green microalgae (known as desmids) systematically placed in the family Mesotaeniaceae of the order Zygnematales (Zygnematophyceae). It accommodates ecologically versatile photosynthetic microorganisms inhabiting a wide range of environments. For example, *Cylindrocystis* is commonly found in acidic oligotrophic freshwaters of the temperate zone, especially peat bogs (Coesel and Meesters 2007, Štěpánková et al. 2012) or in subaerial habitats (Škaloud 2009). It is also considered a typical ice-specialist and has been reported from glaciers in both southern and northern hemispheres (Takeuchi and Kohshima 2004, Stibal et al. 2006, Nedbalová and Sklenář 2008, Yallop et al. 2012, Lutz et al. 2018). In contrast, *Cylindrocystis* has also been detected in hot and dry desert crust communities (Flechtner et al. 1998, Lewis and Lewis 2005).

A number of *Cylindrocystis* species have been described based on morphological characteristics of vegetative cells and zygospore structure (Lütkemüller 1913, Prescott et al. 1972). However, their taxonomic status to this day is unclear. *Cylindrocystis* (C.) *brebissonii*, the type species of the genus, is probably the most frequently reported taxon. Several varieties of this species have been described based on morphological peculiarities and habitat type. For

example, an infraspecific epithet *cryophila* was proposed by Kol (1942) to distinguish the cryophilic *C. brebissonii* thriving on ice fields and snow banks from the aquatic one, whereas Flechtner et al. (1998) established a variety *deserti* to accommodate *C. brebissonii* occurring in dry Mexico soils. Other described varieties include, for example, epithets *minor* (now *C. gracilis*), *curvata*, *jenneri* or *turgida* referring to different cell dimensions and zygospore shape of *C. brebissonii* (Prescott et al. 1972, Lenzenweger 2003).

Despite the wide distribution, *Cylindrocystis* still stays a cryptic taxon aiming for enlightening. For example, only very few studies based on molecular data provided insights into the genetic diversity of the genus *Cylindrocystis* and showed its complex phylogenetic structure (Hall et al. 2008, Gontcharov and Melkonian 2010). The authors demonstrated that *Cylindrocystis sensu lato* represents a group of polyphyletic taxa, intermixed with members of the traditional family Zygnemataceae, encompassing filamentous genera, such as *Zygnema*, *Zygnemopsis* or *Mougeotia*. In addition, there is no molecular data of the cultured polar *Cylindrocystis* and knowledge of *Cylindrocystis* morphotypes in the High Arctic is restricted mostly just to the aforementioned *C. brebissonii* var. *cryophila* (Kol 1942). However, *Cylindrocystis* might not be that simple and veil hidden biodiversity. For example, Pichrtová et al. (2018) revealed extremely high molecular diversity of the Arctic *Zygnema*, a close relative of the genus *Cylindrocystis*. On the other hand, Ryšánek et al. (2016) demonstrated relatively low diversity of another streptotytic alga *Klebsormidium* (Klebsormidiophyceae) in the polar regions.

Since Arctic *Cylindrocystis* has not been studied in detail yet, the establishment of its cultures is a valuable approach for biodiversity studies, enabling to connect morphology, phylogeny and ecology. Here we have isolated and studied seven *Cylindrocystis* strains from different cold habitats in the High Arctic (Svalbard). We used genetical (18S rDNA and *rbc*L) and morphological/cytological (light, confocal laser scanning and Raman microscopy) attributes to compare the new isolates. We focused on the following questions: (i) How many

genotypes and morphotypes do new isolates from Svalbard represent? (ii) Do they share a monophyletic origin or have evolved independently? (iii) Are the uncovered genotypes unique to the Arctic or have they been detected elsewhere? (iv) Finally, what observed morphological/cytological traits could make them successful in the harsh Arctic habitats?

Material and methods

Origin, isolation, and cultivation of strains

Seven strains with *Cylindrocystis*-like morphology were newly isolated from soil and water samples collected in 2016 and 2018 in Spitsbergen (78°N, 16° E) (Table 1). It is the main and largest island of the Svalbard archipelago located in the Arctic Ocean and characterized by relatively mild climate compared to regions at the same latitudes due to the warm Atlantic West Spitsbergen Current (Walczowski and Piechura 2011). The mean temperatures vary from –12 ºC in winter to +5 ºC in summer and the area receives between 200 and 400 mm of precipitation annually (Førland et al. 2011). Five samples originated from Petuniabukta located in Billefjorden, where the Czech field camp "Nostoc" is established. The bay is surrounded by the Pyramiden, Mumien, and Svenbrehøgda mountain ranges and five small valley glaciers (Ferdinandbreen, Svenbreen, Hørbyebreen, Ragnarbreen and Ebbabreen). Two samples were taken on the Breinosa mountain close to the Foxfonna Glacier located on the southern side of the Adventdalen valley in Adventfjorden (Table 1). Small quantities of the collected field material were incubated for several weeks in BBM medium (Bischoff and Bold 1963) in CELLSTAR® 12 well multiwell plates (Greiner Bio-One, Kremsmünster, Austria). Cells of *Cylindrocystis* were isolated using a micropipette and the Nikon Diaphot 200 inverted phase contrast microscope (Tokyo, Japan). The isolated cells were continued to be grown in the multiwell plates in a Q-Cell 200 incubator (PolLab, Wilkowice, Poland) at 20 °C with a constant light of 20–30 μmol photons m^{-2} s^{-1}. For comparison, we have also purchased an additional strain from the Culture Collection of Algae at the University of Texas at Austin,

Cylindrocystis brebissonii var. *deserti* UTEX B 2684, isolated from the soil surface in the Baja California Desert, Mexico. All newly established strains are housed at the working collection at the Department of Ecology, Charles University, Prague.

DNA extraction, PCR, and DNA sequencing

For the total genomic DNA extraction, algal cells were disrupted using the bead-beating method with the Precellys®24 homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). The DNA was isolated using the Geneaid Plant Genomic DNA Mini Kit (New Taipei City, Taiwan). The ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) was amplified with primers RH1 and 1385R (McCourt et al. 2000). The 18S ribosomal DNA (rDNA) was amplified with primers NS1 and 18L (Hamby et al. 1988). The PCRs were done using the Qiagen Multiplex PCR Plus Kit (Hilden, Germany) in a total volume of 10 µl using the GenePro thermal cycler (BioER, Hangzhou, China). The PCR products were quantified on a 1% agarose gel, stained with the GelRed® (Biotium Inc., Fremont, CA), cleaned with ethanol and measured by the Thermo Scientific™ NanoDrop™ One UV-Vis spectrophotometer (Waltham, Massachusetts). The purified PCR products were Sanger sequenced by Macrogen (Amsterdam, the Netherlands) in both directions using the same primer pairs, including additional 18S rDNA sequencing primers 895R (Remias et al. 2012) and 1422R (5′-CTA AGG GCA TCA CAG ACC TG-3′; T. Friedl, unpublished). Sequencing reads were assembled and edited using the SeqAssem programme (Hepperle 2004). The sequences were submitted to the GenBank and are available under accession numbers XX000000 to XX000000.

Table 1. Origin of the Arctic *Cylindrocystis* strains.

Strain	Habitat	Locality	Coordinates
P2016	soil	BSC in abandoned settlement Pyramiden	78°39.40741', 016°23.83477'
P2018	soil	BSC in abandoned settlement Pyramiden	78°39.40640', 016°23.84109'
CWM	soil	wet hummock meadow in Petuniabukta	78°43.81667', 016°26.68333'
“Nostoc”	soil	Czech field station "Nostoc"	78°41.13321', 016°27.40408'
Sven	glacier	glacier Svenbreen	78°43.59360', 016°21.05400'
Fox6	freshwater/glacier	meltwater close to glacier Foxfonna	78°08.63810', 016°05.59993'
Fox7	freshwater/glacier	meltwater close to glacier Foxfonna	78°08.57846', 016°05.80122'

Sequence alignment, model selection and phylogenetic analyses

The BLAST algorithm (Altschul et al. 1997) was employed to search the GenBank database for sequences closely related to the newly sequenced strains. The previously published data by Gontcharov and Melkonian (2010) were also taken into account. For phylogenetic analyses 27 chloroplast *rbc*L sequences and 24 nuclear 18S rDNA sequences were acquired. Multiple sequence alignments of the *rbc*L and 18S rDNA sequences were computed separately using MAFFT 7.0 algorithm (Katoh et al. 2017). The alignments were checked and modified manually in the program BioEdit 7.2.5 (Hall 1999). The *rbc*L alignment comprised 34 sequences/1293positions and the 18S rDNA alignment comprised 31 sequences/1766 positions. We partitioned the *rbc*L dataset by the three codon positions, and the best nucleotide substitution models were estimated using the PartitionFinder2 (Lanfear et al. 2016) as implemented in the CIPRES Science Gateway 3.3 (Miller et al. 2010). The Bayesian Information Criterion (BIC) and the "greedy" heuristic algorithm along with the option of "linked branch lengths" were employed. The outcome suggested models TIM+I for the first codon position, TVMEF+I for the second codon position and HKY+I+G for the third codon position. Meanwhile, the nucleotide substitution model best fitting the 18S rDNA dataset was estimated with Modeltest 3.7 (Posada and Crandall 1998) in conjunction with PAUP* 4.0 (Swofford 2003). Based on the Akaike Information Criterion (AIC), the model MIFef was chosen as the best-fit model for the dataset. All phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI) on XSEDE user portal via CIPRES. The ML analysis for the partitioned *rbc*L dataset was computed using Garli 2.01 (Zwickl 2006) with 100 bootstrap replicates. The ML analysis for the non-partitioned 18S rDNA dataset was performed using RAxML 8.2.10 (Stamatakis 2014) with 1000 bootstrap replicates. The BI analyses were computed using MrBayes 3.2.6 (Ronquist et al. 2012). The set of proposed nucleotide substitution models was thus accordingly modified (e.g., RAxML applies only GTR+I and GTR+I+G models). Two Markov chain Monte Carlo runs (MCMC)

for 500,000 generations with trees sampled every 100 generations were performed in the BI. The convergence of parameters was checked with Tracer v1.7.1 (Rambaut et al. 2018). For each run, the first 25% of sampled trees were discarded as burn-in. Bayesian posterior probabilities were used to assess branch support of the Bayesian tree. For visualization of the phylogenetic trees, the program FigTree v1.4.2 was used (Rambaut 2007).

Microscopy

Vegetative cells were observed and documented using one-month-old cultures. Light microscopy was carried out using an Olympus BX43 light microscope (Tokyo, Japan). Microphotographs were taken with an Olympus DP27 digital camera (Tokyo, Japan). The Olympus micro imagining software cellSens 1.15 (Tokyo, Japan) was used for morphometric measurements of the strains. One hundred cells of each strain were measured for size comparisons. The length and width of the strains were graphed using Prism8 (GraphPad Software, San Diego, CA).

The 3D morphology of chloroplasts of *Cylindrocystis* strains was reconstructed using the laser scanning confocal microscope Leica TCS SP8 (Wetzlar, Germany) equipped with the data processing software Leica Application Suite (LAS X) with the final visualization in the form of a maximum projection and an optical cross-section through the middle of the cell. The diode-pumped solid-state (DPSS) laser was used as the source of excitation at the wavelength of 561 nm with the acquisition of the emission light of the chlorophyll *a* and *b* fluorescence in the range of 650–750 nm.

For *in situ* determination of the chemical composition of intracellular structures, a confocal Raman microscopy was used (Moudříková et al. 2017a, b). Algal cells harvested by centrifugation were mixed with 1% w/v solution of low-gelling agarose (T = 39 °C), immediately spread as a single-cell layer between a quartz slide and a coverslip and sealed with a CoverGrip sealant (Biotium, Fremont, CA). The agarose immobilization was used to

prevent movements of the cells during acquisition of Raman spectra. Two-dimensional Raman maps were obtained by a confocal Raman microscope WITec alpha300 RSA (Ulm, Germany), laser excitation 532 nm (20 mW power at the focal plane) and an Olympus oil-immersion objective UPlanFLN 100×, NA 1.30 (Tokyo, Japan). Using a scanning step of 200 nm in both directions, and an integration time of 100 ms per a cell voxel, Raman map of the cell of *Cylindrocystis* size was acquired within 30–45 min. To remove the strong autofluorescence of chlorophyll obscuring Raman spectra of photosynthetic microorganisms, a wide-area, low-power photobleaching of the entire cell by a defocused 532-nm laser beam was applied prior to the mapping, according to Moudříková et al. (2016). Raman chemical maps have been constructed by multivariate decomposition of the baseline-corrected spectra into the spectra of pure chemical components by using WITec Project Plus 5.1 software (Ulm, Germany).

Results

Phylogenetic analyses

Seven new strains with *Cylindrocysis*-like morphology isolated from terrestrial and freshwater habitats in the High Arctic were sequenced and analyzed. The performed phylogenetic analyses (ML and BI) based on the separate *rbc*L and 18S rDNA datasets were congruent in terms of uncovered clades, lineages and sister relationships (Figs 1, 2). Nevertheless, both tree topologies are presented to show that the *rbc*L molecular marker was more variable than the 18S rDNA (Figs 1, 2). For example, strains P2016 and CWM differed from each other by 30 nucleotides in the *rbc*L sequences but were identical in their 18S rDNA sequences. Similarly, strains Sven and Fox6 were different by eight nucleotides in the *rbc*L sequences but had identical 18S rDNA sequences. Another reason for presenting the two phylogenetic trees is that the two *Cylindrocystis* strains (ACOI 310 and UTEX 1259) revealed by the BLAST algorithm as close relatives of the three newly sequenced strains were represented by *rbc*L

sequences only (Fig. 1). Since one of our aims was to compare the new isolates with all available data, we considered it useful to analyze the two separate datasets with as many found close relatives as possible.

The Arctic *Cylindrocystis* strains investigated in this study, split into two main clades (tentatively named soil clade and freshwater/glacier clade based on the habitats from where the new strains originated) and four lineages. The soil clade encompassed two well-supported lineages, including the four *Cylindrocystis* isolates from Petuniabukta (Figs 1, 2). The first lineage of the soil clade was composed of the three new strains (P2018, P2016, CWM) and *Cylindrocystis brebissonii* var. *deserti* UTEX B 2684 isolated from the desert soil crust in Mexico. The most similar *rbc*L sequence to the UTEX strain was strain P2018 differing by 25 nucleotides. On the contrary, when 18S rDNA sequences were compared, strains P2016 and CWM were more similar to the UTEX strain (three nucleotide difference) than the strain P2018 (seven nucleotide difference). The second lineage of the soil clade included the new "Nostoc" strain along with two isolates (M 3004 and M 3019) from unknown habitats in Germany (though generally specified as freshwater; CCAC <www.ccac.uni-koeln.de>) and a desert isolate BCP-LG2-VF30 from the USA (Figs 1, 2). The two European strains were more similar to the "Nostoc" strain than the American one. That is to say, strains M 3004 and M 3019 differed from the "Nostoc" strain by 33 and 35 nucleotides, respectively, in the *rbc*L sequence and by 21 nucleotides in the 18S rDNA sequence. Meanwhile, the desert isolate BCP-LG2-VF30 showed 46 and 30 nucleotide differences in the chloroplast and nuclear sequences, respectively, when compared to the "Nostoc" strain.

The freshwater clade contained new strains isolated from the glacier ice (Sven) and meltwater (Fox6 and Fox7) close to the glacier. The strains Sven and Fox6 were much more closely related to each other than to the strain Fox7 and thus represented two independent phylogenetic lineages. The closest revealed relative of the strain Fox7 was the strain ACOI 55 originating from Portugal. They differed from each other by 12 and one nucleotides in the

*rbc*L and 18S rDNA sequences, respectively. Meanwhile, strains Sven and Fox6 grouped together with the strains M 2853 isolated from a peat bog in Austria, M 3025 originating from Germany, ACOI 310 isolated from a pond in Portugal, UTEX 1259 of unknown origin and SAG 615-1 from England. This grouping of strains was fully statistically supported (ML/BI: 100/1.00) in the *rbc*L phylogenies (Fig. 1). However, the same data cluster (excluding two strains) did not obtain any statistical support in the 18S rDNA phylogenetic trees (Fig. 2). The number of total variable sites within the aforementioned clade encompassing seven strains was 49 nucleotide positions in the *rbc*L alignment, and 20 positions among the five sequences in the 18S rDNA alignment. The most similar *rbc*L sequence to the strains Sven and Fox6 was the sequence of the strain UTEX 1259 differening by 11 and five nucleotides from the former strains, respectively. The 18S rDNA data comparisons revealed strain M 2853 as the most similar to the two Arctic strains with a difference of four nucleotides.

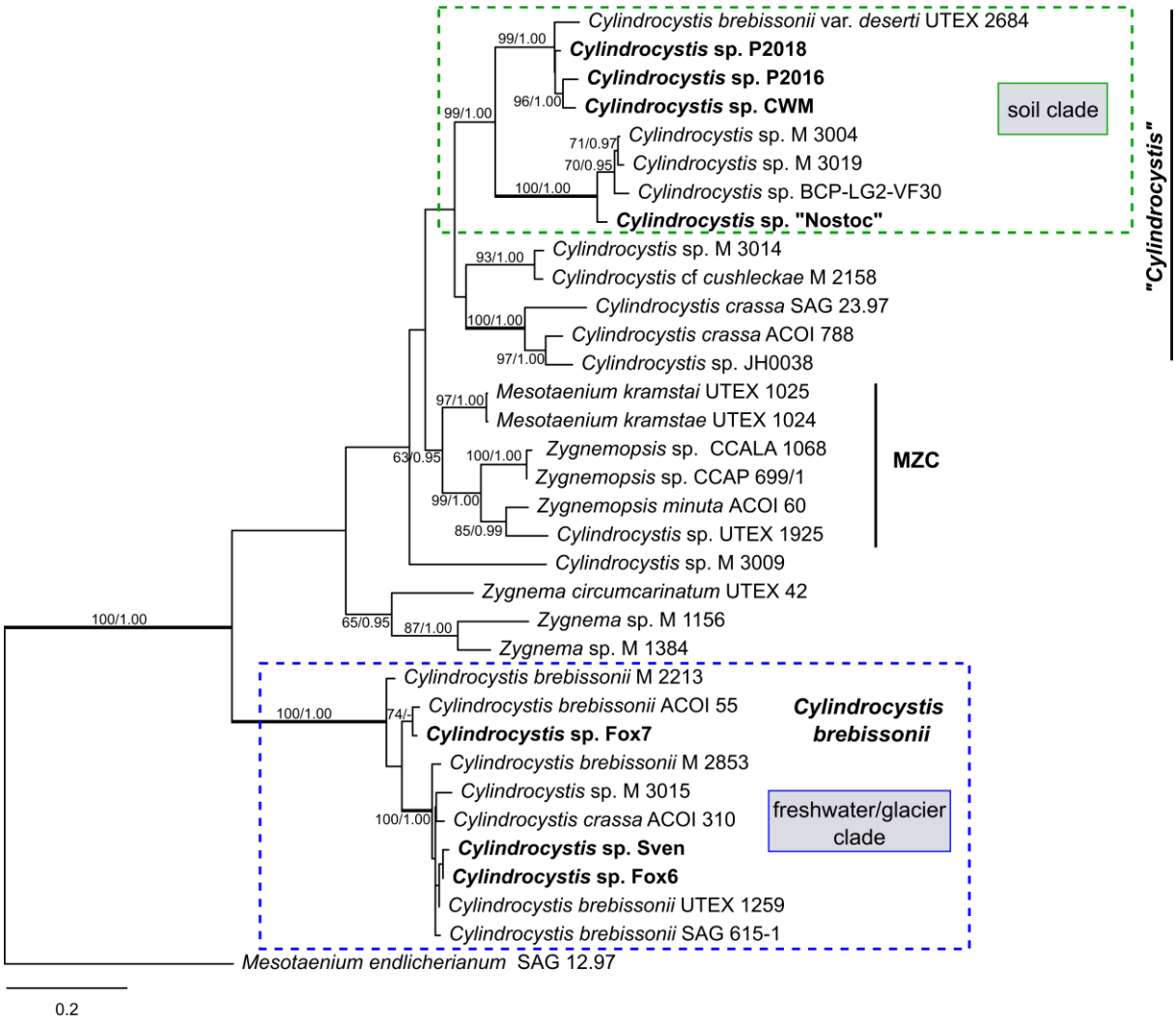

Fig. 1. Maximum-likelihood phylogenetic tree of the *rbc*L sequences. Numbers next to branches indicate statistical support values (maximum-likelihood/Bayesian posterior probabilities). "*Cylindrocystis*", MZC and *Cylindrocystis brebissonii* clades were delimitated according to Gontcharov and Melkonian (2010). New sequences are in bold.

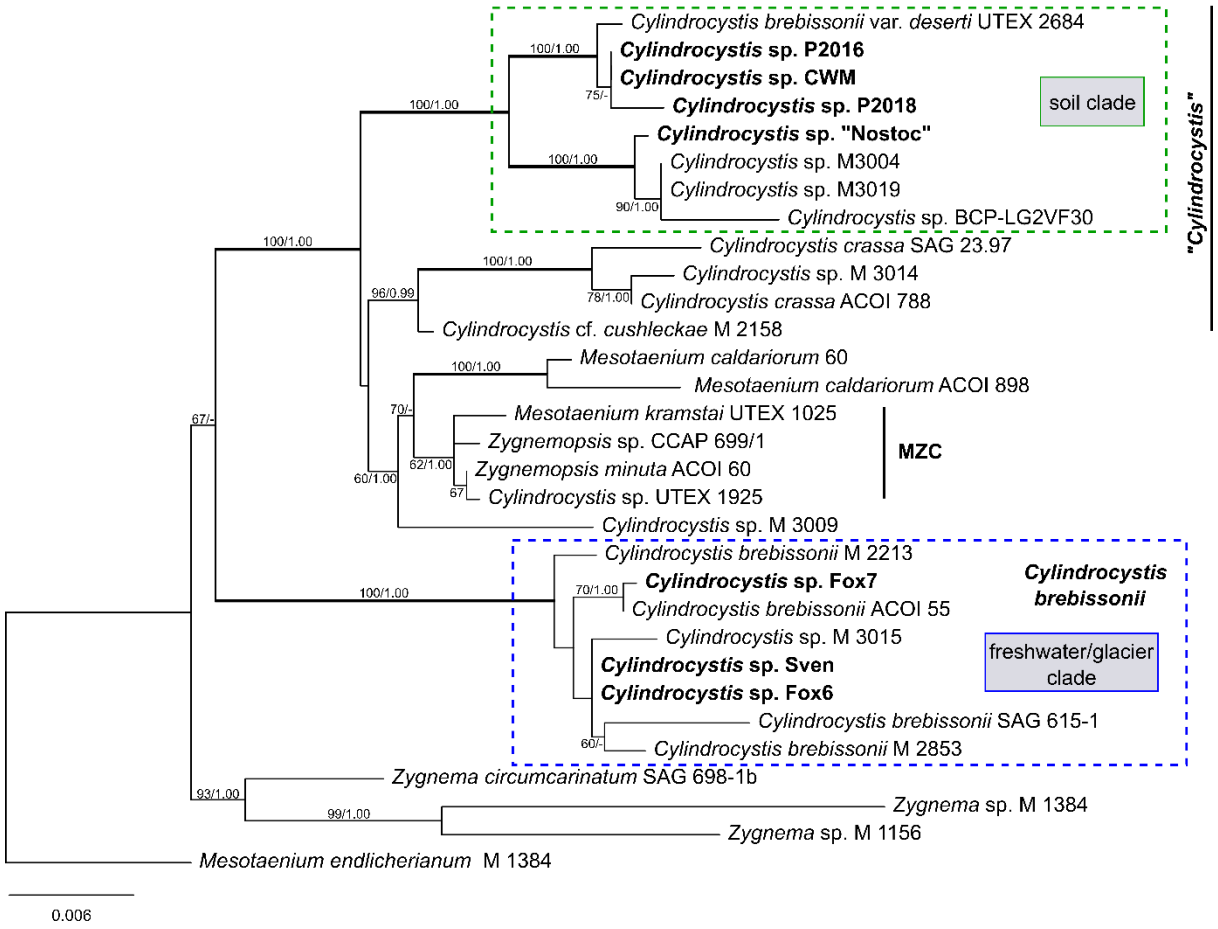

Fig. 2. Maximum-likelihood phylogenetic tree of the 18S rDNA sequences. Numbers next to branches indicate statistical support values (maximum-likelihood/Bayesian posterior probabilities). "*Cylindrocystis*", MZC and *Cylindrocystis brebissonii* clades were delimitated according to Gontcharov and Melkonian (2010). New sequences are in bold.

Morphological observations

The vegetative cells of all strains investigated in this study had a cylindrical shape with broadly rounded ends (Fig. 3). The morphometric parameters (length and width) of the six studied strains (excluding glacier strain Sven which did not survive but including desert strain UTEX B 2684) are summarized in Fig. 4. The cells contained either a single chloroplast occupying most of the cell's volume or a chloroplast composed of two parting halves, eventually detaching from each other and becoming two separate chloroplasts (Fig. 5). Each of the chloroplast halves contained at least one round pyrenoid, surrounded by starch plates (Fig. 3). Big empty holes within the chloroplast mark a pyrenoid position in the images from

the confocal laser scanning microscopy (Fig. 5A–C). All studied strains had a robust cell wall with a smooth surface. The nucleus was located in the cell's center, surrounded by the chloroplasts. The algae reproduced by vegetative cell division and no conjugation or zygospores were observed in the cultures.

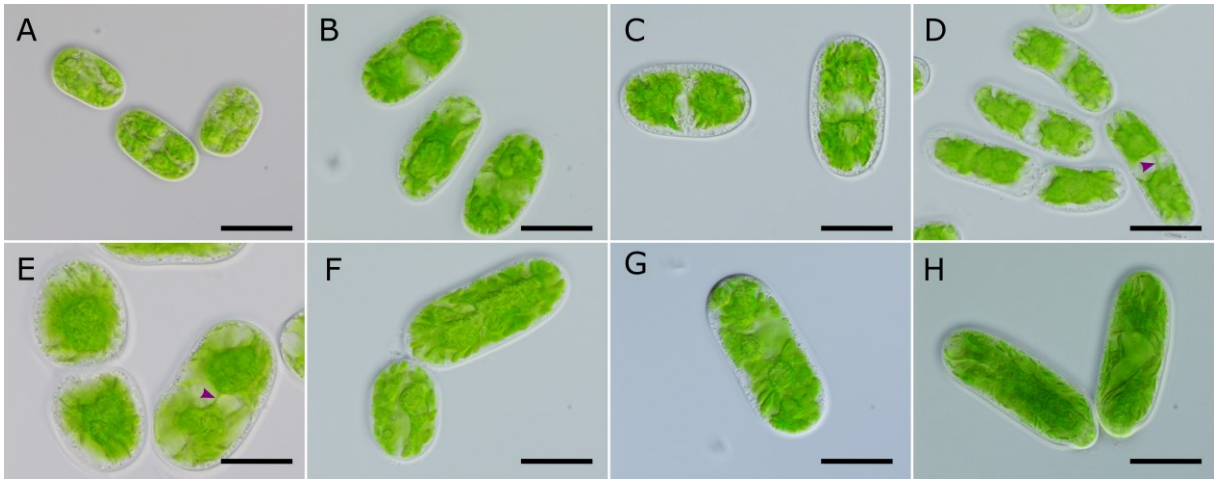

Fig. 3. Ligh microscopy photographs of the studied *Cylindrocystis* strains: A – UTEX B 2684, B – P2016, C – P2018, D – CWM, E – "Nostoc", F – Fox6, G – Sven, H – Fox7. The purple arrow points to the "Charles bridge". Scale bars represent 20 µm.

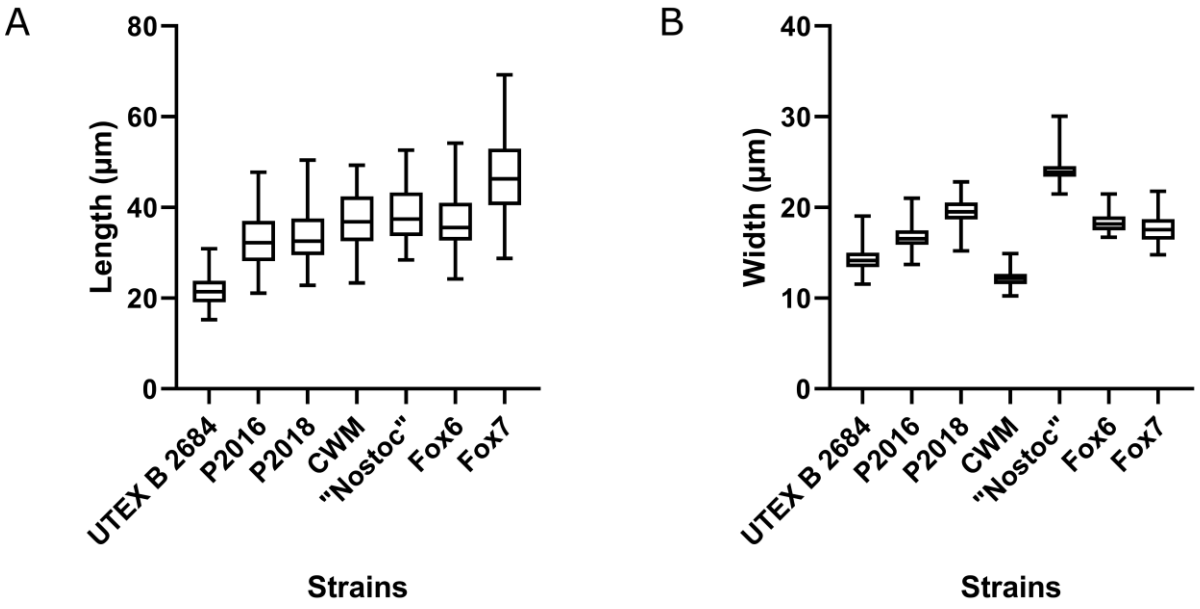

Fig. 4. Cell length and width of investigated strains (n = 100). The line within the box marks the median, whiskers mark the lowest and the highest measurements.

More detaily, the desert strain UTEX B 2684 had short broadly cylindrical or sometimes almost spherical cells. The chloroplast was plate-like with undulating ridges, often composed of two or even four parts (Fig. 5A). Sometimes chloroplast thylakoids were whirled, giving them a “rosy” look. Two or even three small pyrenoids were often observed within a single part of the divided chloroplast. The two Arctic strains P2016 and P2018 also had rather short to medium size cells that were straight and often possessed a central thylakoid bridge (which we referred to as “Charles bridge” – an allusion to the oldest bridge in Prague, dating back to 1357) joining two stellate chloroplasts (Figs 3B, C, 5B, C). The rare longer cells of the strain P2016 appeared slightly curved. In contrast, strain CWM encompassed slimmer cells (Fig. 4B) that were often notably curved. They also possessed the “Charles bridge” connecting two plate-like chloroplasts with irregularly radiating ridges (Fig. 3D). The “Nostoc” strain also contained a plate-like chloroplast with radiating ridges, and thylakoids sometimes arranged in a net-like manner. The formation of the single delicate “Charles bridge” was not common. Instead, the two halves of the chloroplast were connected by several thylakoid extensions (both central and parietal). The freshwater strain Fox6 included cylindrical straight cells with two stellate chloroplasts. The central chloroplast core contained a big pyrenoid surrounded by numerous starch granules. It also contained large radial lobes arranged vaguely. The glacier strain Sven showed a similar chloroplast structure. On the contrary, the strain Fox7 had a somewhat reticulate chloroplast composed of longitudinal delicate twisting ridges and pyrenoid, if visible, was subellipsoidal in shape. The chloroplast was rarely divided into two conspicuous parts (Figs 3H, 5G).

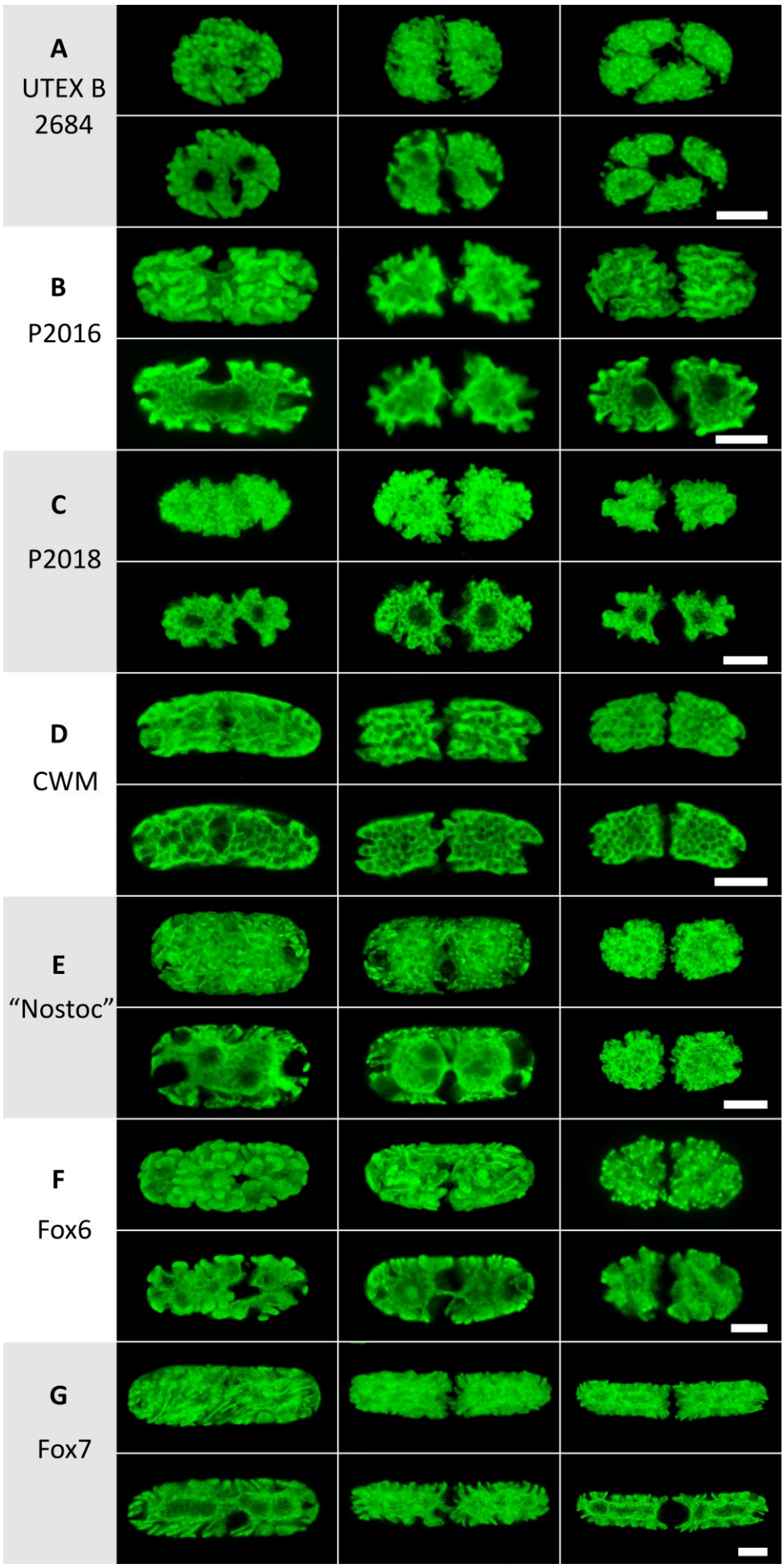
A
UTEX B 2684
B
P2016
C
P2018
D
CWM
E
“Nostoc”
F
Fox6
G
Fox7

Fig. 5. Confocal laser scanning microscopy of the studied strains showing chloroplast morphology and ontogeny (single plate – semi-divided – divided chloroplasts are shown in columns, respectively). The top row shows maximum projection, the bottom row optical cross-section in the middle of the same cell. Scale bars represent 10 μm.

As seen in the fluorescence images of chlorophyll (Fig. 5), chloroplasts of the Arctic *Cylindrocystis* strains (especially notable in CWM, Fig. 5D) contained large numbers of non-fluorescent spherical objects distributed within the chloroplast bodies as grains in pomegranate. To elucidate their chemical nature, a confocal Raman microscopy was used. Raman spectra of the spectrally orthogonal (i.e., linearly independent) components that fully describe spectral variability of Raman maps of seven *Cylindrocystis* strains are shown in Fig. 6. The spherical objects inside the chloroplasts were identified as polyphosphate (PolyP) grains, based on the characteristic Raman bands centered at 698 and 1165 cm^{-1} (Fig. 6A; Moudříková et al. 2016, 2017a). The chemical map of the PolyP in CWM cell is a negative copy of Raman image of chloroplasts visualized by their characteristic Raman spectrum consisting mainly of the bands attributable to glycolipids and proteins (Fig. 7). Furthermore, Raman chemical map of chloroplasts resembles the fluorescence image of CWM chloroplasts (Fig. 5D). The same complementarity of Raman maps of PolyP and chloroplasts can be seen in other *Cylindrocystis* strains, except for UTEX B 2684 (Supporting Information Figs S1–S6).

According to characteristic Raman spectra, other intracellular structures can be visualized by their Raman chemical maps, e.g., starch granules, lipid droplets containing triacylglycerols, cell wall or nuclei (e.g., strain CWM, Fig. 7; also see Supporing Information Figs S1–S6).

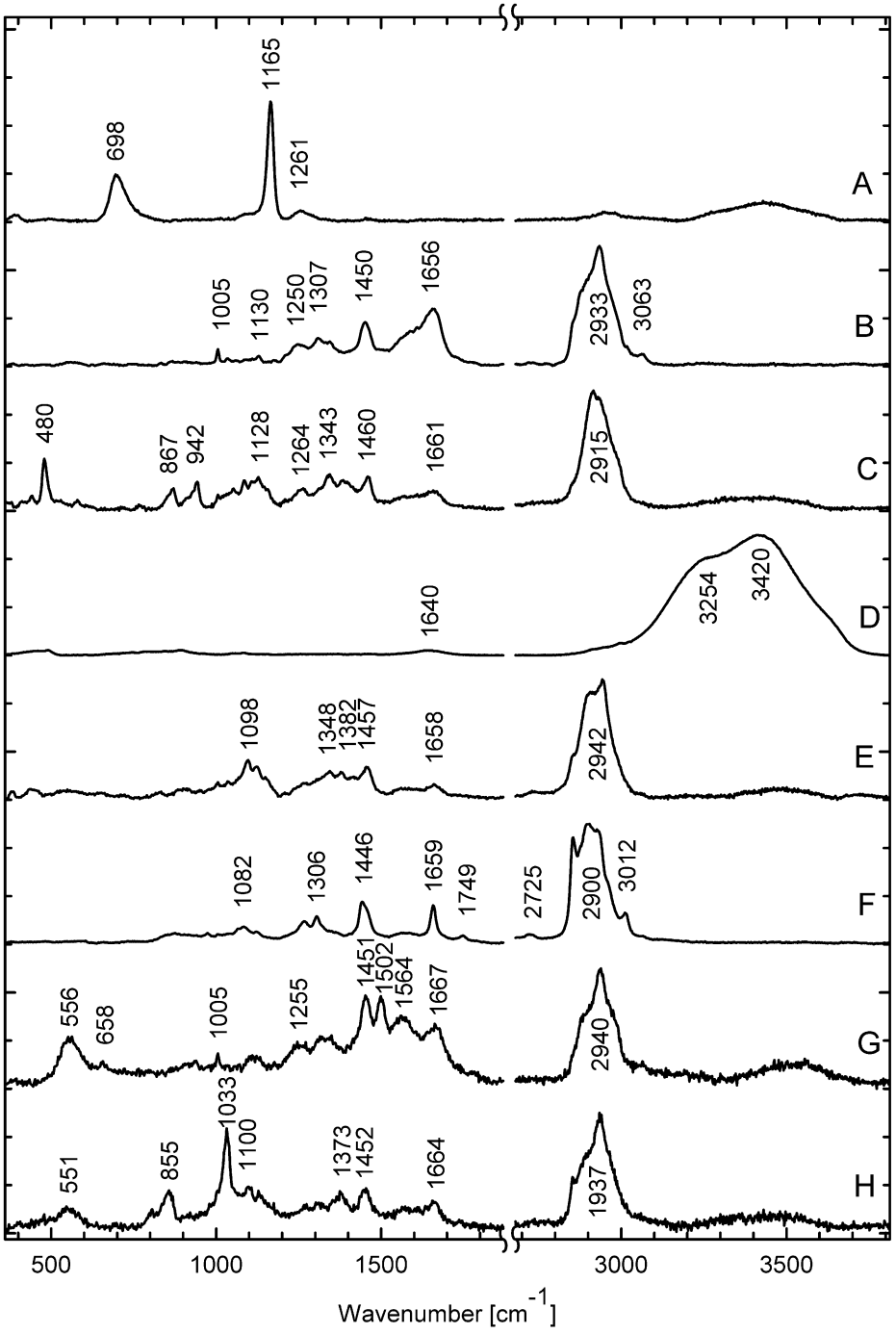

Fig. 6. Raman spectra of linearly independent spectral components fully describing spectral variability of Raman maps of seven *Cylindrocystis* strains. Polyphosphate (A), chloroplasts (B), starch (C), water (D), cell wall (E), neutral lipids (F), nucleus (G), and inclusions of unknown chemical composition (H).

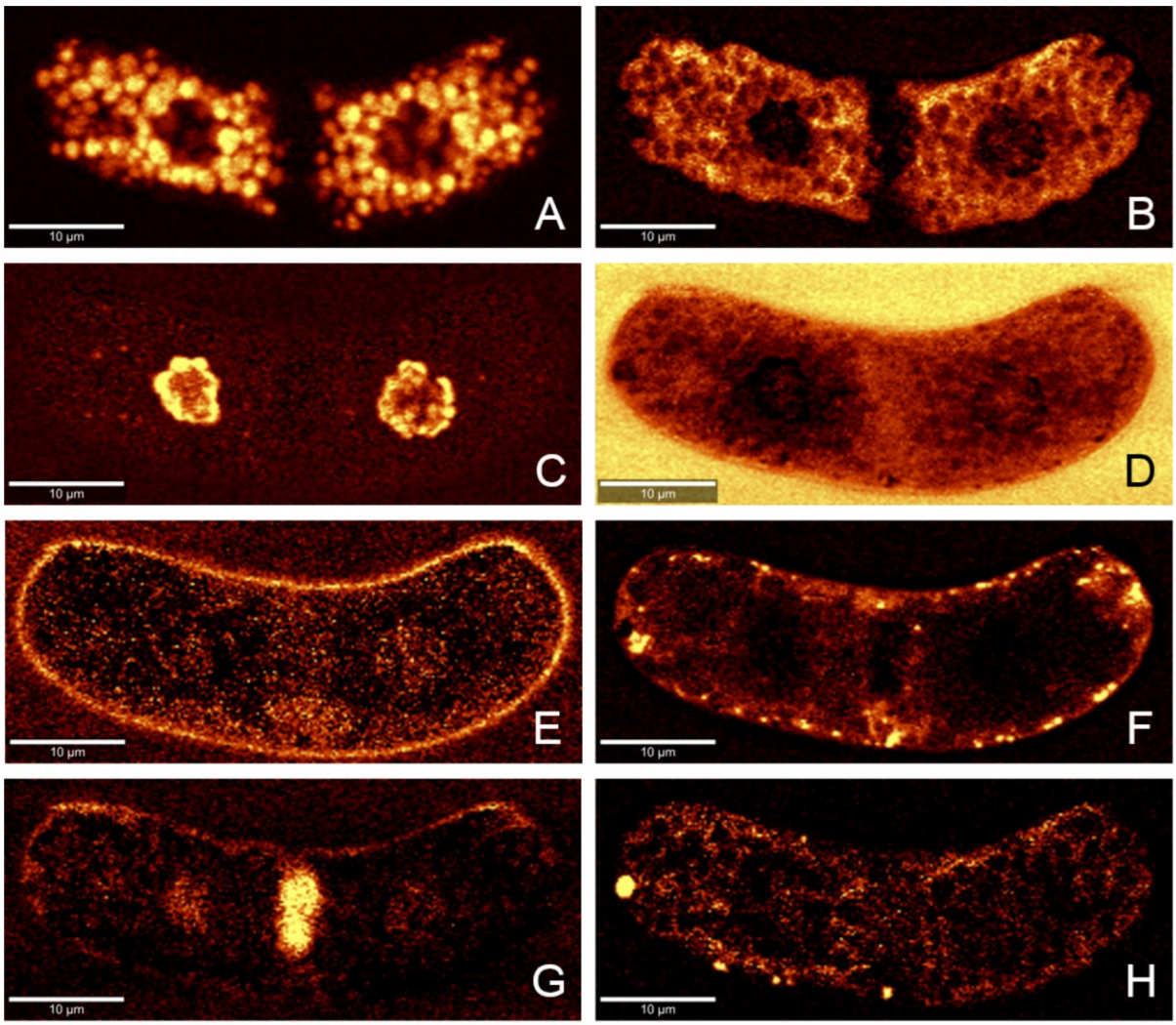

Fig. 7. Raman chemical maps of the CWM cell highlighting polyphosphate grains (A), chloroplasts (B), starch granules (C), water content (D), cell wall (E), lipid droplets (F), nucleus (G), and inclusions of unknown chemical composition (H). The corresponding Raman spectra are shown in Fig. 6.

Dicussion

Diversity. This study represents the first attempt to look at the diversity of the Arctic *Cylindrocystis* using a culture-based approach. We have found five different genotypes on the 18S rDNA level and seven different genotypes on the *rbc*L level out of seven studied strains morphologically resembling the genus *Cylindrocystis*. The origin of the new isolates was not monophyletic, confirming the complex phylogenetic structure of *Cylindrocystis*-like microalgae (Hall et al. 2008, Gontcharov and Melkonian 2010). The new isolates fell within the two previously uncovered clades, i.e. "*Cylindrocystis*" clade and *C. brebissonii* clade

(Gontcharov and Melkonian 2010). The "*Cylindrocystis*" clade was composed of four main lineages, including species *C. crassa* and *C. cushleckae* and numerous other strains without a species affiliation. The four Arctic isolates constituted two separate lineages within that clade (Figs 1, 2). The three freshwater isolates clustered within the clade of *C. brebissonii*. However, we refrained from assigning them to the single species of *C. brebissonii*, since this group of organisms likely veils cryptic species. Overall, our phylogenetic analyses suggested that the seven new isolates could constitute at least five different taxa, what was also corroborated by their different morphology (see discussion below). For the more detailed genetic relationship estimation among close relatives and especially species delineation, more variable molecular markers, such as ATP synthase beta subunit (*atpB*) or internal transcribed spacer 2 (ITS2), could be eligible (e.g., Stancheva et al. 2013, Mikhailyuk et al. 2018).

Apart from the first insights into the genetic diversity of the Svalbard *Cylindrocystis*, this study also revealed morphological diversity by showing that different genotypes could be discerned by their morphological appearance. However, this might not necessarily be true for closely related or cryptic species (e.g., P2016 *versus* P2018; Figs 1, 2). By the use of the confocal laser scanning microscopy, this work has also demonstrated that the chloroplast of the studied strains was not stable (Fig. 5). This could, potentially, be the age-related trait. The strains of our defined soil clade (Figs 1, 2) generally had short and broadly cylindrical cells, except the CWM strain (Fig. 3D). The strains of the freshwater/glacier clade were usually two or three times longer than wide (Fig. 4). The re-examined Mexican desert strain UTEX B 2684 not only had the shortest cells (Fig. 4A), but it also was the only strain that did not posses the cental thylakoid extension. Meanwhile, the "bridge" was easily visible in the bright field of the Arctic strains (e.g., strains CWM and "Nostoc"; Fig. 3D, E) or detected using a cross-section through the middle of the cells in the confocal microscopy (e.g., strain Fox6; Fig. 5F). In addition, UTEX B 2684 occasionally contained a chloroplast composed of four equal parts (Fig. 5A), what was not observed in any other strain included in this study. Such

morphological pecularities of the UTEX B 2684 support the phylogenetic results, showing its separation from the Arctic soil strains (Figs 1, 2).

Based on our observations, cell's length and width could indeed be an important *Cylindrocystis* "species" feature as considered in the past (Lütkemüller 1913; Prescott et al. 1972; Lenzenweger 2003). For example, the strain CWM had the narrowest cells (Fig. 4B) that were also curved (Fig. 3D). Meanwhile,"Nostoc" strain had the broadest cells (Fig. 4B). These features distinguished them well from the other two closely related soil strains P2016 and P2018 (Figs 1, 2). The freshwater strain Fox7 had the longest cells (Fig. 4a). It also had the most delicately lobed chlorplast (Fig. 5G). Meanwhile, its closest revealed relative, strain Fox6, had chloroplast with rather big blunt lobes, resembling buds (Fig. 5F). The similar type of lobes was also present in the soil strain P2016 (Fig. 5B). For comparison, the 3D chloroplast morphology of the Ecuadorian glacier *Cylindrocystis* (Nedbalová and Sklenář 2008) also rather resembled strain Fox6, though had deeper incisions.

To conclude, our molecular and morphological data showed that diversity of the Arctic *Cylindrocystis*-like organisms is not restricted just to the single species *C. brebissonii*. Our results point to a much larger number of deeply divergent phylogenetic lineages with *Cylindrocystis*-like morphology. As already noted by Gontcharov and Melkonian (2010), the "*Cylindrocystis*" clade represents a new genus or even several new genera. Therefore, *Cylindrocystis* now awaits a thoughtful investigation and taxonomic reclassification what could be a challenging task considering that the conjugating green algae do not willingly reproduce sexually and form zygospores – a crucial morphological trait for recognizing taxa described in the past century – while grown in cultures. Finally, this study provided the first reference sequences of the Arctic *Cylindrocystis* which could be further used for studying Arctic ice and soil eukaryotic communities using culture independent approaches, i.e. Next Generation Sequencing (Lutz et al. 2018, Rippin et al. 2018). However, we predict even

higher diversity of microalgae with *Cylindrocytis*-like morphology in the High Arctic than uncovered in this study.

Ecology. *Cylindrocystis brebissoni* represents one of the three main glacier algae species. Strain Sven was isolated directly from the ice sample, whereas, Fox6 and Fox7 were isolated from the meltwater close to the glacier, suggeting their glacier origin as well. In Svalbard, *Cylindrocystis* has been reported from glacier surfaces before (Stibal et al. 2006). Studies conducted in Greenland found *Cylindrocystis* either as a dominant (Yallop et al. 2012) or insignificant (Lutz et al. 2018) constituent of the cryoflora based on the direct cell counting and oligotyping, respectively. However, despite its known occurrence in cold habitats, *Cylindrocystis* is much less studied compared to its counterparts thriving on bare ice in both polar and alpine glacier ecosystems. For example, other glacier zygnematophytes *Mesotaenium berggrenii* and *Ancylonema nordenskiöldii* have gained attention in terms of physiology, ultrastructure and compounds produced (Remias et al. 2009, 2011, 2012). Apart from ice, *Cylindrocystis* has been detected in the biological soil crusts in Svalbard before (Borchhardt et al. 2017). Arctic soil *Cylindrocystis* algae could easily be recognized in the plain Arctic habitats because they formed macroscopic gelatinous colonies – a well know feature of desmids. In terrestrial habitats, such gelatinous coating is useful for the retention of the limiting water and protection against quick desiccation. In addition, mucilaginous gels could also be important in protecting cells against high UV radiation, predation, and parasitism as known, for example, in Cyanobacteria (e.g., Ehling-Schulz et al. 1997). Since we have found *Cylindrocystis* in the same locality (soil surface) after two years (2016 and 2018), local occurrence could be important in the success of this algal group in terrestrial environments, in contrast to the widespread minute coccoid trebouxiophytes (Hodač et al. 2016). Life in mucilaginous coatings could ensure much stable presence in otherwise unstable Arctic habitats. First of all, the cells are not so easily blown away. Secondly, the colonies and populations can be established in microhabitats with sufficient nutrient supply, e.g., close to

the glaciers (Fox6 and Fox7) or wet meadows (CWM) where meltwater could bring the nutrients. On the other hand, the two isolates (P2016 and P2018) found in the same spot were phylogenetically distinct from each other what also suggests that the colonies are likely composed of several similar species what we could have overlooked while isolating the strains and assuming the colonies were composed of the single species. For example, the mats of *Zygnema* or *Trentepohlia* (Chlorophyta) which were usually perceived as homogeneous were demonstrated to be composed of several different genotypes (Klimešová et al. 2018, Pichrtová et al. 2018).

Up until now, zygnematophytes were generally considered as freshwater organisms. However, the Arctic soil strains were closely related to the two desert isolates (UTEX B 2684 and BCP-LG2-VF30) confirming the presence of the true terrestrial nature within the Zygnematophyceae. As also revealed from molecular data, the strains isolated from terrestrial and freshwater habitats were phylogenetically separated. The *C. brebissonii* clade accommodates true freshwater/glacier species, meanwhile "*Cylindrocystis*" clade encompasses species from both freshwater and terrestrial habitats. However, to better unravel ecological speciation of this algal group, an increased taxon sampling is necessary. Nevertheless, *Cylindrocystis* could be an excellent model organism to study terrestrialization since freshwater, limnoterrestrial and terrestrial algae with *Cylindrocystis*-like morphology have been described.

Distribution. Our new data confirmed that the genus *Cylindrocystis* (in its broad traditional understanding) is not highly selective of habitats and similar genotypes are widely distributed, occurring from cold polar to arid deserts. However, the polar and desert isolates likely represent different species. Same as terrestrial, similar freshwater genotypes are also widely distributed (e.g., strains Fox7 and ACOI 55). Up to now, there are no sequences of the Antarctic *Cylindrocystis* what could confirm bipolar distribution of the uncovered soil and freshwater/glacier clades (Figs 1, 2). For example, Ryšánek et al. (2016) showed that both

Arctic and Antarctic *Klebsormidium* strains fell within the same clade and comprise cosmopolitan species. Pichrtová et al. (2018) also demonstrated that polar *Zygnema* strains did not form any endemic lineages and were intermixed with strains originating from different geographic regions. The current knowledge allows us to conclude that the two discussed *Cylindrocystis* clades show at least polar-temperate distribution and that newly isolated Arctic strains originated likely from temperate communities. Furthermore, terrestrial genotypes naturally might be better adapted to desiccation, which is a crucial physiological trait for a long-distance dispersal. On the other hand, local conditions might have a strong influence on their diversification, resulting in more variable genotypes (e.g., soil clade; Figs 1, 2). Since water environment is more stable and more similar worldwide than terrestrial habitats, same or similar freshwater genotypes are expected to be more widespread (e.g., freshwater/glacier clade; Figs 1, 2).

Polyphosphate. The most apparent feature seen in the Raman chemical maps of all *Cylindrocystis* strains (except for UTEX B 2684) is the presence of a large number of small spherical PolyP grains (diameter 1–2 μm) nested within chloroplasts (Fig. 7A). Our results likely constitute the first proofs of the presence of PolyP in the cells of zygnematophytes. Inorganic PolyP is a linear polymer consisting of many tens or hundreds of orthophosphate residues (P_i) linked by high-energy phosphoanhydride bonds (Kornberg 1995, Kulaev et al. 2004). PolyP, known in biology from the beginning of the 20th century as volutin granules (Meyer 1904), was found in all kinds of living organisms, from bacteria, fungi, protozoa, insects up to vertebrates and mammals, naturally including microalgae (Kulaev et al. 2004). This still enigmatic polymer was shown to play multiple roles in basic metabolism of prokaryotic and eukaryotic cells, and its newly emerging functions in the microbial world have been reviewed repeatedly (Harold 1966, Albi and Serrano 2016, Jiménez et al. 2017, Xie and Jakob 2019). PolyP was shown to serve as a reservoir of phosphorus (Harold 1966), divalent cations (Siderius et al. 1996), and energy (Kornberg et al. 1999), and to play a role in

pH buffering (Pick et al. 1991), detoxification (Keasling et al. 1998), and as an antioxidative agent (Gray and Jakob 2015). PolyP was found to be a potent protein-like chaperone that protects cells against stress-induced protein aggregation (reviewed recently by Xie and Jakob 2019), thus potentially playing an important role in stress resistance (Gray and Jakob 2015).

Within intact cells, PolyP can be visualized by a specific staining (toluidine blue) or fluorescence labelling (DAPI) (Gomes et al. 2013), however it can also be unambiguously identified and quanified in a label-free manner by Raman microscopy due to characteristic Raman bands at around 698 and 1165 cm^{-1} (Fig. 6A; Moudříková et al. 2016, 2017a). The simplest explanation of the presence of PolyP grains in Arctic *Cylindrocystis* may stem from their role as effective long-term reservoir of phosphorus. The algal cell isolated from their natural but nutrient-poor Arctic habitats have been cultivated in a relatively P_i-rich BBM medium ($[P_i] \sim 1.72$ mM). Consequently, the cells might accumulate a considerable amount of PolyP in expectation of the phosphate shortage. Such an excessive accumulation of phosphorus in the form of PolyP is known as a luxury uptake (Cembella et al. 1982, 1984). The microalgae mostly dwelling in P_i-poor environments are naturally equipped to take up and to store P_i whenever it becomes available (Cembella et al. 1982, 1984).

Another hypothesis explaining the presence of PolyP grains within chloroplasts may relate the PolyP function in the stress resistance. *Cylindrocystis* strains were cultivated at significantly higher temperatures compared to the temperatures they were isolated from (20°C *versus* below 5°C). Therefore, it is reasonable to assume that the cells could have suffered from the temperature stress. Furthermore, to keep the studied strains alive, they had to be cultivated in the small volumes of plates with a repeated process of re-inoculation, thus frequently transferred into a fresh medium rich in P_i. The response to the possibly experienced stress was connected with the increased P_i uptake and biosynthesis of PolyP, the reserve of which was stored as PolyP granules in chloroplasts. The temperature stress could also explain why, for example, the desert strain UTEX 2684 has not accumulated the polyP under the same

cultivation conditions. However, it is clear that more systematic study on this particular phenomenon is necessary to clarify a real reason of such excessive accumulation of polyP by the Arctic *Cylindrocystis* strains.

Nevertheless, our results suggest that exceptional ability of Arctic *Cylindrocystis* strains to accumulate PolyP could be interesting for the better understanding of biomolecular mechanism of luxury P_i uptake, as well as the search for microalgae more suitable for biotechnological applications related to the microalgae-based P removal. For example, for the phosphorus uptake from nutrient-rich wastewaters and the potential application of the PolyP-enriched algal biomass to fertilize crop soils (Solovchenko et al. 2016).

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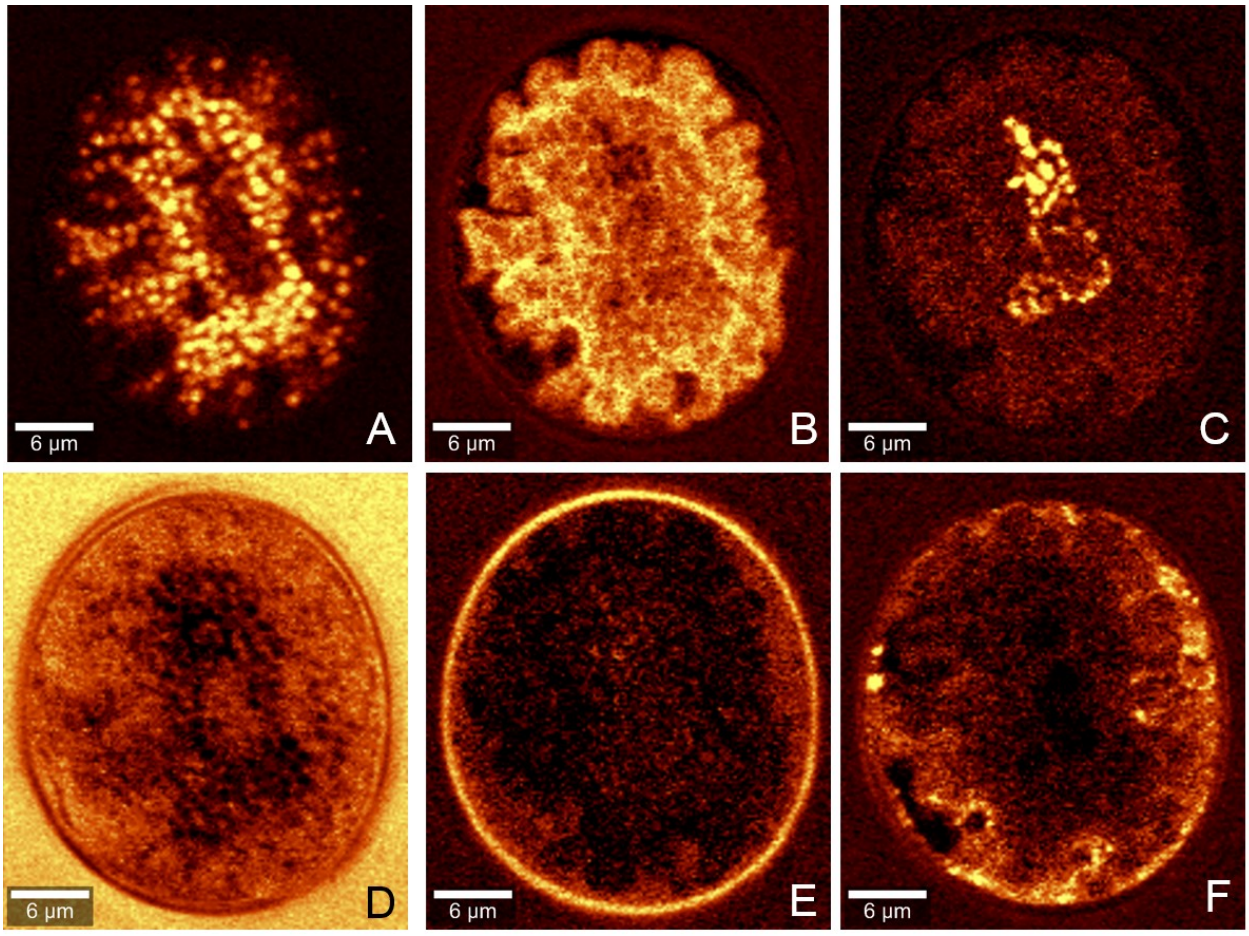

Fig. S1. Raman chemical maps of the "Nostoc" cell highlighting polyphosphate grains (A), chloroplasts (B), starch granules (C), water content (D), cell wall (E), and lipid droplets (F).

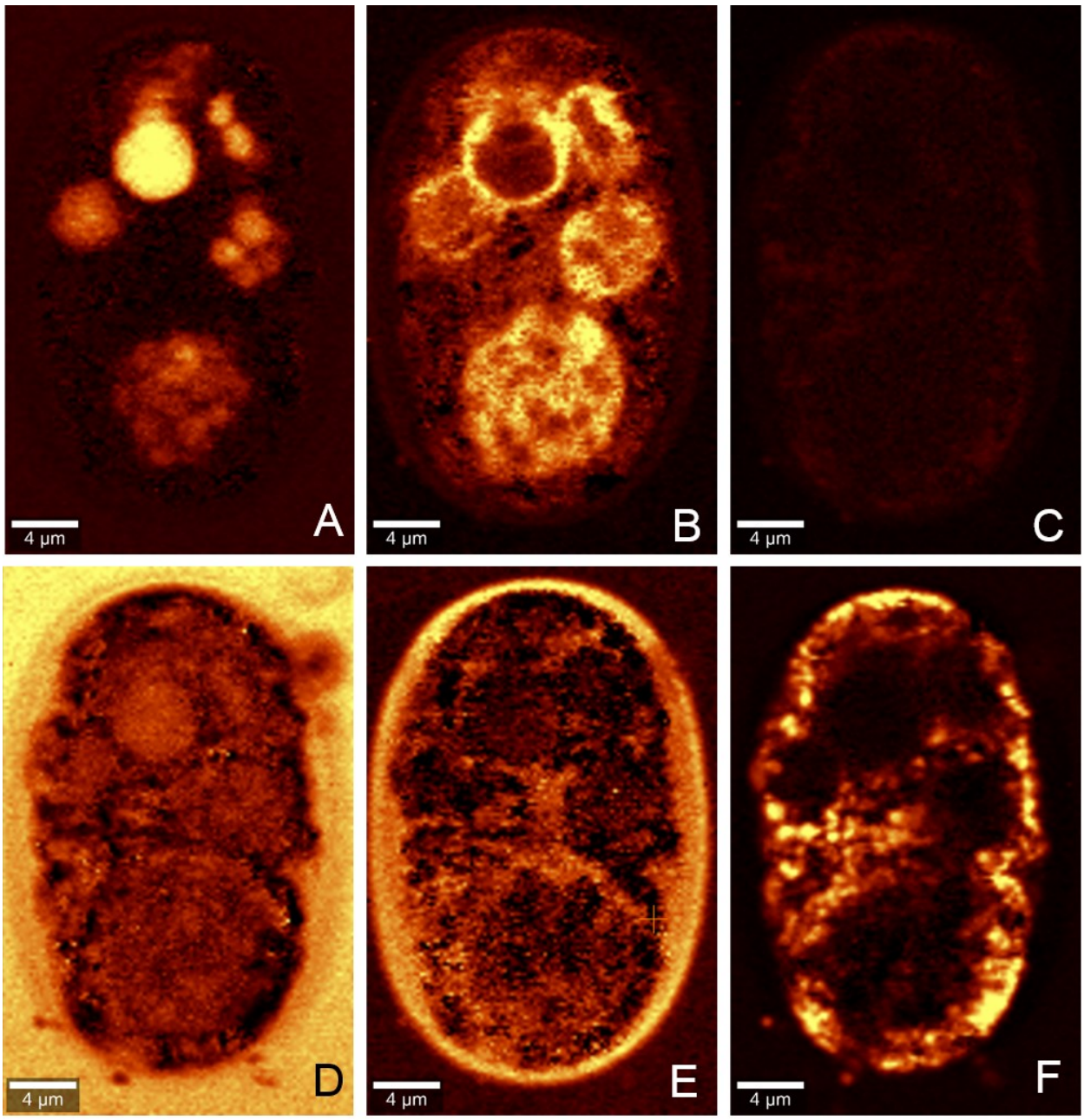

Fig. S2. Raman chemical maps of the Fox6 cell highlighting polyphosphate grains (A), chloroplasts (B), starch granules (C), water content (D), cell wall (E), and lipid droplets (F).

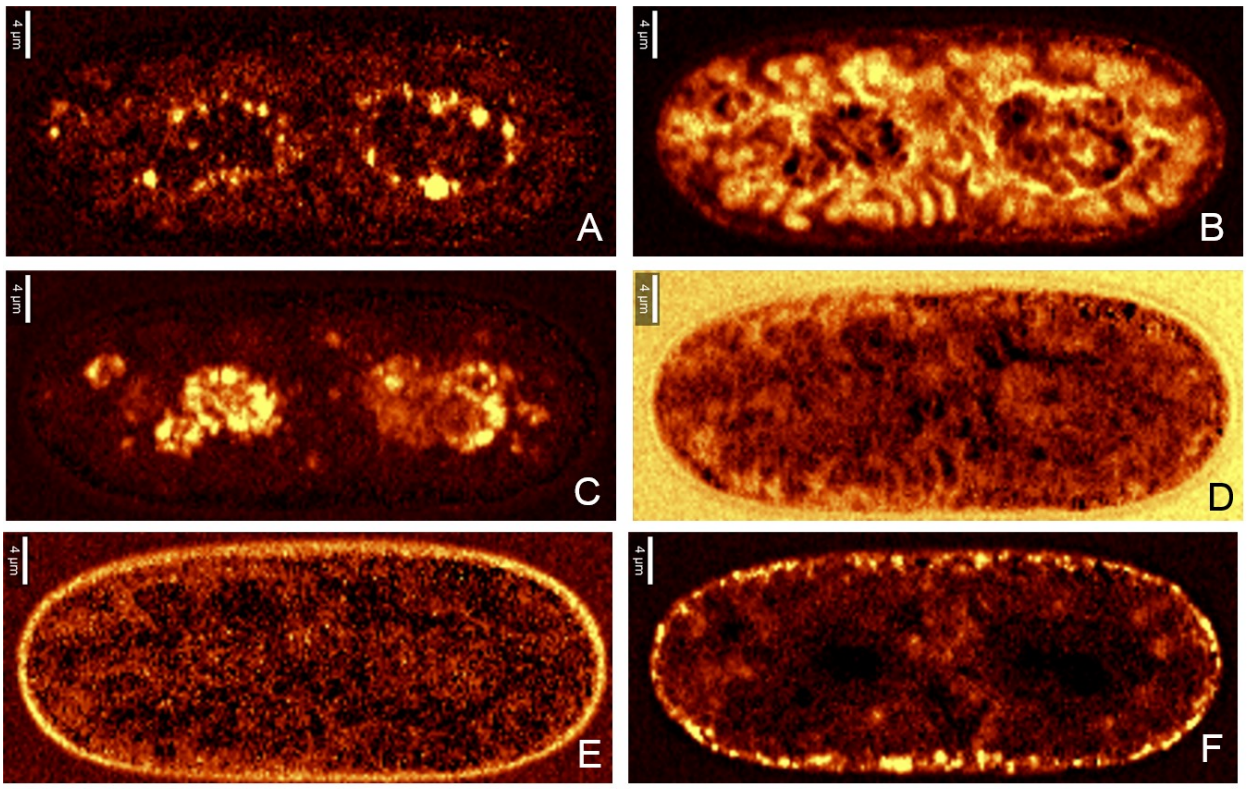

Fig. S3. Raman chemical maps of the Fox7 cell highlighting polyphosphate grains (A), chloroplasts (B), starch granules (C), water content (D), cell wall (E), and lipid droplets (F).

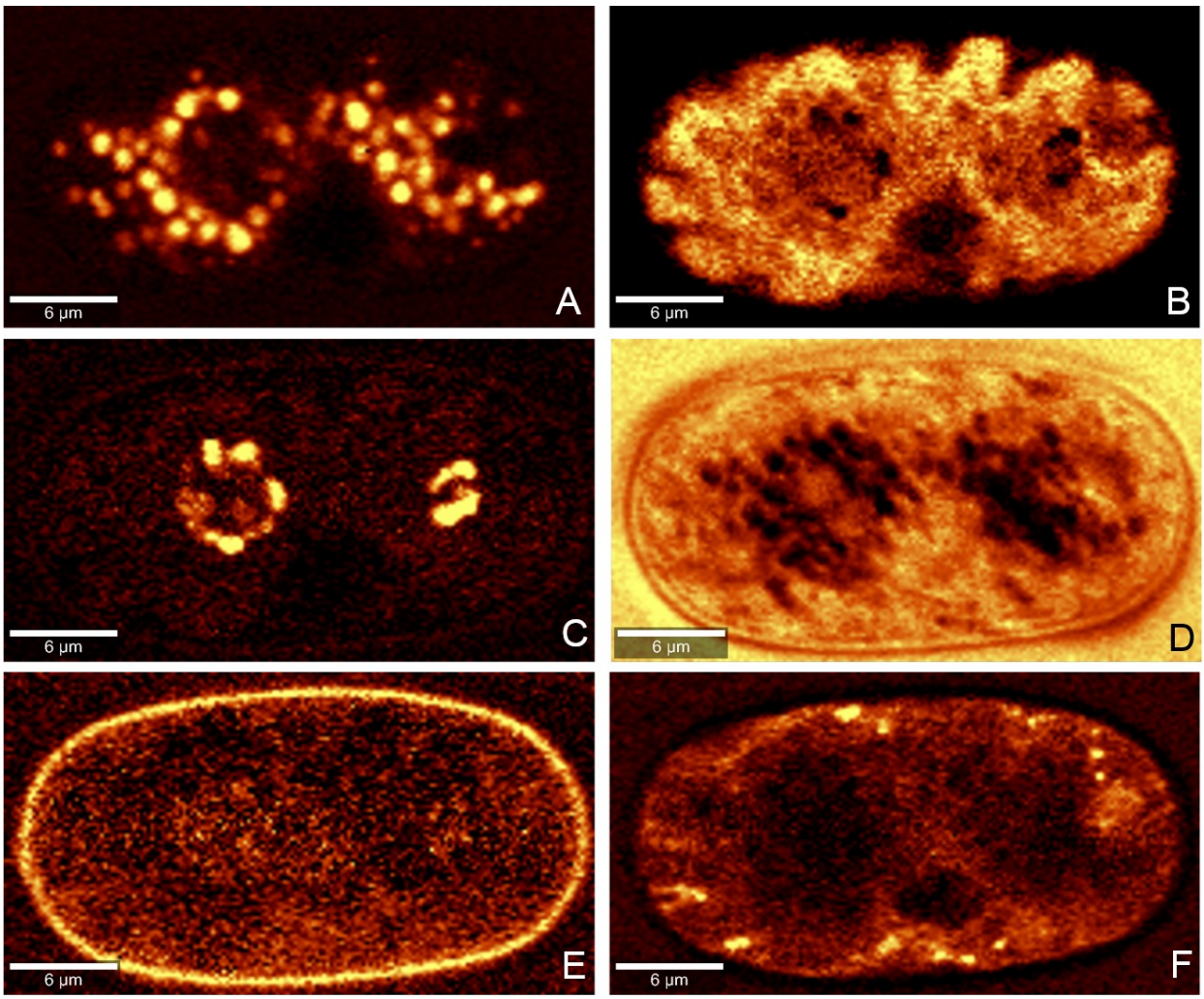

Fig. S4. Raman chemical maps of the P2016 cell highlighting polyphosphate grains (A), chloroplasts (B), starch granules (C), water content (D), cell wall (E), and lipid droplets (F).

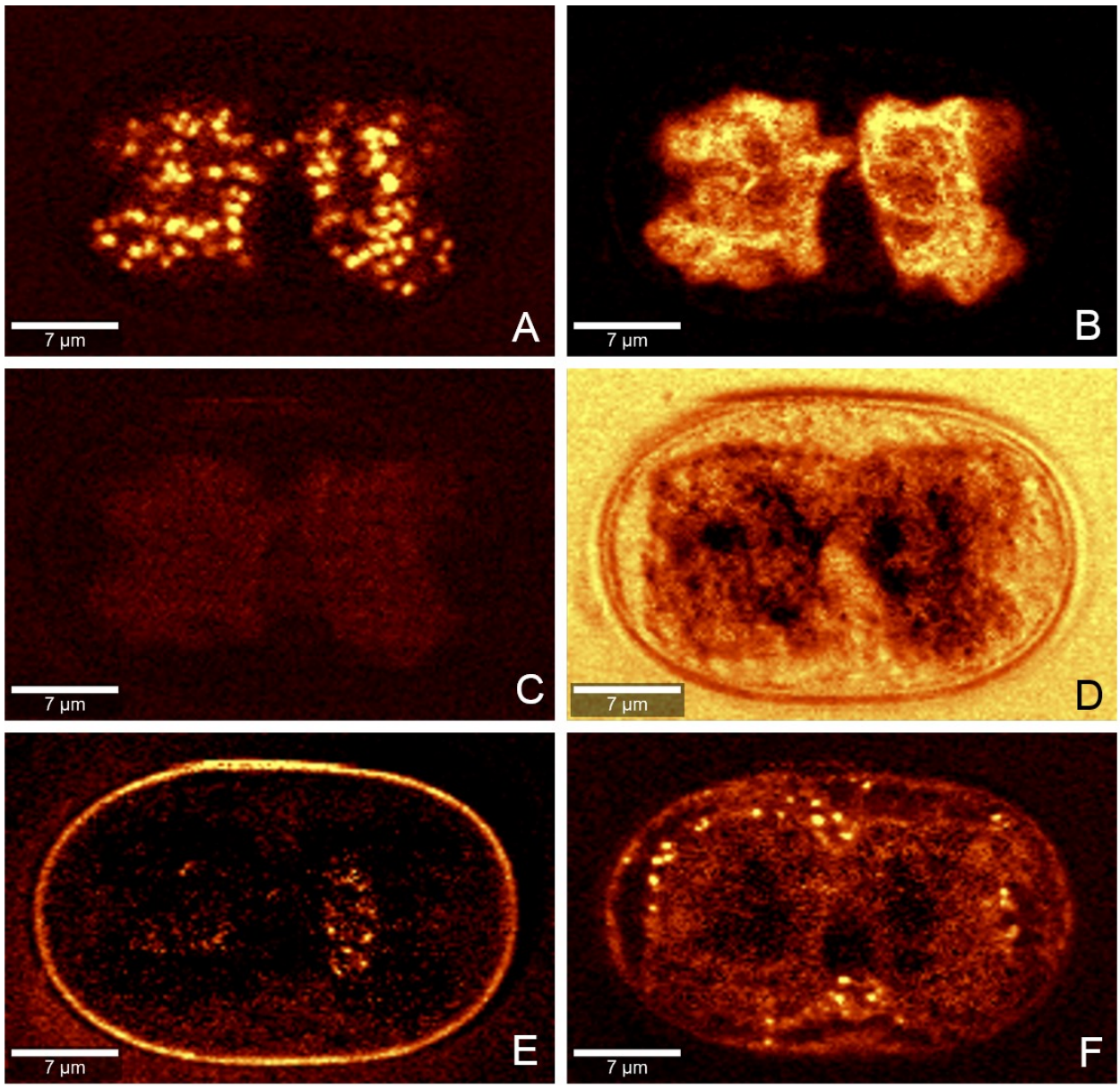

Fig. S5. Raman chemical maps of the P2018 cell highlighting polyphosphate grains (A), chloroplasts (B), starch granules (C), water content (D), cell wall (E), and lipid droplets (F).

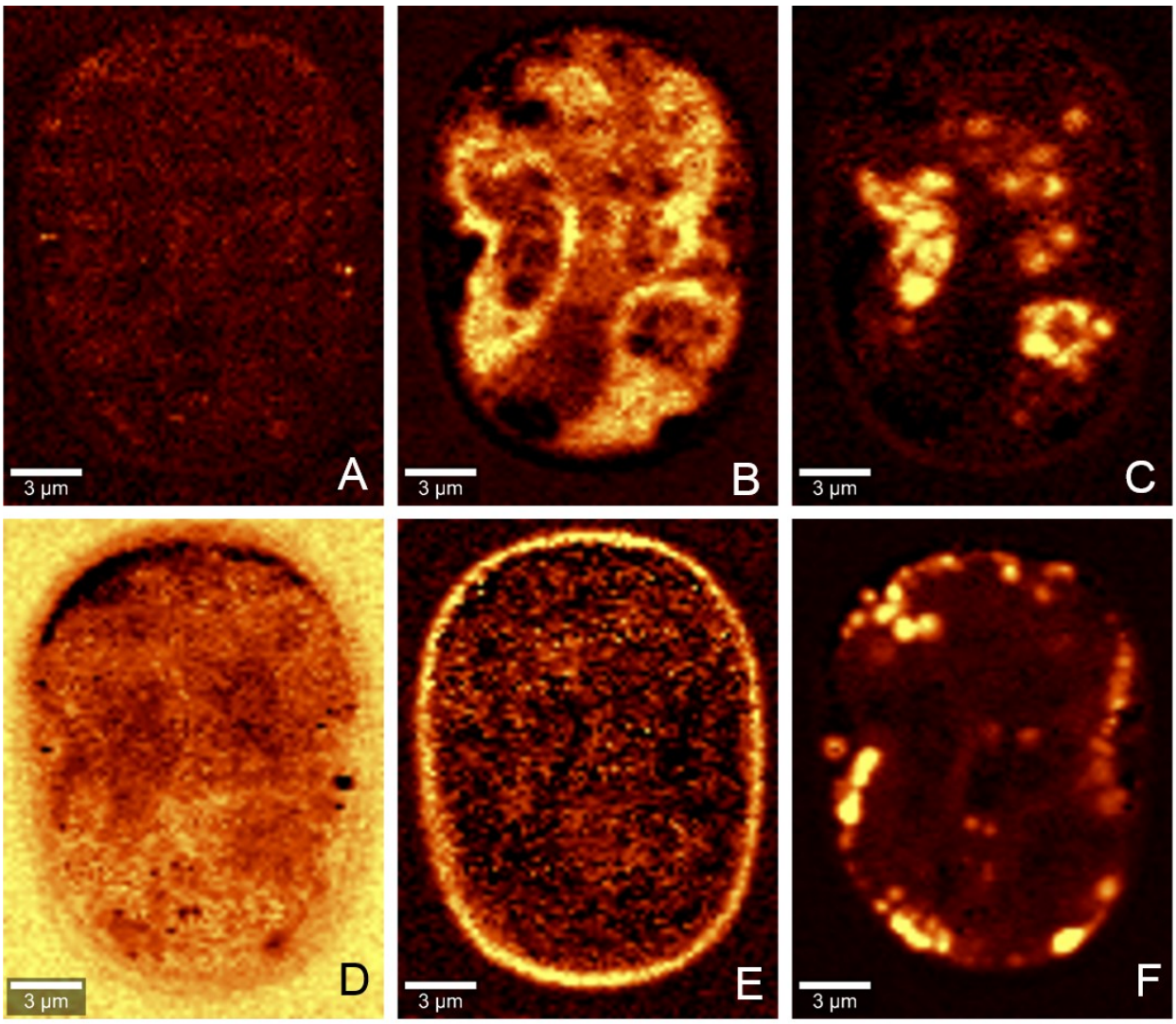

Fig. S6. Raman chemical maps of the UTEX B 2684 cell highlighting polyphosphate grains (A), chloroplasts (B), starch granules (C), water content (D), cell wall (E), and lipid droplets (F).

Appendices

Paper IX

BARCYTĖ D, HODAČ L & NEDBALOVÁ L (2017) *Lunachloris lukesovae* gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), a novel coccoid green alga isolated from soil in South Bohemia, Czech Republic. *European Journal of Phycology* 52: 281–291

Authors' contributions:

DB obtained the data, LH analyzed the molecular data; DB and LH jointly wrote the paper; LN read the draft of the paper

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Lunachloris lukesovae gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), a novel coccoid green alga isolated from soil in South Bohemia, Czech Republic

Dovilė Barcytė[a], Ladislav Hodač[b] and Linda Nedbalová[a]

[a]Department of Ecology, Faculty of Science, Charles University, Prague, Czech Republic; [b]Department of Systematics, Biodiversity and Evolution of Plants (with Herbarium), Georg-August-University of Göttingen, Göttingen, Germany

ABSTRACT

Culture collections of microorganisms can still hold undiscovered biodiversity; with molecular techniques, considerable progress has been made in characterizing microalgae which were isolated in the past and misidentified due to a lack of morphological features. However, many strains are still awaiting taxonomic reassessment. Here we analysed the phylogenetic position, morphology and ultrastructure of the strain CCALA 307 previously identified as *Coccomyxa* cf. *gloeobotrydiformis* Reysigl isolated in 1987 from field soil in South Bohemia, Czech Republic. Molecular phylogenetic analyses based on SSU rDNA and the plastid *rbc*L gene revealed that the strain CCALA 307 formed a distinct sister lineage to *Neocystis* and *Prasiola* clades within the Trebouxiophyceae. We describe this strain as a new genus and species, *Lunachloris lukesovae*. Multiple conserved nucleotide positions identified in the secondary structures of the highly variable ITS2 rDNA barcoding marker provide further evidence of the phylogenetic position of *Lunachloris*. Minute vegetative cells of this newly recognized species are spherical or ellipsoid, with a single parietal chloroplast without a pyrenoid. Asexually, it reproduces by the formation of 2–6 autospores. Since the majority of recent attention has been paid to algae from the tropics or extreme habitats, the biodiversity of terrestrial microalgae in temperate regions is still notably unexplored and even a 'common' habitat like agricultural soil can contain new, as yet unknown species. Moreover, this study emphasizes the importance of culture collections of microorganisms even in the era of culture-independent biodiversity research, because they may harbour novel and undescribed organisms as well as preserving strains for future studies.

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Introduction

The diversity of small unicellular green coccoid algae has always been underestimated owing to their simple and uniform morphology, which has made precise identification extremely challenging given the limitations of technological resources and previously available equipment. The ongoing application of molecular methods has not only helped to reveal the actual biodiversity of many 'little green balls' (Komárek & Fott, 1983) but has also enabled a more acceptable delimitation of species. In particular, the green algal class Trebouxiophyceae has recently been enriched with many new lineages (Zhang *et al.*, 2008; Eliáš & Neustupa, 2009; Neustupa *et al.*, 2009, 2011, 2013*a*, 2013*b*; Bock *et al.*, 2010; Fučíková *et al.*, 2014; Song *et al.*, 2015) and has gone through a number of revisions, encompassing the genera *Chloroidium* (Darienko *et al.*, 2010), *Chlorella* (Bock *et al.*, 2011), *Auxenochlorella* (Darienko & Pröschold, 2015), *Coccomyxa* (Darienko *et al.*, 2015; Malavasi *et al.*, 2016), *Elliptochloris* and *Pseudochlorella* (Darienko *et al.*, 2016) and *Dictyochloropsis* (Škaloud *et al.*, 2016), with descriptions of new species and/or new taxonomic combinations. Unsurprisingly, their molecular diversity greatly exceeded the morphological diversity previously established. For example, the widely distributed genus *Coccomyxa* appears to be phylogenetically extremely diversified and impossible to identify just by visual examination (Darienko *et al.*, 2015; Malavasi *et al.*, 2016).

More and more new species continue to be described based on a combination of phylogenetic, morphological and ecological data and the majority of recently described terrestrial green algae (Chlorophyta) have been isolated from various habitats, mostly in the tropics (Zhang *et al.*, 2008; Eliáš & Neustupa, 2009; Neustupa *et al.*, 2009, 2011; Eliáš *et al.*, 2010; Němcová *et al.*, 2011), or in extremely dry deserts (Fučíková *et al.*, 2014). Because these poorly explored and often extreme environments are more attractive for current biodiversity research, the terrestrial green microalgae of temperate regions have long been neglected. Only a few examples of new genera and species, accurately described using a polyphasic approach, are known from temperate Europe, e.g. *Leptochlorella* and *Kalinella apyrenoidosa* (Neustupa *et al.*, 2013*a*), *Parachloroidium* (Neustupa *et al.*, 2013*b*) and *Jenufa aeroterrestrica* (Procházková *et al.*, 2015), while numerous other new species from Europe have been defined via the revision of the cryptic genera as mentioned above.

CONTACT Dovilė Barcytė dovile.barcyte@gmail.com

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Many newly reported species and genera of terrestrial green algae fall within well-established clades. Prominent examples are the *Watanabea* clade (including the terrestrial genera *Heveochlorella* Zhang, Huss, Sun, Chang & Pang, *Kalinella* Neustupa, Němcová, Eliáš & Škaloud, *Parachloroidium* Neustupa & Škaloud, *Desertella* Fučíková, Lewis & Lewis and *Polulichloris* Song, Zhang, Liu & Hu) or the *Prasiola* clade (e.g. *Pseudomarvania* Eliáš & Neustupa). So far, modern molecular studies have revealed only a handful of novel terrestrial lineages within the green algal classes Chlorophyceae (e.g. *Jenufa* Němcová, Eliáš, Škaloud & Neustupa) and Trebouxiophyceae (e.g. *Xylochloris* Neustupa, Eliáš & Škaloud, *Eremochloris* along with *Xerochlorella* Fučíková, Lewis & Lewis and *Leptochlorella* Neustupa, Veselá, Němcová & Škaloud). However, the molecular diversity of terrestrial algae in Central Europe is still far from understood, and new lineages or clades might be expected in aero-terrestrial habitats such as tree bark (Neustupa *et al.*, 2013*a*, 2013*b*; Procházková *et al.*, 2015) and particularly in soils (Hodač, 2016). Although such potentially undescribed microorganisms can be obtained from current field sampling, re-examination of established algal cultures is also required. In particular the public collections of algal strains, e.g. Culture Collection of Algae at University of Göttingen, Germany (SAG), Culture Collection of Algae of Charles University in Prague (CAUP) and Culture Collection of Autotrophic Organisms in Třeboň, Czech Republic (CCALA), preserve isolates from Central Europe and hold numerous terrestrial taxa waiting for a taxonomic reassessment. Recently, multiple algal strains deposited in public culture collections have been described as new species or even genera based on molecular phylogenetics. For example, the strain CAUP H 5502, isolated in 1975, was later described as *Ooplanctella* Pažoutová, Škaloud & Nemjová (Pažoutová *et al.*, 2010). Similarly, SAG 12.86 isolated in 1983 was recently circumscribed as *Symbiochloris* (Škaloud *et al.*, 2016) and *Chlorella vulgaris* KIEG 1904 isolated in 1977 (and stored in a private culture collection) was described as *Planktochlorella* Škaloud & Němcová (Škaloud *et al.*, 2014).

CCALA is one of the world's oldest culture collections of algal strains, containing many terrestrial coccoid green algae deposited before the advent of molecular phylogenetics. Many strains are still waiting for inspection and here we aim to extend this effort and focus on a *Coccomyxa*-like strain CCALA 307 isolated in the late 1980s.

Materials and methods

Origin of the strain and culturing technique

The algal strain 307 was obtained from CCALA where it was referred to as *Coccomyxa* cf. *gloeobotrydiformis* Reisigl. It was isolated in 1987 during a 3-year study of secondary succession of abandoned arable fields near Chelčice, South Bohemia, Czech Republic. The strain was isolated from a wet meadow close to Dlouhá Ves (~ 49° 06'N, 14°07'E), formed after several years of field abandonment (Lukešová, 1993). We cultivated the strain in Bold's Basal Medium (BBM; Bischoff & Bold, 1963) in a Q-Cell 200 incubator (PolLab, Bielsko-Biała, Poland). Light (20 µmol photons m^{-2} s^{-1}) was provided continuously by a cool white fluorescent tube (Standard 8W, Sylvania, USA) and the cultivation temperature was 20°C. Aliquots of liquid monocultures were used for microscopic observations and genetic analysis.

Light and electron microscopy

The morphology of the strain was investigated using both Nikon Eclipse E400 (Nikon Inc., Tokyo, Japan) and Olympus BX-51 (Olympus Corp., Tokyo, Japan) light microscopes. Chloroplast morphology was investigated using a Zeiss LSM 880 laser scanning confocal microscope (Zeiss, Jena, Germany) equipped with a Helium-Neon laser. We used a 633 nm excitation line collecting emitted light between 645 and 721 nm. A C-Apochromat 63x/1.2 W Korr water immersion objective with a M 27 adapter was employed. A series of optical sections through the chloroplast were captured and used for three-dimensional (3D) morphology reconstruction. Chlorophyll autofluorescence was used for visualization of chloroplast structure. For reconstruction of the chloroplast, 3D morphology ImageJ version 1.50g (Schneider *et al.*, 2012) was used.

For transmission electron microscopy (TEM), the sample was fixed for 24 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and post-fixed in 2% OsO_4 in the same buffer. Fixed cells were dehydrated in a graded ethanol series (35%, 50%, 70%, 80%, 96%, 100% for 15 min), transferred to acetone (3 × 100% for 15 min) and finally embedded in Araldite – Poly/Bed® 812 mixture (Polysciences Inc., Hirschberg an der Bergstraße, Germany). Ultrathin sections were cut on a Reichert-Jung Ultracut E ultramicrotome and stained using uranyl acetate and lead citrate. Sections were examined using a JEOL JEM-1011 electron microscope (JEOL Ltd, Tokyo, Japan). Photomicrographs were obtained using a Veleta CCD camera (EMSIS GmbH, Münster, Germany) equipped with image analysis software Olympus Soft Imaging Solution GmbH (Münster, Germany) and later modified by Inkscape 0.91 (Free Software Foundation Inc., Boston, USA).

DNA isolation, PCR and sequencing

DNA extraction from fresh material was performed with the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). PCR reactions were done using PPP Master Mix (Top-Bio s.r.o., Prague, Czech Republic) in a total volume of 25 µl. 18S rDNA region was amplified using forward eukaryote specific primer 20F (Thüs *et al.*, 2011) and reverse green algae specific primer CH1750R (Hallmann *et al.*, 2013). Sequencing of this segment used standard sequencing primers 34F, 370R, 1122F (Pažoutová *et al.*, 2010), 895R, 1422F (Remias *et al.*, 2012), 891F, 1122R, 1422R (Friedl, unpublished). The ITS1-5.8S-rDNA-ITS2 region was amplified using forward primer AL1500af (Helms *et al.*, 2001) and reverse primer LR3 (Vilgalys & Hester, 1990). The region was sequenced with primers ITS1, ITS4 (White *et al.*, 1990), 5.8SbF (Mikhailyuk *et al.*, 2008) and nr-SSU-1780-5´Algal, nr-LSU-0012-3´Algal (Piercey-Normore & DePriest, 2001). The large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) was amplified and sequenced using a set of primers rbcL1F, rbcL2F, rbcL4R, rbcL7R, rbcL8F, rbcL9R, rbcL10F, rbcL14R, rbcL19F, rbcL20F (Hoham *et al.*, 2002). Detailed primer information is given in Supplementary Table S1. All PCR reactions were performed in a thermocycler GeneTouch (BioER, Hangzhou, China) using the following program for the primer set 20F/CH1750R: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 3 min and final extension at 72°C for 10 min. For ITS2 primer set AL1500af/LR3, an initial denaturation at 95°C for 5 min was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 25 s and final extension at 72°C for 5 min. For *rbc*L primer sets we used a PCR cycle as indicated by Hoham *et al.* (2002) with a slightly modified annealing temperature (52.8°C). Aliquots of 2 µl of PCR products were quantified on a 1% agarose gel stained with GelRed in 0.5 TBE buffer (130 V 30 min). PCR products were purified using ethanol and sent for sequencing to Macrogen (Amsterdam, the Netherlands). Sequences were deposited in GenBank under accession numbers KX620913 and KX620914.

Phylogenetic analyses

SSU rDNA and rbcL phylogenetic analyses

The closest related SSU, ITS and *rbc*L sequences to the strain CCALA 307 and other representatives of the class Trebouxiophyceae were acquired from GenBank employing the BLAST algorithm (Altschul *et al.*, 1997). All new sequences were checked for chimeras using Bellerophon (Huber *et al.*, 2004). Two separate trebouxiophycean datasets were compiled, one for SSU sequences and one for *rbc*L sequences; the sequence alignments were computed using MAFFT v.6 (Katoh & Toh, 2008). The aligned sequences were checked for possible misaligned positions in BioEdit 7.0.9.0 (Hall, 1999). The SSU alignment of the Trebouxiophyceae included 87 sequences/1755 positions (686 variable, 487 parsimony informative). The *rbc*L alignment comprised 57 sequences/1173 positions (623 variable, 489 parsimony informative). Based on the AIC criterion in jModelTest 0.1.1 (Posada, 2008), the GTR+Γ+I nucleotide substitution model was selected as best fitting both the datasets. A maximum-likelihood phylogeny was computed in RAxML 7.0.4 (Stamatakis *et al.*, 2008) under the proposed model, and statistical support values were derived from rapid bootstrapping (1000 replicates) in the same program. For additional statistical support, Bayesian posterior probabilities were computed in MrBayes 3.2.1 x64 (Ronquist *et al.*, 2012). We carried out two MCMC runs for one million generations each with one cold and three heated chains under the GTR+Γ+I evolutionary model (parameters were estimated from the data); trees were sampled every 100 generations. The final trees were visualized using FigTree (Rambaut, 2007). For additional sequence comparisons, Kimura-2-parameter p-distances were computed in MEGA6 (Tamura *et al.*, 2013).

ITS2 rDNA secondary structure analysis

Precise annotation of the internal transcribed spacer 2 including the 5.8 and 28S flanking regions was accomplished by the ITS2 online database (Schultz *et al.*, 2006; Selig *et al.*, 2008; Keller *et al.*, 2009; Koetschan *et al.*, 2010, 2012). The annotation of the ITS1 spacer was assessed via a comparison with annotated *Neocystis* sequences available in GenBank. Minimum energy secondary structure model of ITS2 was computed with RNAstructure 5.3 (Reuter & Mathews, 2010) and visualized by Varna 3.8 (Darty *et al.*, 2009). Subsequently, a sequence + structure alignment including CCALA 307, *Neocystis brevis* CAUP D 802, *N. mucosa* KR 1989/14, *Gloeocystis polydermatica* CCAP 31/5 and *Stichococcus bacillaris* SAG 379-1b was built employing the ClustalW algorithm implemented in 4SALE 1.7. (Seibel *et al.*, 2006, 2008). The same software computes compensatory base changes (CBCs; Wolf *et al.*, 2013) among sequences.

Results

Cell morphology and ultrastructure

Strain CCALA 307 was coccoid unicellular with a thin, smooth and unornamented cell wall. The cell shapes varied from spherical to ellipsoid (Figs 1–6).

Mature vegetative cells were (3.0–) 4.3–5.4 (–6.7) µm in diameter. Old, mature cells were mostly globular, had few lipid droplets in the cytoplasm and reached up to 7.7 µm in diameter. A single parietal moon-shaped chloroplast occupied the majority of the cell volume (Figs 1–15). Starch granules were observed in the inter-thylakoid space (Fig. 11) but no pyrenoids. The chloroplast was usually divided into two lobes separated by a narrow incision (Figs 3–5, 7, 8, 12, 15). The cells had one nucleus which was located in the middle and embraced by a chloroplast (Figs 10–12). The space between the nucleus and the chloroplast was occupied by mitochondria and small vacuoles (Figs 10–12). Plastoglobuli were visible in cross-sections (Fig. 15). The cells reproduced by autosporulation, forming 2–6 autospores of the same size (Figs 6, 9, 13, 14) which were later released through a rupture of a mother cell wall at one side of the cell (Fig. 14). The daughter cells stayed covered by the persistent remains of the mother cell wall (Figs 4, 5, 15). Neither zoospore formation nor sexual reproduction were observed.

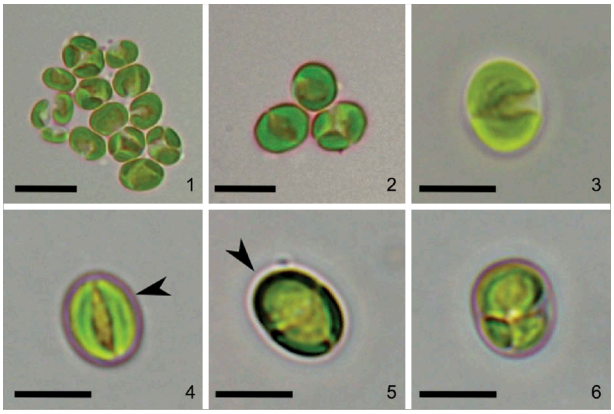
Molecular phylogeny and ITS2 secondary structure analysis

The strain CCALA 307 showed 97% SSU sequence similarity to *Neocystis brevis* SAG 850-1 (KM020044), *Neocystis mucosa* SAG 40.88 (JQ920367; *Neocystis*-clade) and *Coenocystis inconstans* (AB017435) (the closest cultured species available in GenBank (October 2016)). The *rbc*L comparisons revealed a relatively low similarity of 89% with the closest match, '*Chlorella*' *mirabilis* SAG 38.88 (KM462865; *Prasiola* clade). Both the SSU (Fig. 16) and *rbc*L (Fig. 17) analyses congruently pointed out that strain CCALA 307 represents a phylogenetically isolated lineage within the Trebouxiophyceae, possibly a sister branch of the *Neocystis* and *Prasiola* clades (Figs 16–17), yet with low statistical support. The phylogenetic placement of the studied strain was identical in the SSU and *rbc*L tree topologies, for both the maximum-likelihood and the Bayesian inferences. Considering the genetic similarity of CCALA 307 with GenBank sequences of uncultured organisms, the closest SSU relative was Uncultured Dunaliellaceae clone Amb_18S_930 (EF023670; 97%) and the closest *rbc*L relative was Uncultured *Trebouxia* photobiont clone L-68 (AM158969; 89%).

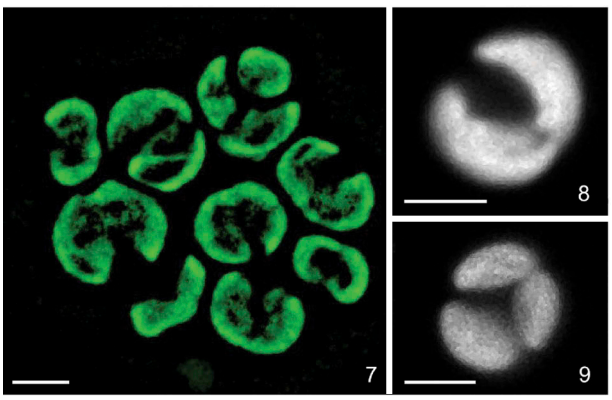
Regarding the ITS1-5.8S-ITS2 spacer region, the closest GenBank relatives of CCALA 307 comprised several *Neocystis* species (e.g. *Neocystis mucosa* SAG 40.88) with 81% similarity. The ITS2 secondary structure model of CCALA 307 showed features characteristic of other green algae, consisting of four helices and well identifiable conserved motifs (U-U in helix II and UGGU in helix III; Fig. 18). Particularly helix II and helix III are highly conserved among CCALA 307 and both *Neocystis* species (blue and red coloured nucleotide positions in Fig. 18). The strain CCALA 307 also shares some structural features with the representatives of the *Prasiola* clade (blue and green coloured nucleotide positions in Fig. 18), but the major parts of helix I and helix IV are specific for CCALA 307 and divergent from both the *Neocystis* and *Prasiola* clade representatives (grey coloured nucleotide positions in Fig. 18). The closer relationship of CCALA 307 to the *Neocystis* clade than to the *Prasiola* clade was supported by the analysis of compensatory base changes (CBCs) within the ITS2 secondary structure. CCALA 307 differed by four CBCs from *Neocystis* spp. (black dots in Fig. 18) and by seven CBCs from the members of the *Prasiola* clade (grey and white dots in Fig. 18).

Lunachloris Barcytė & Hodač, gen. nov.

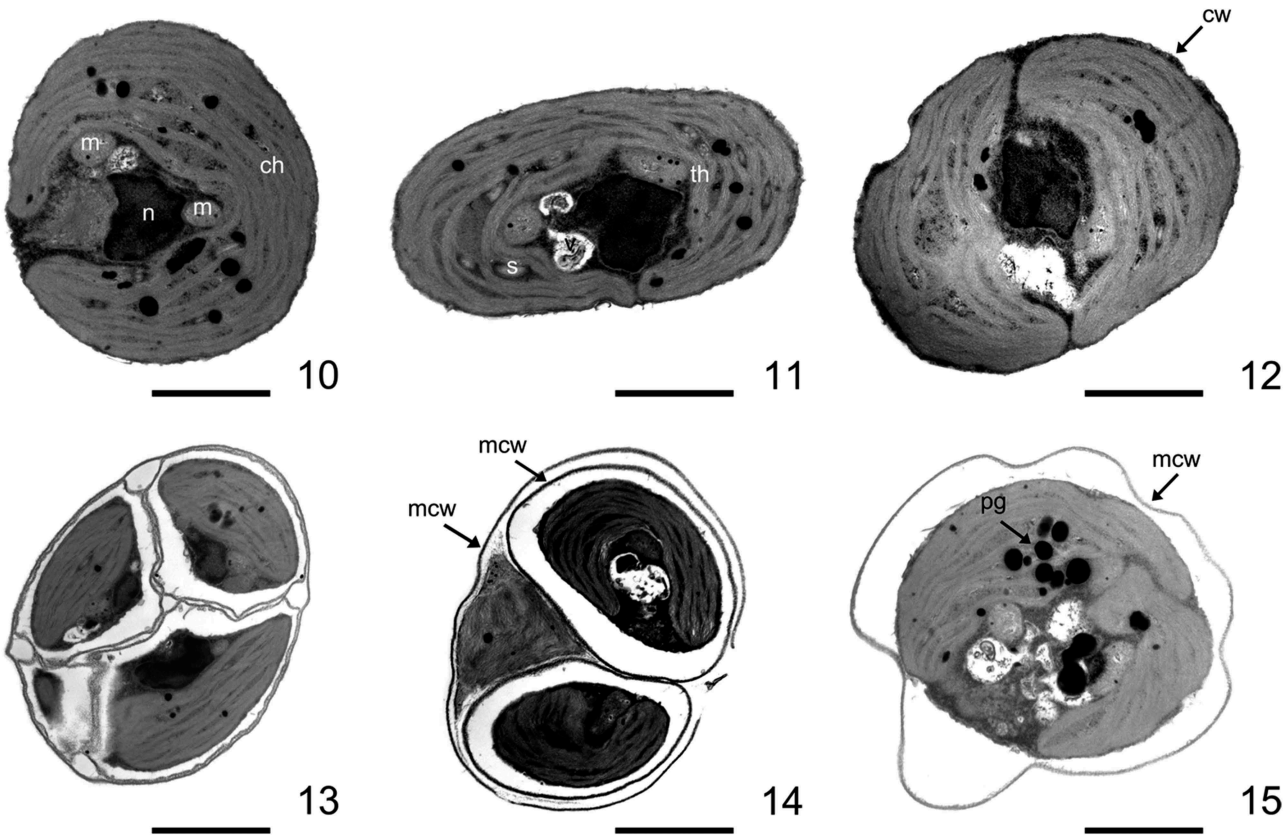
DIAGNOSIS: Vegetative cells solitary, spherical or ellipsoid, uninucleate. Single parietal smooth two-lobed chloroplast lacks pyrenoid. Asexual reproduction via



Figs 1–6. Morphology of *Lunachloris lukesovae* gen. et sp. nov. CCALA 307. **Figs 1–5.** mature cells **Fig. 6.** autosporangium. Arrowheads point to the margins of the parental cell wall. Scale = 5 µm.



Figs 7–9. Confocal reconstructions of the chloroplast structure of *Lunachloris lukesovae* CCALA 307. **Figs 7–8** mature vegetative cells. **Fig. 9.** autosporangium. Scale = 3 µm.



Figs 10–15. Ultrastructure of *Lunachloris lukesovae* CCALA 307. **Figs 10–12**. Vegetative cells (remnants of mother cell wall are not shown). **Figs 13–14.** Autosporangia. **Fig. 15.** Vegetative cell surrounded by mother cell wall after autospore release. ch, chloroplast; m, mitochondrion; n, nucleus, s, starch; th, thylakoids; cw, cell wall; mcw, mother cell wall; pg, plastoglobuli. Scale = 1 μm.

2–6 autospores which are liberated through rupture of mother cell wall at one side. Size of autospores is the same within single autosporangium. Sexual reproduction or production of zoospores were not observed. Lipid droplets may be present in cytoplasm. The genus differs from other genera in SSU, ITS2 and *rbc*L sequences.

Type species: *Lunachloris lukesovae* Barcytė & Hodač.

Etymology: Genus name comes from a Latin word *luna* meaning 'moon', emphasizing moon-shaped chloroplast and Greek word *khloros*/χλωρός meaning 'pale green'.

Lunachloris lukesovae Barcytė & Hodač, sp. nov.

Diagnosis: Vegetative cells are solitary, uninucleate with spherical or ellipsoid outline, (3.0–) 4.3–5.4 (–6.7) μm in diameter and covered by remnants of mother cell wall when cultivated in liquid (BBM) media. Old cells are mostly globular. No flagella were observed. The single chloroplast is smooth, parietal and composed of two lobes. No pyrenoid. The nucleus is central, 2–4 mitochondria in the cytoplasm. Asexual reproduction is via 2–6 autospores. Sexual reproduction and production of zoospores were not observed. Lipid droplets present in older cells. The species differs from other species in SSU, ITS2 and *rbc*L sequences. DNA sequences available for the type strain: nuclear SSU, ITS1-5.8S-ITS2 rDNA KX620913 and plastid *rbc*L KX620914.

Holotype: The authentic strain CCALA 307 is permanently cryopreserved at CCALA in the metabolically inactive stage. Figures 1–6 show the morphology of the holotype.

Type locality: Meadow soil by Dlouhá Ves (near Chelčice), Czech Republic.

Etymology: The species name is after Dr Alena Lukešová, a prominent Czech soil biologist, who isolated the strain.

Discussion

Morphological characteristics of the strain investigated in this study fit with the typical description of many coccoid green algae and, therefore, we were not able to reveal its precise taxonomic position based merely on morphological and ultrastructural characters. Among the known algal genera it resembles, for example, *Coccomyxa* Schmidle (as it was originally assigned) and *Choricystis* (Skuja) Fott or *Neocystis* Hindák, all with very similar size and shape of the cells (Komárek & Fott, 1983; Ettl & Gärtner, 2014)

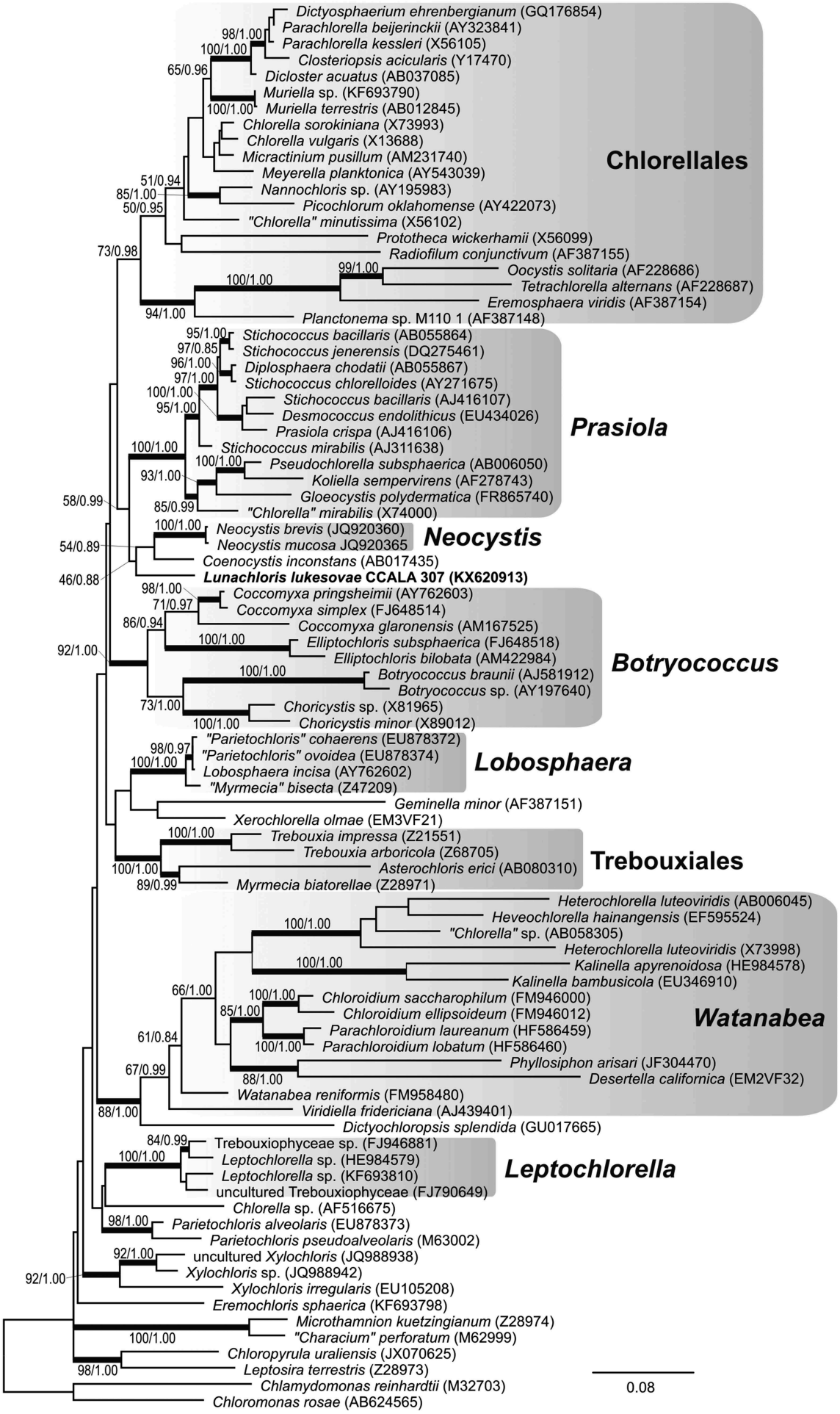


Fig. 16. Maximum-likelihood tree of SSU sequences from *L. lukesovae* CCALA 307 and other Trebouxiophyceae. Numbers next to branches indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities). Thick lines indicate branches with high statistical support.

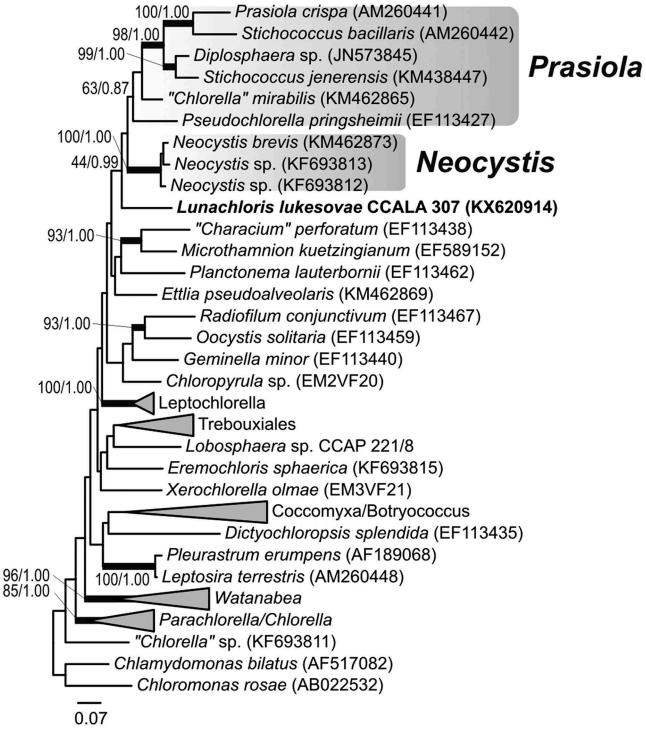


Fig. 17. Maximum-likelihood tree of *rbc*L sequences from *L. lukesovae* CCALA 307 and other Trebouxiophyceae. Numbers next to branches indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities). Thick lines indicate branches with high statistical support.

and a parietal chloroplast without a pyrenoid. However the shape of the chloroplast is most similar to *Neocystis* (Eliáš *et al.*, 2013) but no mucilage covering the cells was detected as is typical for this genus. Interestingly, the cells of *Lunachloris* were always surrounded by the persistent remnants of a mother cell wall as is also known, for example, in *Coenochloris* Korshikov and some species in *Radiococcus* Schmidle. This feature was seen not only using a light microscope but also confirmed by TEM microphotographs. However, this character can be induced only while growing the alga in liquid medium as, for example, noticed in *Hylodesmus* (Eliáš *et al.*, 2010). The strain reproduced by autosporulation and we did not detect any zoospores.

The nuclear 18S-ITS1-5.8S-ITS2 rDNA region of *Lunachloris* was highly divergent compared with other trebouxiophycean taxa, indicating an early split from the nearest neighbours and thus the isolated phylogenetic position (Fig. 16). These findings were in agreement with phylogenetic analysis of the chloroplast *rbc*L gene (Fig. 17). The alga was placed on a solitary branch as a sister group to *Neocystis* and *Prasiola* (Karsten *et al.*, 2005; Krienitz *et al.*, 2011) clades and the distinct, lineage-forming *Coenocystis inconstans* (Hanagata & Chihara, 1999), belonging to core trebouxiophyceans (Lemieux *et al.*, 2014). Given that the affiliation to the above-mentioned clades and species was not statistically supported in any analyses, *Lunachloris* might represent a novel lineage most closely related to the *Neocystis* clade and *Coenocystis inconstans*, known from tree bark (Hanagata & Chihara, 1999). In contrast to *Lunachloris*, both genera exhibit a mucilaginous layer covering the cells. In addition, *C. inconstans* also has a pyrenoid. Therefore, these three phylogenetically closely related genera could be discriminated by their appearance, although not by very conspicuous characteristics, especially in the case of *Lunachloris* and *Neocystis*. Moreover, they are an example of how vegetative morphology can develop relatively quickly in different directions within close relatives. On the other hand, the features seen in cultures, e.g. mucilage production, could be induced by cultivation conditions, as is known, for example, in *Coccomyxa* (Darienko *et al.*, 2015). Therefore, the morphology of many coccoid autosporic algae should be interpreted with caution.

Trebouxiophytes are extremely diverse ecologically (Leliaert *et al.*, 2012), however, the majority of them are reported from various terrestrial or aeroterrestrial habitats, including soil, tree bark, wet rocks or artificial hard substrates (Hallmann *et al.*, 2013 and references therein). For example, *Chloroidium* belonging to the *Watanabea* clade, *Myrmecia* and *Trebouxia* in the Trebouxiales, *Coccomyxa* and *Elliptochloris* in the *Elliptochloris* clade, *Stichococcus, Diplosphaera* and *Pseudochlorella* from the *Prasiola* clade, and *Dictyochloropsis* are all well-known from both aerophytic substrata and soil (Darienko *et al.*, 2010, 2015, 2016; Hallmann, 2015; Hodač *et al.*, 2016; Malavasi *et al.*, 2016; Škaloud *et al.*, 2016). The majority of *Neocystis* strains, which are the closest relatives to *Lunachloris*, were isolated from soil (Eliáš *et al.*, 2013) while *Coenocystis inconstans* is an aerial alga (Hanagata & Chihara, 1999). *Lunachloris* was isolated from a wet meadow where water is usually at, or near, the surface of the soil throughout most of the year. However, considering the ecology of its closest relatives, we speculate that *Lunachloris* might be a true terrestrial/soil alga rather than an aquatic species occurring in soil water.

Generally, minute soil algae tend to disperse over long distances and, therefore, are expected to be common (Hodač *et al.*, 2016). However, *Lunachloris* has not been detected before, even by culture-independent environmental sequencing. Multiple microalgae have been re-detected by both culture-dependent and culture-independent approaches. However, *Lunachloris lukesovae* might represent an example of a rare or rarely accessible species. On the other hand, the total biodiversity of soil microalgae is still poorly understood and requires extended sampling for diversity

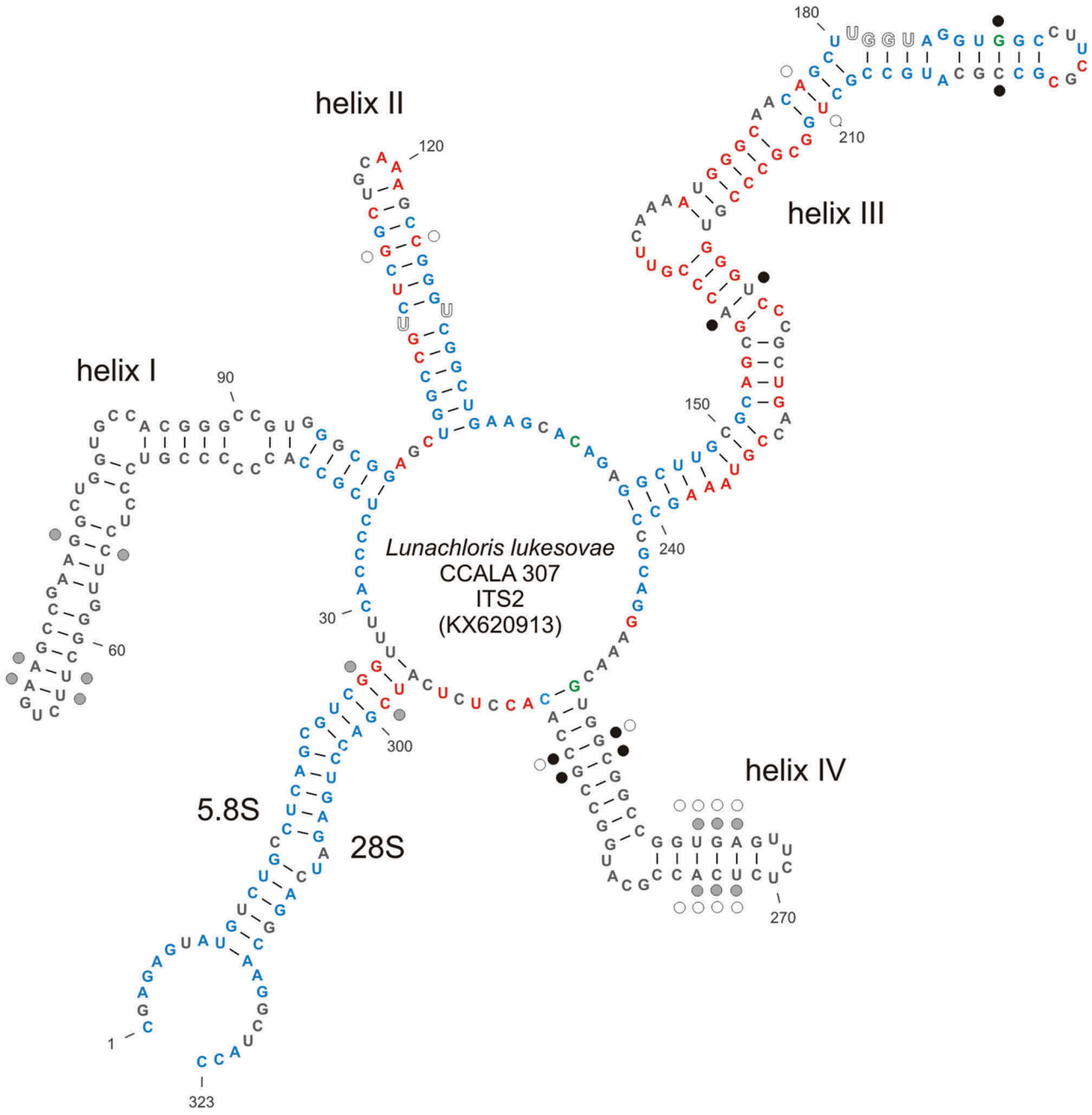


Fig. 18. Secondary structure of the ribosomal internal transcribed spacer 2 (ITS2 rDNA) of *L. lukesovae* CCALA 307. Nucleotide sites which are conserved among *L. lukesovae* CCALA 307 and relatives from the *Neocystis* clade (*Neocystis brevis* CAUP D 802, *N. mucosa* KR 1989/14) and from the *Prasiola* clade (*Gloeocystis polydermatica* CCAP 31/5, *Stichococcus bacillaris* SAG 379-1b) are indicated by blue bold type. Nucleotides highlighted in green are common to *L. lukesovae* CCALA 307 and the *Prasiola* clade and are not present in *Neocystis* spp. Nucleotides highlighted in red are common only to *L. lukesovae* CCALA 307 and *Neocystis* spp. Dots near to some nucleotide sites mark compensatory base changes (CBCs). Black dots represent CBCs among *L. lukesovae* and *Neocystis* spp. Grey and white dots represent CBCs among *L. lukesovae* and *G. polydermatica* and *S. bacillaris*, respectively.

evaluation (Hodač *et al.*, 2016). Since the recently described genus *Chloropyrula* (isolated from soil in the Ural Mountains and represented by a single isolate; Gaysina *et al.*, 2013) has since been detected again in a soil crust in the Californian desert (Fučíková *et al.*, 2014), *Lunachloris* also could be found elsewhere in the future. Due to the common problem of morphological misidentification, we cannot exclude the possibility that *Lunachloris* has been isolated and preserved elsewhere. Consequently, the molecular reassessment of historical strains might reveal rare or as yet undiscovered species (Hoshina, 2014).

For many years, the discovery of new algal species has relied on the isolation and cultivation of unialgal strains. Many of these are still accessible thanks to various microbial culture collections whose fundamental aim is to collect, preserve and make strains available to the public. Moreover, culture collections serve as biodiversity stores, that are especially important in light of recent, rapid

biodiversity loss (Pimm & Raven, 2000; Cary & Fierer, 2014). Traditional isolation methods discriminate against very small, rare or difficult-to-culture algal species, however, many culture-independent methods, i.e. DNA metabarcoding and metagenomics, have recently been developed to study microbial communities directly in their environment and without doubt generally show a much more quantitatively complete view of the existing biodiversity. Nevertheless, these novel, advanced methods are not ideal and also have limitations. For example, next-generation sequencing (NGS) technologies generally produce shorter sequences (with higher error rates) in comparison to traditional genomic sequencing, leading to lower taxonomic resolution. Consequently, in this paper we have demonstrated that isolation and cultivation of strains could still provide a valuable complementary approach for biodiversity studies even in the era of culture-independent biodiversity research.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

D. Barcytė: original concept, molecular lab work, microscopy, drafting and editing of manuscript; L. Hodač: phylogenetic analyses, drafting and editing of manuscript; L. Nedbalová: financial support and writting of manuscript.

Supplementary Information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at http://dx.doi.org.10.1080/09670262.2017.1283541

Supplementary table S1. List of primers included in the study.

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Table S1. List of primers included in the study

Primer name	Sequence	Authority
18S rDNA		
20F	GTA GTC ATA TGC TTG TCT C	Thüs *et al* ., 2011
CH1750R	CTT CCT CTA RTG GGA GG	Hallmann *et al* ., 2013
34F	GTC TCA AAG ATT AAG CCA TGC	Friedl, in: Pažoutová *et al* ., 2010
370R	AGG CTC CCT CTC CGG AAT C(AG)A ACC C	Friedl, in: Pažoutová *et al* ., 2010
891F	GTC AGA GGT GAA ATT CTT GGA	Friedl, unpubl.
895R	AAA TCC AAG AAT TTC ACC TC	Remias *et al* ., 2010
1122F	GGC TGA AAC TTA AAG GAA TTG	Friedl, in: Pažoutová *et al* ., 2010
1122R	CAA TTC CTT TAA GTT TCA GCC	Friedl, unpubl.
1422F	CAG GTC TGT GAT GCC CTT AG	Remias et al., 2010
1422R	CTA AGG GCA TCA CAG ACC TG	Friedl, unpubl.
ITS1-5.8S-rDNA-ITS2		
AL1500aF	GCG CGC TAC ACT GAT GC	Helms *et al* ., 2001
LR3	GGT CCG TGT TTC AAG ACG G	Vilgalys & Hester, 1990
ITS1	TCCGTAGGTGAACCTGCGG	White *et al* ., 1990
ITS4	TCC TCC GCT TAT TGA TAT GC	White *et al* ., 1990
5,8 SbF	CGA TGA AGA ACG CAG CG	Mikhailyuk *et al* ., 2008
nr-SSU-1780-5' Algal	CTGCGGAAGGATCATTGATTC	Piercey-Normore & DePriest, 2001
nr-LSU-0012-3´Algal	AGTTCAGCGGGTGGTCTTG	Piercey-Normore & DePriest, 2001
rbcL		
rbcL1F	GCTGG TGTTA AAGAT TATCG	Hoham *et al* ., 2002
rbcL2F	GTCGT GGTCT ATTAG GTTG	Hoham *et al* ., 2002
rbcL4R	GAAAA TGAAA CGGTC TCTCC	Hoham *et al* ., 2002
rbcL7R	AAATA AATACC ACGGC TACG	Hoham *et al* ., 2002
rbcL8F	GGTCT TTCAG CTAAA AACTA CGG	Hoham *et al* ., 2002
rbcL9R	CCG TA GTT TT TAG CT GAA AG RCC	Hoham *et al* ., 2002
rbcL10F	GGTAA CGTWT TTGGT TTCAA AGC	Hoham *et al* ., 2002
rbcL14R	CGTTC WCCTT CAAGT TTACC	Hoham *et al* ., 2002
rbcL19F	CTCAA TCGTT CATGC GTTGG	Hoham *et al* ., 2002
rbcL20F	CGTTC ATGCG TTGGA GAGAC	Hoham *et al* ., 2002

Paper X

BARCYTĖ D, FOTT J & NEDBALOVÁ L (2019) A molecular approach to identification of protonemata helps assess biodiversity of extremely acidic freshwaters. *Limnology* 20: 225–231

Authors' contributions:

JF, LN and DB designed the study; DB obtained and analyzed the data, and wrote the paper; JF contributed in writing of the manuscript; LN read the draft of the paper

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RESEARCH PAPER

A molecular approach to identification of protonemata helps assess biodiversity of extremely acidic freshwaters

Dovilė Barcytė[1,2] · Jan Fott[1] · Linda Nedbalová[1]

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Abstract

Macroscopic fuzzy clumps of green filaments resembling filamentous algae were found on multiple sampling occasions in the water close to the shore of the extremely acidic (pH < 3) Hromnice Lake in Czechia. Microscopic investigation revealed that these filaments were moss protonemata. In order to identify the moss, we sequenced chloroplast (*rbc*L), mitochondrial (*nad*5), and nuclear (ITS2) molecular markers of these filaments. In addition, we sampled adult mosses growing on the wet substrate soaked with lakewater. The sequences of protonemata matched those of the adults, which were morphologically identified as *Dicranella* sp. Phylogenetic analysis of the *rbc*L gene showed a sister relationship with *D. heteromalla*, generally known for growing in acidic habitats, and other protonemata occurring in acidic rivers in Japan. The *nad*5-based phylogeny revealed that the studied protonemata belonged to the species *D. cerviculata*, and the same taxonomic affiliation was confirmed by the ITS2 rDNA sequence and its secondary structure. The extreme environment of Hromnice Lake prevents the further development of protonemata which, in turn, are capable of surviving acidic conditions in the prolonged protonemal stage. Due to their macroscopic similarity to filamentous algae, protonemata might be more common in extremely acidic waters than originally thought.

Keywords Acidic pit lakes · Protonema · *Dicranella* · Molecular phylogeny

Introduction

The majority of acidic pit lakes (pH 2.0–4.5) are characterized by high levels of iron (Fe^{3+}), sulfate (SO_4^{2-}), and various other heavy metals, especially aluminum (Al^{3+}). These unfavorable environmental conditions prevent the establishment of typical lake ecosystems with high species diversities and complex biotic interactions. Only well-adapted acidophilic or opportunistic acidotolerant species can colonize acidic pit lakes (Geller et al. 2013).

Planktic prokaryotic and eukaryotic microorganisms are the main biotic constituents of extremely acidic pit lakes. The plankton of such lakes have been well studied, especially in Europe (Lessmann et al. 2000; Moser and Weisse 2011; Hrdinka et al. 2013; Falagán et al. 2014; Pociecha et al. 2018). However, studies of the benthos, periphyton, or littoral species of the extremely acidic pit lakes are relatively scarce. Therefore, our knowledge of the organisms that invade extremely acidic waters is incomplete and they require further investigation. The application of molecular techniques, ranging from Sanger to high-throughput sequencing, could help to fill this gap, especially for organisms which are difficult to identify or to detect.

The present study aimed to identify and explain the nature of the protonemata in Hromnice Lake, an acidic pit lake that has already been mentioned by Hrdinka et al. (2013). We hypothesized that adult bryophytes growing on the lake shore might be conspecific with the protonemata living in the water. We used a molecular approach to identify the protonemata and to test our hypothesis.

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✉ Dovilė Barcytė
dovile.barcyte@gmail.com

[1] Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2 128 44, Czechia

[2] Department of Zoology, National Museum, Cirkusová 1740, Prague 9 193 00, Czechia

Materials and methods

Location

Geographical, geological, historical, and limnological characteristics of Hromnice Lake (Czechia, 49°51′02.5″N, 13°26′39.3″E) were described in detail in Hrdinka et al. (2013). The lake is characterized by water of pH ~ 2.6 and by water chemistry dominated by sulfate, iron, and other heavy metals typical of pit lakes. Hromnice Lake is the most acidic pit mining lake in Czechia, with a low species diversity (Fig. S1 in the Electronic supplementary material, ESM).

Sampling

Early samples of protonemata and adult mosses were taken during the years 2010–2012 from a spit of land only a few centimeters above the water level in the shallow part of the lake (Fig. S2 in the ESM). The clumps of protonemata were sampled using a strainer. Upon arrival at the laboratory they were cultivated for several months (Petri dish, fluorescent light, 10 °C, diluted lakewater enriched by Bold's Basal Medium for algae; see Fig. S3 in the ESM).

Samples for molecular identification were taken in May 2017. The green fuzzy filaments were placed in a sterile 50-mL Falcon tube and immediately transported to the molecular genetics laboratory in the National Museum in Prague for DNA extraction. We also took three adult mosses growing on the lake shore and a leafy liverwort from the water. Morphological identification was carried out using a magnifying glass and a Nikon Eclipse E400 light microscope (Nikon Corp., Tokyo, Japan).

The protonemata and related mosses were deposited in the Herbarium Collections at the Charles University in Prague (international acronym: PRC), Czechia.

DNA extraction, PCR, and sequencing

The total genomic DNA was extracted from the collected plants using the Geneaid (New Taipei City, Taiwan) Plant Genomic DNA Mini Kit. The filaments of the protonemata and the tissue of the adult organisms were disrupted using the bead-beating method. The plant material was transferred to bead-beating tubes containing 2.8-mm zirconium oxide beads and 100 µl of lysis buffer and beaten four times for 20 s at 6500 rpm in a bead-based Precellys 24 homogenizer (Bertin Instruments, Motigny-le-Bretonneux, France). We used three molecular markers for the molecular identification of the bryophytes. A partial fragment of the plastid ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) region was amplified and sequenced with primers RH1 and 1385R (Manhart 1994). The mitochondrial intron sequence of the NADH protein-coding subunit 5 (*nad*5) gene was amplified with the primers *nad5*F4 and *nad5*R3 (Fedosov et al. 2016). Primers ITS1 and ITS4 were used for the nuclear internal transcribed spacer 2 (ITS2) amplification (White et al. 1990). Polymerase chain reactions (PCRs) were done using PPP Master Mix (Top-Bio, Prague, Czechia) in a total volume of 25 µl. For the chloroplast and mitochondrial markers, the cycling conditions were 95 °C for 10 min of initial denaturation, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 45 s, and elongation at 72 °C for 2 min 50 s, with a final extension for 10 min at 72 °C. The ITS2 region was amplified as follows: initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. The amplification products were purified with ethanol and sequenced by Macrogen (Amsterdam, the Netherlands). The obtained sequences were submitted to GenBank under accession numbers MK353893–MK353897.

Phylogenetic analyses

The BLAST search algorithm (Altschul et al. 1997) was employed to search the GenBank database for sequences closely related to our newly sequenced plants. For phylogenetic comparisons, 36 chloroplast *rbc*L sequences and 10 mitochondrial *nad*5 sequences were acquired. Sequences of the *rbc*L and *nad*5 genes were aligned separately by MAFFT v7 (Katoh et al. 2017) and modified manually in BioEdit v7.2.5 (Hall 1999), resulting in final alignments of 1296 and 1089 positions, respectively. Best-fit nucleotide substitution models for both datasets were estimated using PartitionFinder 2 (Lanfear et al. 2017) with the Bayesian information criterion (BIC) and the "greedy" algorithm. Phylogenetic relationships were inferred by maximum likelihood (ML) and Bayesian approaches. ML analysis was performed with RAxML 8.2.10 (Stamatakis 2014) using the GTR + I + G model for the *rbc*L dataset (including all three codon positions) and GTR + G for the *nad*5 alignment. The robustness of the nodes was evaluated by a bootstrap procedure with 1000 and 100 replications, respectively. Bayesian analysis was performed by a Markov chain Monte Carlo (MCMC) method in MrBayes v3.2.1 (Ronquist et al. 2012). Two heated and one cold chains were employed in all analyses, and runs were initiated from random trees. Two independent runs were conducted with one million generations per run, and trees and parameters were sampled every 100 generations. Convergence of parameters was checked with Tracer v1.7.1 (Rambaut et al. 2018). For each run, the first 25% of the sampled trees were discarded as burn-in. Bayesian posterior probabilities were used to assess the branch support of

the Bayesian tree. The program FigTree v1.4.2 was used to visualize the phylogenetic trees (Rambaut 2007).

ITS2 rDNA secondary structure analysis

The internal transcribed spacer 2 (ITS2) located between the 5.8S and 26S rRNA genes was annotated using the ITS2 database (Koetschan et al. 2012). The ITS2 secondary structure was computed based on the minimum energy model in the program RNAstructure 5.4 (Reuter and Mathews 2010) and drawn by VARNA 3.9 (Darty et al. 2009). In the search for compensatory base changes (CBCs: mutations that occur in both nucleotides of a paired structural position while retaining the paired nucleotide bond) with respect to its closest revealed relative, the ITS2 sequences along with their secondary structures were aligned using the ClustalW algorithm implemented in the program 4SALE 1.7 (Seibel et al. 2008).

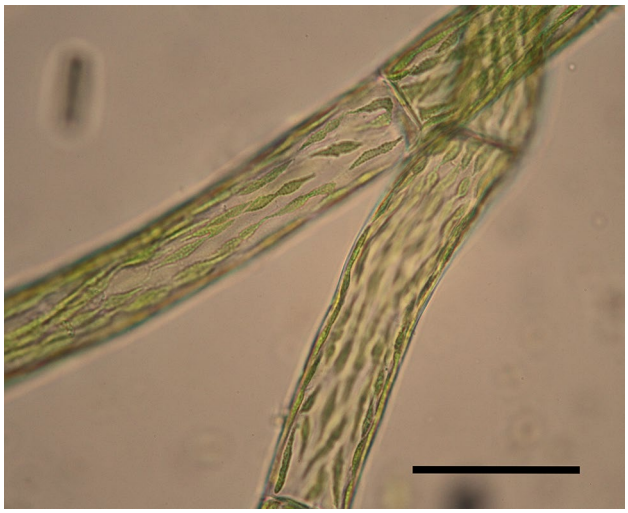

Fig. 1 Protonemata of *Dicranella cerviculata* that occur in Hromnice Lake. *Scale bar* = 50 µm

Results and discussion

Protonema is a short-lived filamentous undiferentiated multicellular structure of a bryophyte that develops into a leafy or thallose gametophyte. However, in Hromnice Lake we have observed long-lasting, thread-like macroscopic structures (Fig. S2 in the ESM) that were previously identified as protonemata (Hrdinka et al. 2013). The green filaments were found during each visit to the lake (since 2010), even after the ice melt. This points to successful asexual reproduction of the protonemata under the harsh environmental conditions.

It is known that bryophytes occur in highly acidic environments as protonemata. However, this phenomenon is greatly underestimated and rarely discussed. Whitton and Diaz (1981) noted the presence of protonemata in acidic waters (pH ≤ 4.0) in Europe and USA, while Wehr and Whitton (1983) reported protonemata from acidic springs in British Columbia (Canada). Higuchi et al. (2003) found macroscopic mat-forming aggregates of protonemata in three acidic rivers (pH 1.9–2.4) in Japan. These aggregates, which they called "Misuzugoke," remained in a prolonged protonemal stage for several growing seasons. We also detected protonemata in algal samples from the acidic (pH ~ 2.5) and heavy-metal-rich Tinto River in Spain (data not shown). Indeed, due to their morphological similarity to filamentous algae, protonemata might often be overlooked and occur in extremely acidic sites more commonly than reported.

The presence of numerous small chloroplasts (Fig. 1) distinguishes protonemata from green filamentous algae, which are commonly encountered in similar types of habitats. For example, streptophytic green algae from the order Zygnematales successfully occur in periphyton or metaphyton of acidified freshwaters. In particular, members of the genera *Zygogonium* were reported from acidic freshwaters in Europe and North America (Whitton and Diaz 1981; Zettler et al. 2002; Kleeberg et al. 2006).

The studied protonemata were found together with the liverwort *Gymnocolea inflata*, the only submerged macrophyte detected in Hromnice Lake. *Gymnocolea inflata* is commonly associated with acidic habitats, especially peat bogs. Since *G. inflata* does not arise from a filamentous protonema, we rejected the possibility that they were conspecific and focused on the mosses growing on the lake shore. Morphological identification based on the arrangement and structure of leaves revealed that the sampled individuals belonged to the two haplolepideous mosses (subclass Dicranidae) from the genera *Dicranella* and *Dicranum*.

In order to molecularly match the protonemata with the adult mosses, we sequenced the partial fragment of the *rbc*L at both life cycle stages. The phylogenetic analysis revealed that the studied protonemata were genetically identical to the two sampled adult mosses, identified as *Dicranella* sp. (Fig. 2). This confirmed our hypothesis that the green threads living freely in the water were the initial stages of one of the mosses growing in the terrestrial habitat nearby. The newly sequenced *Dicranella* mosses formed a sister lineage to the species *D. heteromalla* and the Japanese protonemata "Misuzugoke" (Fig. 2). The results indicated that both Czech and Japanese protonemata occurring in acidic freshwaters belonged to the same genus but two different species, and that acidotolerance is associated with at least two *Dicranella* species (Fig. 2). Unfortunately, neither gametophytic morphological features nor phylogenetic analyses based on the chloroplast-encoded *rbc*L gene were sufficient to be able to identify the exact taxonomic affiliation of the acidotolerant

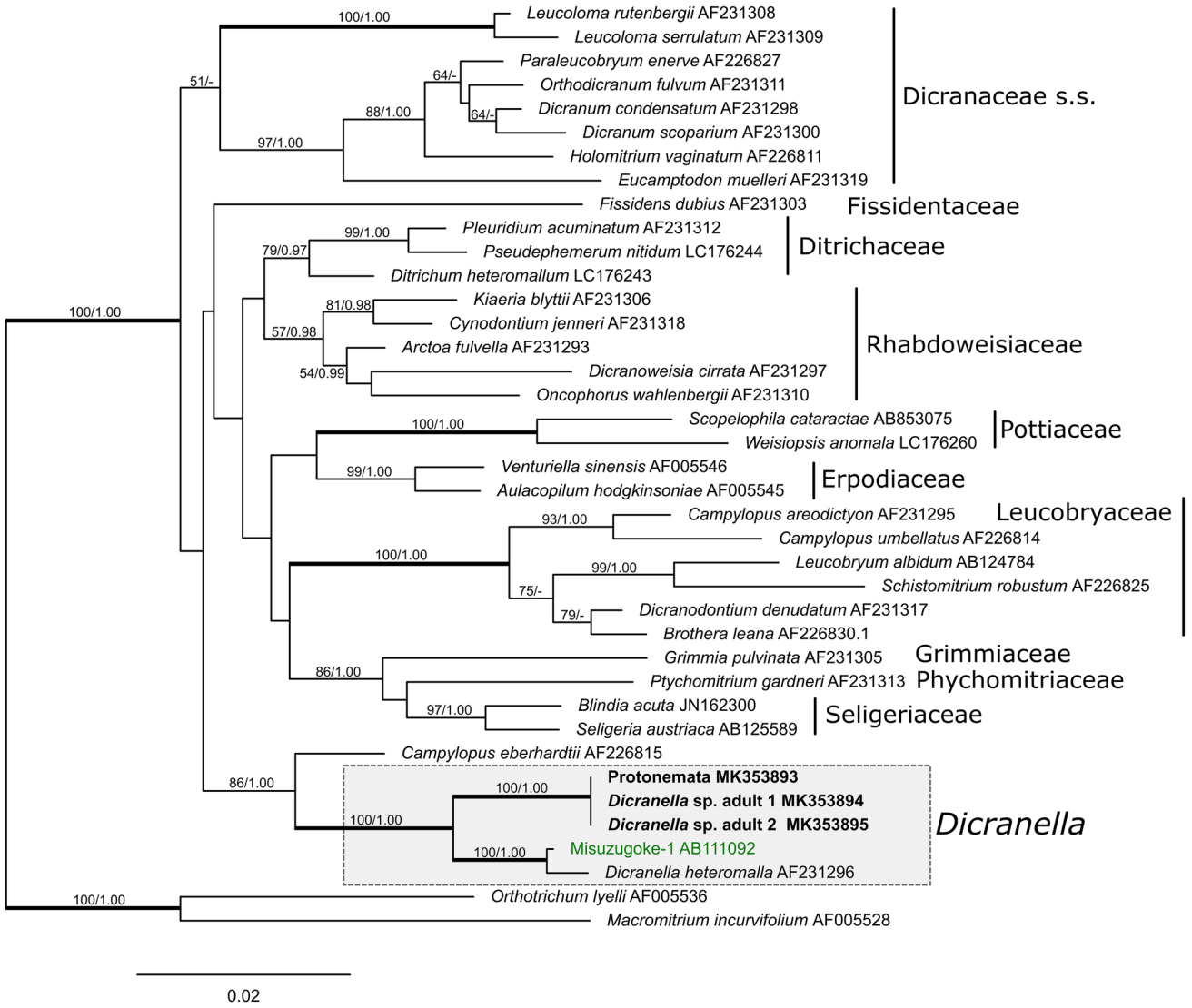

Fig. 2 Maximum-likelihood tree of the Dicranidae mosses based on *rbc*L sequences. *Numbers next to branches* indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities). *Thick lines* indicate full statistical support. New sequences are in *bold*; the protonemata occurring in acidic rivers in Japan are shown in *green*. *Scale bar* indicates the expected number of substitutions per site

mosses growing on the shore of Hromnice Lake. Therefore, we employed additional molecular markers, *nad*5 and ITS2, to improve protonema recognition (as well as that of the adults). Phylogenetic analysis of the mitochondrial *nad*5 dataset revealed that the Czech protonemata belonged to the species *D. cerviculata* (Fig. 3). It matched the *D. cerviculata* specimen Stech B970824.1 with one ambiguous nucleotide (out of 1083 bp) in the latter sequence. As in *rbc*L analysis, *D. heteromalla* was shown to be a sister species, and this relationship was highly statistically supported (Fig. 3). The Hromnice Lake protonemata and the two adult mosses had identical *nad*5 sequences. Meanwhile, the closest BLAST hit for the newly obtained ITS2 sequence (356 nucleotides) of the studied protonemata was *D. cerviculata* voucher Stech B970824.1 (KM594586), with 94% identity (identical at 338 out of 358 positions), including 3% gaps. However, despite such a low similarity coefficient, no CBCs were detected in the ITS2 secondary structure (Fig. 4), suggesting that the protonemata in Hromnice Lake should indeed be assigned to the widespread species *D. cerviculata*. The ITS2/CBC approach has been proven to correlate with the biological species concept in different eukaryotic groups (Wolf et al. 2013), and is now commonly used for species delimitation.

The genus *Dicranella* (Müll. Hal.) Schimp. *sensu lato* includes generally small mosses with slightly branching stems as well as long narrow smooth leaves with a wider, often clasping, base along with plane margins and a broad vein (Marshall 1919). However, recent molecular

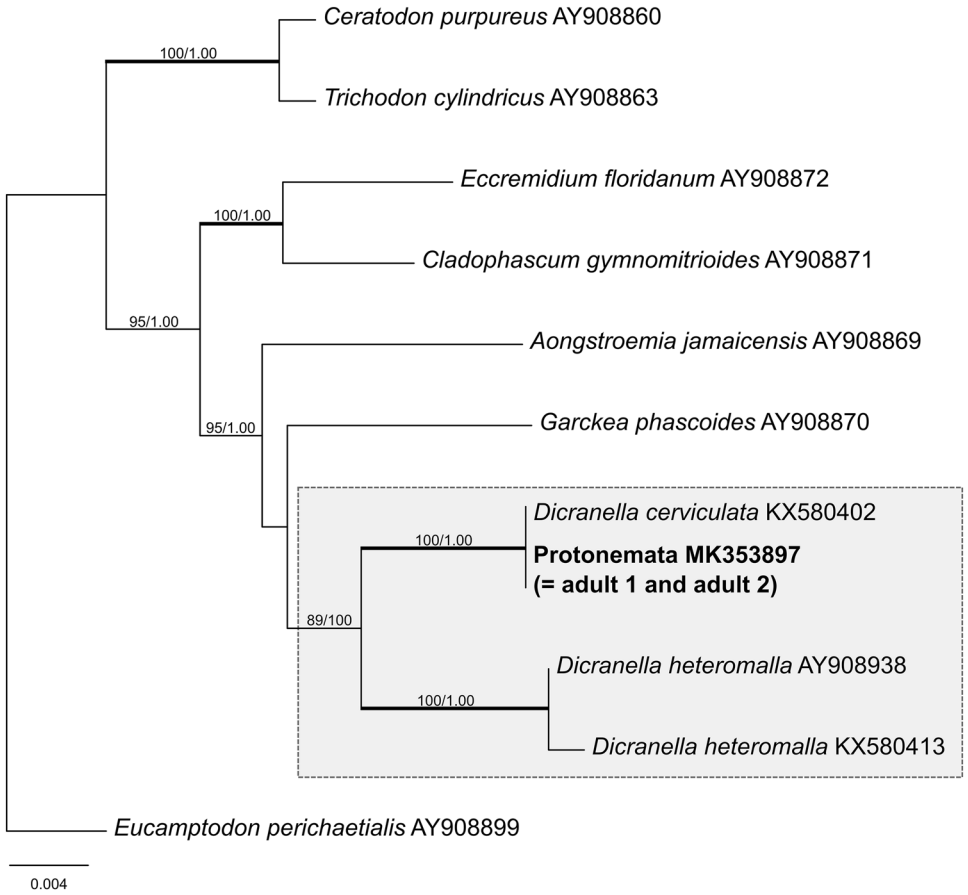

Fig. 3 Maximum-likelihood tree of the Dicranidae mosses based on *nad*5 sequences. *Numbers next to branches* indicate statistical support values (maximum-likelihood bootstraps/Bayesian posterior probabilities). *Thick lines* indicate full statistical support. New sequences are shown in *bold*. *Scale bar* indicates the expected number of substitutions per site

phylogenetic analyses discovered that this genus is polyphyletic (Bonfim Santos et al. 2017), and *Dicranella* now awaits taxonomic reclassification. In Czechia, a total of nine conventional *Dicranella* species have been reported, including the two taxa discussed here (Kučera et al. 2012).

Dicranella cerviculata has been found in acidic habitats before, especially in mining areas (Robinson and Reed 1987). Its sister species, *D. heteromalla*, is also commonly reported from a variety of acidic habitats (Whitton and Diaz 1981; Wehr and Whitton 1983). The morphological difference between the two species relates to the sporophyte capsule, which has a prominent swelling where it joins the seta in *D. cerviculata*. Therefore, examining only the gametophyte stage of the moss could easily lead to confusion between the two species.

Our multigene molecular phylogenetic approach revealed that the third sampled moss growing on the shore of Hromnice Lake belonged to the common species *Dicranum scoparium*, which occurs in a wide range of habitats, including acidic ones (data not shown and not discussed here). This species belongs to the family Dicranaceae *sensu stricto* (Fig. 2).

There are no experimental data on the survival of protonemata under acidic conditions that could serve as an indicator of the acid tolerance of the moss. On the other hand, several studies have focused on the effects of heavy metals on protonemal growth and bud formation. For example, Francis and Petersen (1989) showed that increasing copper (Cu), cadmium (Cd), and zinc (Zn) concentrations and the levels of certain combinations of these metals reduces protonemal filament growth (in length). Rachna and Vashistha (2017) demonstrated that significant levels of different Cd and lead (Pb) salts reduced protonemal branching and caused rounding of the cells. In addition, bud formation was either partially or fully suppressed by elevated metal concentrations. Since concentrations of heavy metals in acidic habitats, especially in mining areas, are highly elevated, their toxicity and synergistic effects, rather than the acidic conditions, probably prevent the further development of protonemata. Since the protonema of *D. cerviculata* can survive in water with high metal concentrations, forming visible biomass, it may be worth studying its metal tolerance and absorption capabilities. This would also be useful considering its potential usage for phytoremediation purposes. For example, protonemata of the moss *Funaria hygrometrica*, a colonist species of bare, disturbed, nutrient-rich soils as well as mine sites contaminated with various heavy metals, were shown to

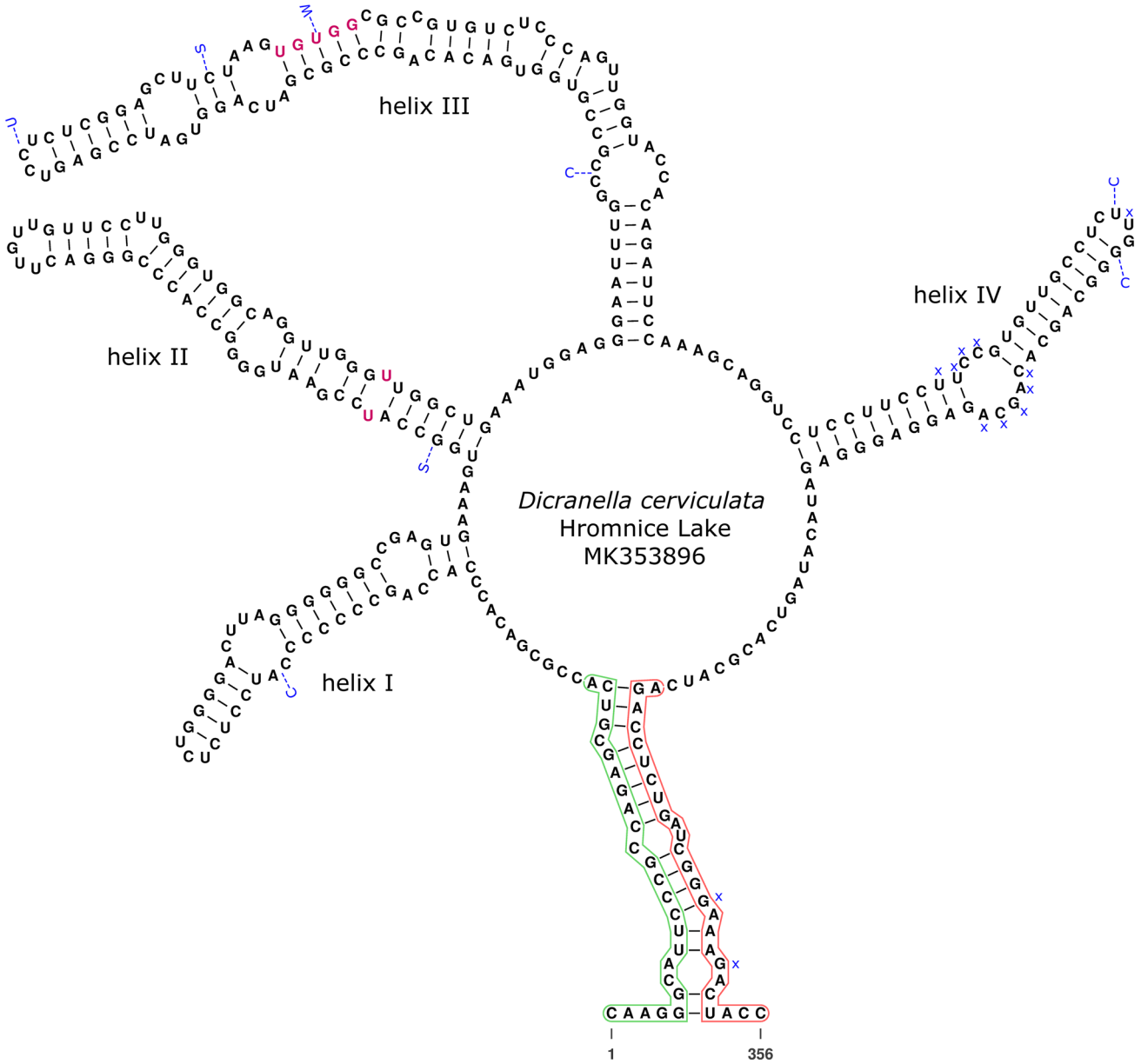

Fig. 4 ITS2 secondary structure model of the Hromnice Lake protonemata. *Nucleotides outside the structure* show the differences between the protonemata and its closest revealed relative *D. cerviculata* Stech B970824.1. Characters follow IUPAC nucleotide codes, except that a lowercase x means that the nucleotide is not present in the latter sequence. The four helices, each with a stem-loop, are labeled *I–IV*. The model includes the 3′ end of the 5.8S rRNA gene and the 5′ end of the 26S rRNA gene. Typical ITS-2 motifs, a pyrimidine–pyrimidine mismatch in helix II and a UGUGU motif on the 5′ side at the apex of helix III, are marked

be useful candidates for the mitigation of Pb toxicity in wastewater (Itouga et al. 2017).

The present study is only the second study to use molecular tools to identify protonemata occurring in extremely acidic freshwaters (the first such study was Higuchi et al. 2003). Such studies can help us to better understand moss biodiversity and colonization of acidic habitats. For example, we speculate that protonemata of only acidotolerant moss species, such as *D. cerviculata* or its relatives, can invade acidic environments. Moreover, the substantial development of protonemata in the lake water may help to colonize the shoreline.

To conclude, the extreme environment of Hromnice Lake prevents the further development of protonemata, which are in turn capable of surviving acidic conditions and increased heavy metal concentrations during the prolonged protonemal stage.

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Limnology

A molecular approach for identification of protonemata helps to assess biodiversity of extremely acidic freshwaters

Dovilė Barcytė[1,2], Jan Fott[1], Linda Nedbalová[1]

[1]Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2, 128 44, Czechia

[2]Department of Zoology, National Museum, Cirkusová 1740, Prague 9, 193 00, Czechia

Correspondence: Dovilė Barcytė, dovile.barcyte@gmail.com

Fig. S1. Hromnice Lake formed as a consequence of mining of pyritic shales. Photo P.J. Juračka.

Fig. S2. Macroscopic clumps of protonemata occurring in the water close to the shore. Adult *Dicranella* grew on the wet substrate beneath. Photo P.J. Juračka.

Fig. S3. Cultivation of protonemata in a Petri dish.