

Identity, ecology and ecophysiology of planktic green algae dominating in ice-covered lakes on James Ross Island (northeastern Antarctic Peninsula)

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Abstract The aim of this study was to assess the phylogenetic relationships, ecology and ecophysiological characteristics of the dominant planktic algae in ice-covered lakes on James Ross Island (northeastern Antarctic Peninsula). Phylogenetic analyses of 18S rDNA together with analysis of ITS2 rDNA secondary structure and cell morphology revealed that the two strains belong to one species of the genus *Monoraphidium* (Chlorophyta, Sphaeropleales, Selenastraceae) that should be described as new in future. Immotile green algae are thus apparently capable to become the dominant primary producer in the extreme environment of Antarctic lakes with extensive ice-cover. The strains grew in a wide temperature range, but the growth was inhibited at temperatures above 20 °C, indicating their adaptation to low temperature. Preferences for low irradiances reflected the light conditions in their original habitat. Together with relatively high growth rates (0.4–0.5 day⁻¹) and unprecedentedly high content of polyunsaturated fatty acids (PUFA, more than 70% of total fatty

acids), it makes these isolates interesting candidates for biotechnological applications.

Keywords *Monoraphidium* · Ice-covered lakes · Antarctica · Phylogeny · Ecology · Temperature · Light · Fatty acids

Introduction

Antarctic lakes are characterised by extreme physical and chemical conditions that offer a natural laboratory in which to study evolution and survival strategies. They are characterised by simplified food webs dominated by microbial life and photoautotrophic microbes are the dominant primary producers. In addition to a general ecological interest in the diversity and adaptation mechanisms to these harsh habitats, cold adapted microorganisms could have a considerable potential as sources of valuable compounds as PUFA or novel biochemicals such as low-temperature enzymes and anti-freeze proteins (Laybourn-Parry and Pearce 2007). However, detailed studies of Antarctic photoautotrophic protists combining molecular, morphological and ecophysiological data are still quite rare (Morgan-Kiss et al. 2006, 2008).

Maritime Antarctic lakes tend to host higher phytoplankton diversity in comparison with continental ones. The dominance of flagellates from various taxonomic groups (mostly cryptophytes, chlorophytes or chrysophytes) is often a characteristic feature of Antarctic lake plankton. Non-motile species are usually less abundant and their dominance is only rarely reported (Butler et al. 2000; Izaguirre et al. 2003; Laybourn-Parry and Wadham 2014).

James Ross Island belongs to a transitory zone between the maritime and continental Antarctic regions (Øvstedal

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and Lewis-Smith 2001). More than 80% of the island surface is covered with ice. Only the northernmost part of the island, the Ulu Peninsula, is significantly deglaciated and represents one of the largest ice-free areas in the northern part of the Antarctic Peninsula. The origin of the lakes on James Ross Island is related to the last glaciations of the Antarctic Peninsula ice sheet and retreat of the James Ross Island ice cap during the late Pleistocene and Holocene (Nedbalová et al. 2013). Interactions between volcanic landforms and glacial geomorphology during previous glacial-interglacial cycles, the Holocene paraglacial and periglacial processes and relative sea level change have resulted in the complex present-day landscape of James Ross Island (Davies et al. 2013). All of these processes have influenced the development of the lakes which are found on the Ulu Peninsula at altitudes from <20 m above sea level (a.s.l.) near the coast to 400 m a.s.l. in the mountain areas (Fig. 1, Nedbalová et al. 2013).

During two Czech research expeditions (2008 and 2009) to James Ross Island, lake ecosystems of the Ulu Peninsula were studied in respect to their origin, morphometry, physical, chemical and biological characteristics (Nedbalová et al. 2013), together with detailed cyanobacterial and microalgal diversity descriptions (e.g., Komárek et al. 2012; Kopalová et al. 2013; Škaloud et al. 2013). As a part of this study, we observed in the open water of deep ice-covered lakes at higher altitudes small immotile green algae as the dominant component of phytoplankton. The algae were morphologically similar to the genus *Monoraphidium* from the Selenastraceae. The ice-covered lakes on James Ross Island are young and unstable and further glacier thawing may induce their decay. As they originated from the last retreat of local glaciers, their maximum expected age could be approximately a century or rather some decades. Their key feature is the development of extensive ice cover, which persists at least partly during summer (Nedbalová et al. 2013).

The aim of the present study was to describe the phylogenetic relationships, ecology and ecophysiology of the two strains of *Monoraphidium* that were newly isolated from the plankton of ice-covered lakes on James Ross Island. To our knowledge, this is the first record of a immotile green alga to dominate in such type of extreme environment.

Materials and methods

Sampling sites

James Ross Island (64°10'S, 57°45'W) is a ~2600 km² large island situated in the northeastern Antarctic Peninsula region (Fig. 1). The island is mostly covered with glaciers, only the Ulu Peninsula is deglaciated. The climate

is characterised by short summers (December–February) when the mean monthly air temperature exceeds 0 °C. The annual mean temperature at the Mendel Station close to sea level was −7.2 °C and mean summer temperatures were above 0 °C for up to 4 months (2004–2009; Láška et al. 2010). The terrestrial vegetation on the island is limited to non-vascular plants and composed of a predominantly bryophyte and lichen tundra. Human presence is limited to the northern side of the island, where the Czech Johann Gregor Mendel Antarctic Research Station has been located since 2006.

A large number of lakes can be found on the Ulu Peninsula, formed by glacial erosion and deposition, followed by glacier retreat during the Holocene. Based on the origin, bedrock geology, geomorphology and hydrological stability of a representative set of 29 lakes, six different lake types were defined. They range from stable shallow lakes that originated several thousand years ago to deep cirque and kettle lakes with the maximum expected age of approximately a century or rather some decades (Nedbalová et al. 2013). Lake water samples were collected in 2008–2009 from the surface layer, the majority of the lakes were sampled from the shore (for details, see Nedbalová et al. 2013). The samples were fixed with Lugol's solution and observed under an inverted microscope (Nikon, Japan) after the transport to the Czech Republic. The remaining sample was used for chlorophyll-*a* and chemical analyses (for methods, see Nedbalová et al. 2013; Elster et al. 2016).

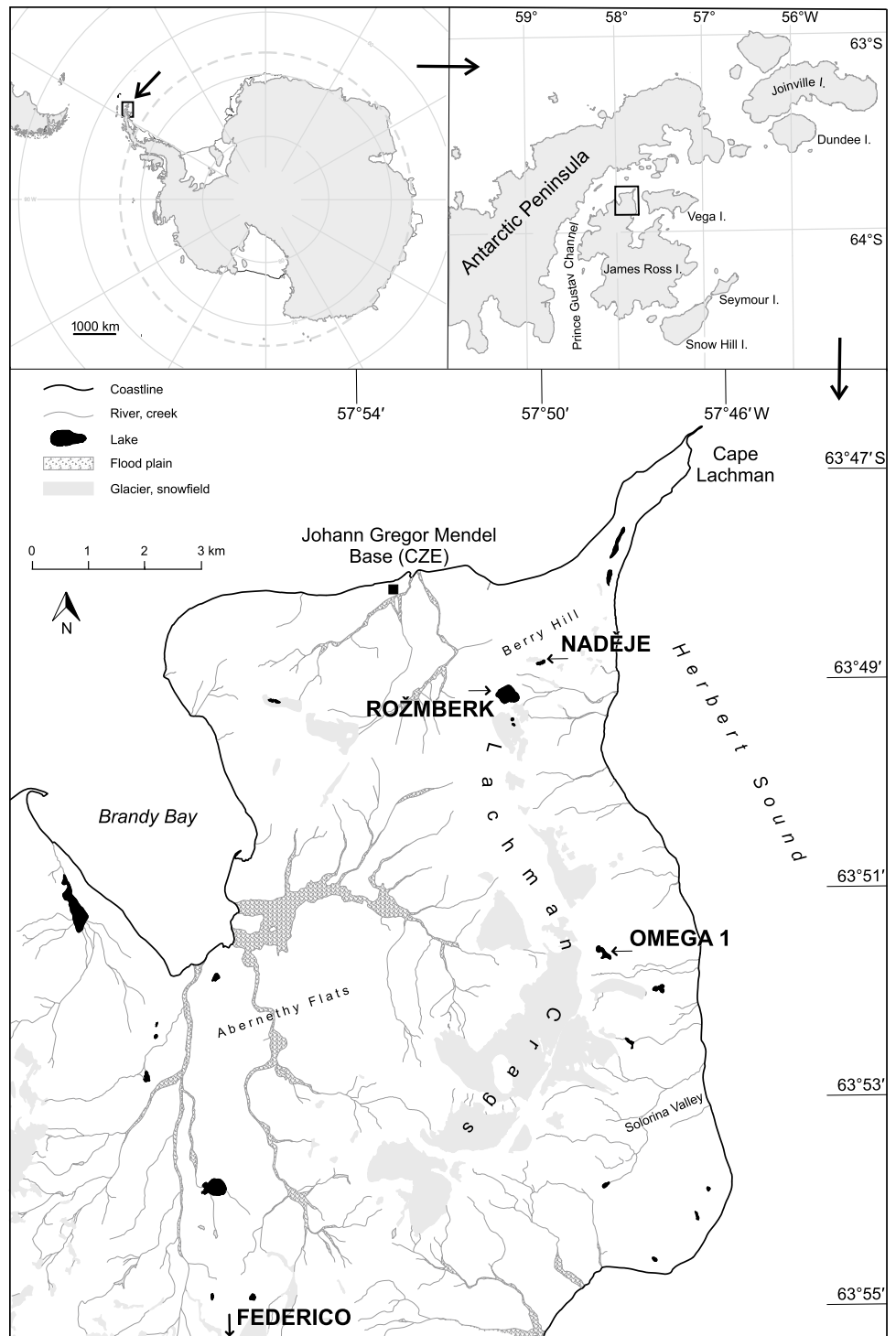
Isolation of algal strains

Phytoplankton samples from surface layer of the lakes Naděje and Omega 1 (Figs. 1, 2) were taken in February 2009. The same day, the samples dominated by *Monoraphidium* were concentrated using syringe and GF/C filters (Whatman, UK) and inoculated on solidified BG11 medium (Allen 1968) in a 96-well microplate. After transport to the Czech Republic, the isolation of strains (strain A from Naděje lake and strain B from Omega 1 lake) was done by serial dilution at the Institute of Botany AS CR in Třeboň. The cultures were grown at 3 °C under continuous fluorescent lighting with photosynthetically active radiation (PAR) intensity of ~20 μmol m⁻² s⁻¹ in liquid circumneutral Bold's Basal Medium (BBM) (Bischoff and Bold 1963). Fresh samples from liquid monocultures were further used for genetic analysis, microscopic observations, inoculation of ecophysiological experiments and lipid analysis.

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from algal cultures following the standard protocol in DNeasy Plant Mini

Fig. 1 Map of the Ulu Peninsula showing the location of lakes sampled in 2008–2009. The names are given for the lakes, where *Monoraphidium* was the dominant in phytoplankton. The inset map shows the tip of the Antarctic Peninsula with the position of James Ross Island and the Ulu Peninsula



Kit (Qiagen, Germany), with minor modifications: At the beginning of the procedure the cells were mechanically disrupted by shaking for 6 min (30 Hz) in the presence of glass beads (3 mm diameter, Sigma-Aldrich, Czech Republic) in the Mixer Mill MM 400 (Retsch, Germany). Afterwards, quality and concentration of DNA was checked on

the NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

The 18S small subunit ribosomal RNA gene (SSU 18S rDNA) and the internal transcribed spacer region 2 (ITS2 rDNA) were amplified from DNA isolates by PCR using existing primers (Table 1).

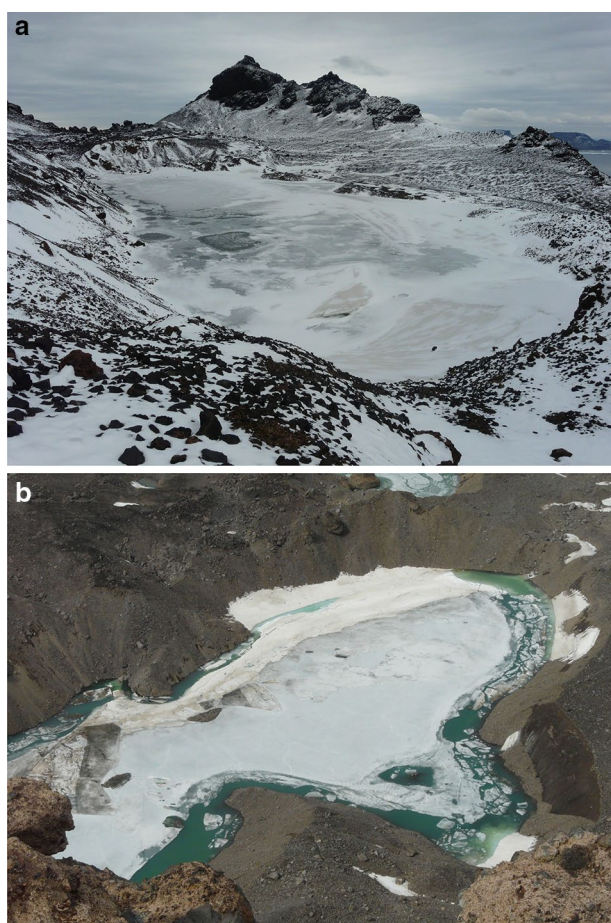


Fig. 2 The original localities of the *Monoraphidium* strains: lakes Naděje (a) and Omega 1 (b), Ulu Peninsula, James Ross Island

Table 1 List of primers used for amplification of 18S rDNA and ITS2 regions

Primer	Sequence 5′–3′	Source
NS1	GTAGTCATATGCTTGCT	White et al. (1990)
18L	CACCTACGGAAACCTTGTTAC-GACTT	Hamby et al. (1988)
402-23F	GCTACCACATCCAAGGAA-GGCA	Katana et al. (2001)
1308-39R	CTCGTTCGTTAACGGAAT-TAACC	Katana et al. (2001)
ITS1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
ITS4	TCCTCCGCTTATGATATGC	White et al. (1990)

Amplification reactions for 18S rDNA were performed using the following cycle parameters: 5 min hot start at 95 °C, followed by 33 cycles (1 min at 94 °C, 45 s at annealing temperature of 59 °C, 3 min at 72 °C) and 7 min at 72 °C.

Each 50 µl PCR reaction for 18S rDNA amplification contained 5 µl of DNA isolates (diluted to concentration of

5 ng/µl), 1 µl of each 10 µM primer, 4 µl of 25 mM MgCl₂, 0.1 µl of 2 mM dNTPs, 5 µl of 10× Taq buffer + KCl-MgCl₂, 31.1 µl sterile Milli-Q water, and 0.4 µl of 5U/µl Taq DNA polymerase (Fermentas, USA).

Amplification reactions for ITS2 rDNA region were performed using the following cycle parameters: 5 min hot start at 97 °C, followed by 37 cycles (1:25 min at 95 °C, 2 min at annealing temperature of 56 °C, 4 min at 72 °C) and 7 min at 72 °C. Each 35 µl PCR reaction contained 1 µl of DNA isolates (diluted to concentration of 5 ng/µl), 1.4 µl of each 10 µM primer, 2.8 µl of 25 mM MgCl₂, 2.6 µl of 2 mM dNTPs, 3.5 µl of 10× Taq buffer + KCl-MgCl₂, 22.2 µl sterile Milli-Q water, and 0.1 µl of 5U/µl Taq DNA polymerase (Fermentas, USA).

The PCR products were stained with bromophenol loading dye, quantified on 1.5% agarose gel, stained with GelRed. The amplification products were purified and sequenced using an Applied Biosystems (Foster City, USA) automated sequencer (ABI 3730xl) at Macrogen (Seoul, Korea).

New sequences are available in the NCBI Nucleotide Sequence Database under accession numbers KX671910–KX671913.

Phylogenetic analyses

Newly obtained 18S rDNA and ITS2 *Monoraphidium* sequences were edited and assembled in the program FinchTV 1.4.0 (Geospiza, USA) and MEGA 6, respectively (Tamura et al. 2013). To cover 18S rDNA diversity of all *Monoraphidium* diversity several sequences were retrieved from available public sequence databases using BLAST (Altschul et al. 1990) at NCBI (<http://www.ncbi.nlm.nih.gov/>). The final 18S rDNA alignment was done using ClustalW (Thompson et al. 1994) in the program MEGA 6 and adjusted manually with exclusion of introns and any ambiguous regions. The matrix contained 62 sequences (1527 bp); *Bracteacoccus aeri*, *Bracteacoccus minor* and *Desmodesmus communis* were selected as outgroup.

The best-fit nucleotide substitution model was estimated with jModeltest 2.0.1 (Posada 2008). Based on the Akaike information criterion (AIC), the GTR + Γ + I model was selected for the 18S rDNA (GTR—general time reversible model, Γ —rate heterogeneity among sites, I—proportion of invariable sites).

The phylogenetic tree of 18S rDNA was inferred by Bayesian inference (BI) using MrBayes version 3.2.6 (Ronquist et al. 2012). Two parallel Markov chain Monte Carlo (MCMC) runs for 2,000,000 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 25% were discarded as burn-in. Bayesian posterior probabilities were

used to assess clade support. The computed phylogenetic tree was visualized using FigTree 1.4.2 (Rambaut 2014) and modified in Inkscape 0.91 (Free Software Foundation Inc., USA). Bootstrap analysis was performed by maximum likelihood (ML) using GARLI 2.0 (Zwickl 2006). ML analysis consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopotermin command set to 100,000). Based on Peksa and Škaloud (2011), Bayesian posterior probabilities and bootstrap values were treated as those with weak (less than 50% for BI and ML), moderate (50–94% for BI; 50–79% for ML) and high (more than 94% for BI; more than 79% for ML) support.

Secondary structure prediction of nuclear rDNA ITS2

Nuclear rDNA ITS2 regions of the *Monoraphidium* strains were identified using published sequence of *Ankistrodesmus gracilis* (GenBank: AB917098) (Hoshina 2014). These sequences were then folded with 5.8S-LSU stem regions using the Mfold server at <http://mfold.rna.albany.edu/?q5mfold> (Zuker 2003) to predict several secondary structure models, from which we selected the model that was consistent with the specific features of nuclear rDNA ITS2, U-U mismatch in helix II and the UGGU motif near the 59-end site apex of helix III (Coleman 2003). The secondary structures of nuclear rDNA ITS2 were drawn using VARNA version 3.7 (Darty et al. 2009) and modified by Inkscape 0.91 (Free Software Foundation Inc., USA).

Light and electron microscopy

Microscopic observations of the strains were done with Olympus BX43 microscope (Olympus Corporation, Japan) equipped with camera. Microphotographs were processed using the QuickPHOTO Camera 3.0 software (Promicra, Czech Republic). The same software was used to measure the size (length and width) of 50 cells of each strain.

For scanning and transmission electron microscopy (SEM and TEM) the cells in actively growing cultures were fixed for 24 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and post-fixed in 2% OsO₄ in the same buffer. Fixed cells were dehydrated through an ascending ethanol and acetone series. Following steps for SEM included transfer of the fixate into chamber where it was carefully dried by method of critical point in Bal-Tec CPD 030 device. Gold-coated specimens were examined with a JEOL 6380 LV scanning electron microscope. Next steps for TEM included embedding of the fixate in Araldite and Poly/Bed® 812 mixture. Ultrathin sections were cut with a diamond knife on a Ultracut E ultramicrotome (Reichert-Jung, Austria) and stained using uranyl acetate and lead citrate. The TEM grids were examined with a JEOL 1011

TEM (JEOL Ltd., Japan). Photomicrographs were obtained using a Veleta CCD camera equipped with image analysis software Olympus Soft Imaging Solution GmbH (Münster, Germany) and later modified by iTEM 5.1 (Olympus Soft Imaging Solution, Germany).

Temperature and light growth requirements

The strains were cultivated in sterile immunological plates (FB type, 9 × 12 cm, 96 wells, polystyrene, Falcon) in a volume of 0.2 ml per well (the initial optical density measured at 750 nm (OD₇₅₀) was set to 0.05). Cultures were inoculated by stock culture in stage of linear growth. After the inoculation, plates were sealed with food wrap polyethylene foil to reduce evaporation and a lid, and incubated in the cultivation unit. The experiments were performed in a cultivation unit for crossed gradients of temperature and light (Labio, Czech Republic; see Kvíderová and Lukavský (2001) for detailed unit description). The irradiance (photosynthetically active radiation in range 400–700 nm) gradient was set from 9 to 69 μmol m⁻² s⁻¹ continuous light using LED lights (IP40, Sikov, Czech Republic), and the temperature gradient was from 1 to 20 °C and from 7 to 30 °C (two runs of the experiment were performed). Light was measured using a LI-250A Light Meter with a quantum sensor LI-190SA (Li-COR, USA) and temperature was measured using a digital thermometer with contact probe (Gryf 310, Czech Republic), CO₂ was supplied to a final concentration of 2% (v/v). The gradients were fitted by linear (temperature) and parabolic (irradiance) functions in Statistica 12 (StatSoft, USA). The plates were placed on a cloth (gauze) filled with water to improve heat transfer.

Growth was evaluated as optical density at 750 nm (OD₇₅₀) over a period of 38 days directly in wells of immunological plates using a Varioscan™ Flash Multimode Reader (Thermo Fisher Scientific, Finland). The OD₇₅₀ values were converted into cell numbers using conversion curves created according to Kvíderová (2010). Growth curves were constructed individually for each well and the relative growth rate (μ) during exponential phase was calculated as the slope of linear regression of dependency of ln cell number on time (Kvíderová and Henley 2005). Finally, a total of 35 different combinations of light and temperature were evaluated. The growth rate data for contour plots (created in Statistica 12, StatSoft, USA) were smoothed by a distance weighted least square method.

Analysis of fatty acids

The biomass for the analysis of fatty acids was obtained by cultivation at 3 °C in a Q-Cell cultivator (PolLab, Poland), where the light intensity was ~20 μmol m⁻² s⁻¹. 100 mg of lyophilized biomass harvested at stationary phase of

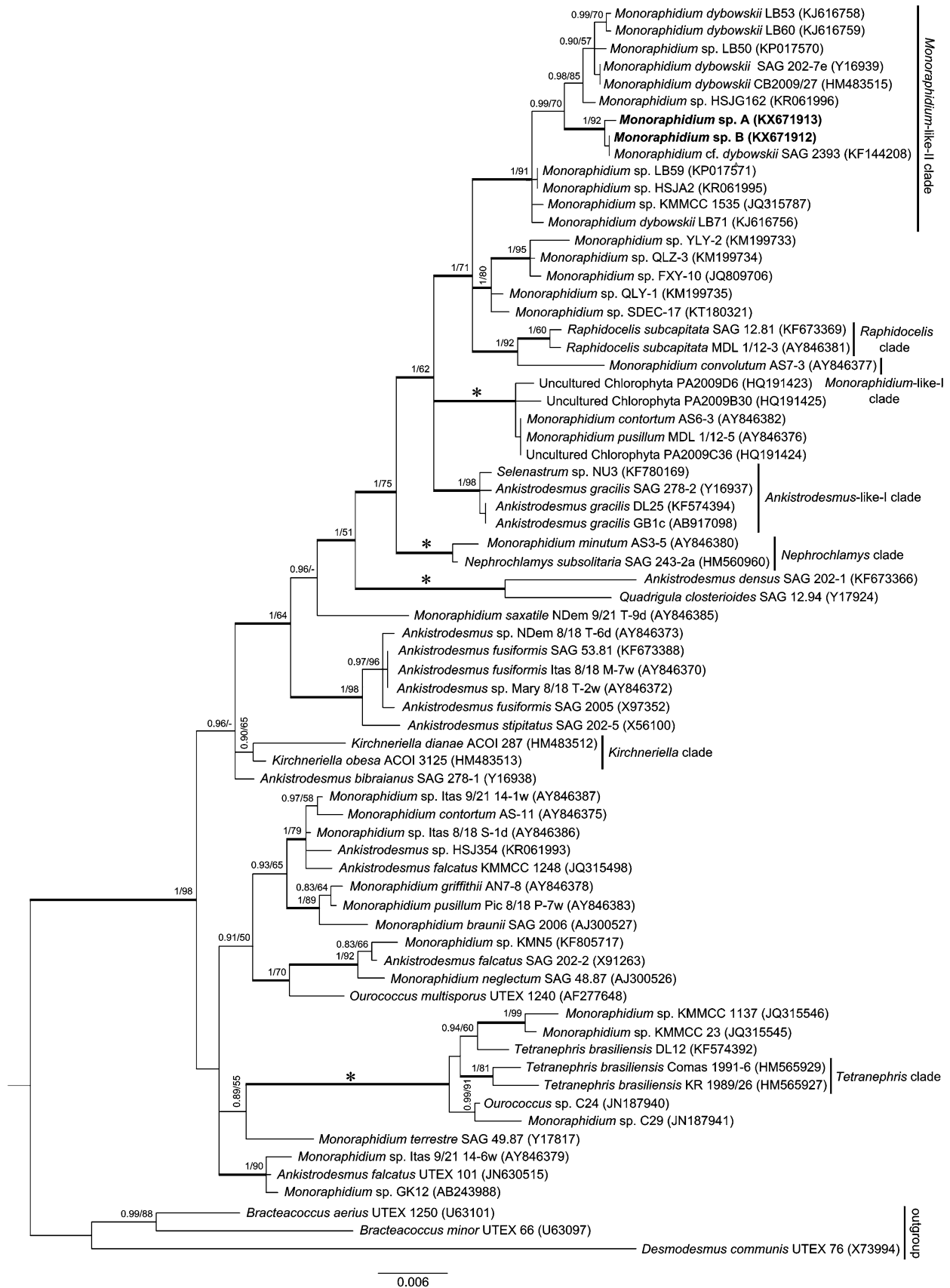


Fig. 3 Bayesian phylogenetic tree of the Selenastraceae based on analysis of 18S rDNA. Numbers are posterior probability and bootstrap values (BI/ML >50). Full statistical support (1.00/100) is marked with an asterisk. Thick branches represent nodes receiving the highest posterior probability support (1.00). Newly obtained sequences of Antarctic *Monoraphidium* strains (A and B) are in bold. Accession numbers are indicated after each species name

growth was saponified with 10% KOH solution in methanol at room temperature overnight. Neutral or basic compounds in a solution of pH 9 were extracted with diethyl ether, the aqueous solution of fatty acids was acidified to pH 2 and the acids were extracted into hexane. Fatty acids were methylated with a BF₃-methanol mixture and gas chromatography–mass spectrometry of FAMES was done on a Varian GC/MS system with the split/splitless injector (250 °C) and a SLB®-IL111 capillary GC Column L × I.D. 100 m × 0.25 mm, df 0.20 μm. Helium was used as carrier gas at 1.0 ml/min. The split/splitless injection port was maintained at 255 °C. The split ratio was 1:90, and the injection volume was 1 μL. For FAME GC/MS analysis with the SLB®-IL111 column, the temperature program was as follows: 150 °C for 1 min, subsequently increasing at 2 °C/min to 180 °C and at 1 °C/min to 250 °C, which was maintained for 1 min. FAMES were identified according to their mass spectra (Dembitsky et al. 1993) and using a mixture of chemical standards obtained from Sigma-Aldrich. All experiments concerning the analysis of FAMES and their derivatives were carried out by electron impact MS.

Results

Phytoplankton of James Ross Island lakes

Chlorophyll-*a* concentrations in the water of the surveyed 29 lakes were in the range 0.2–10.2 μg l⁻¹. Majority of lakes in this survey were shallow and the autotrophic biomass in open water was mostly formed by detached benthic species; no substantial phytoplankton neither floating mats occurred in these lakes. In four deep lakes (Naděje, Omega 1, Rožmberk, Federico; Fig. 1), phytoplankton was dominated by a small immotile species that was tentatively determined according to its morphology as a green alga *Monoraphidium* sp. Its abundances reached ~10⁵ cells ml⁻¹ resulting in relatively high chlorophyll-*a* concentrations in these four lakes (2.7–6.7 μg l⁻¹). In contrast to massive autotrophic mats in the littoral zone of shallow lakes, these assemblages were only poorly developed in deep lakes. The lake biota was further characterized by the common occurrence of a calanoid copepod (*Boeckella poppei* Mrázek) that apparently used the phytoplankton as main food source. These deep lakes are located at higher altitude (>200 m a.s.l.) and their key feature is the development of

extensive ice cover, which persists at least partly during summer. At the time of the sampling, the ice was up to 2 m thick and had a characteristic candle-like structure (up to 30 cm long) causing significant light extinction. The lake water was characterised by low temperature (<1 °C) and an isothermal vertical profile during the period of measurement. Whereas high conductivity and dissolved organic carbon concentrations were characteristic for shallow coastal lakes, there were high soluble reactive phosphorus and nitrate concentrations in deep ice-covered lakes (for details, see Nedbalová et al. 2013).

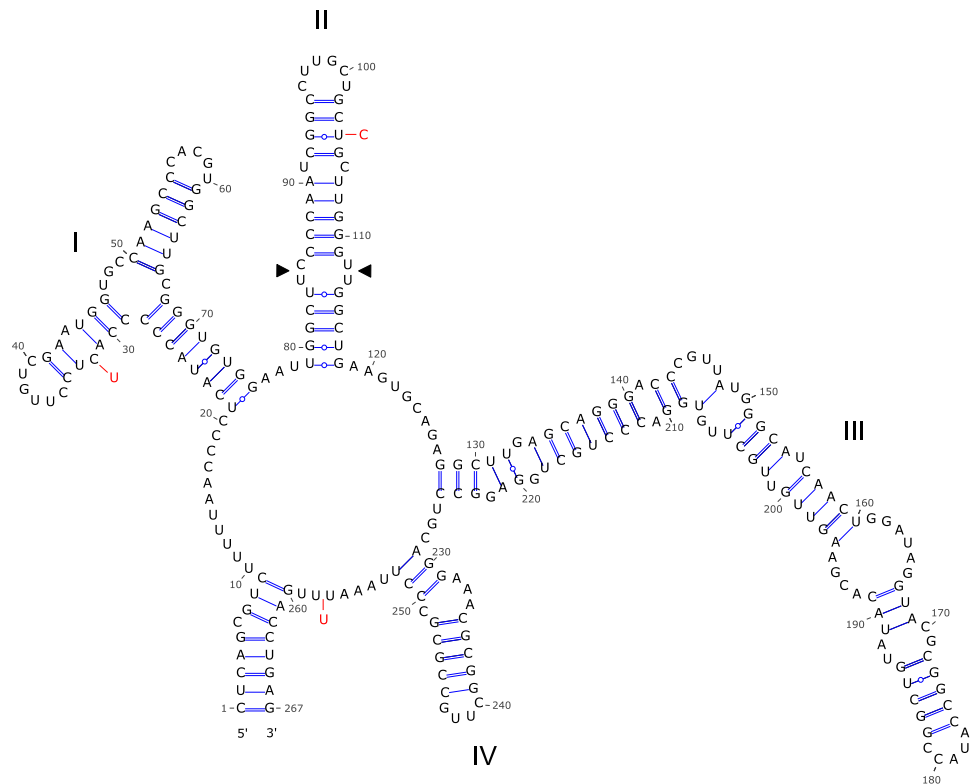
Analysis of molecular data

Molecular phylogeny of the two newly isolated *Monoraphidium* strains was inferred from partial sequences of the nuclear 18S rDNA and confirmed that the strains belong to lineage of green algae within the family Selenastraceae (Chlorophyceae). The analyses (BI and ML) of 1527 aligned nucleotide sites resulted in trees with the same topology (Fig. 3). With a high statistical support, both strains were closely affiliated with organisms that were determined as *M. dybowskii* (Woloszynska) Hindák & Komárkova-Legnerová or *Monoraphidium* sp. The clade assignment was *Monoraphidium*-like-II according to Krienitz et al. (2011). Inside this clade, the closest match to 18S rDNA of the isolates from Antarctic lakes was the strain *M. cf. dybowskii* SAG 2393 being 99.8% identical to the strain B sequence over its entire length (the difference was two indels). *M. cf. dybowskii* SAG 2393 and the strain B revealed sister relationship to the strain A. The strains A and B differed in one nucleotide change and two indels in 18S rDNA. These three isolates formed a highly supported subclade within the *Monoraphidium*-like-II clade.

The 18S rDNA sequences of the strains A and B had different number of introns. The sequence of the strain A contained three introns (inserted between positions 541 and 542, 1143 and 1144, 1242 and 1243), whereas the strain B had two introns (inserted between positions on the positions 1143 and 1144 a 1242 and 1243; related to the sequence of *Chlamydomonas reinhardtii* [GenBank: JN903978]).

Although there were some variations in the secondary structure of ITS2 rDNA between the two new strains A and B, no compensatory base change (CBC-double sided base change of a nucleotide pair in helix, retaining secondary structure) was detected. The comparison showed that there were three differences: one change in terminal part of helix I, at the end of the helix II and in the region between helix IV and LSU stem (Fig. 4). Since there are no freely available ITS2 rDNA sequences of the other members of the newly defined *Monoraphidium*-like-II clade, detailed comparison among them was not possible. Culture material of the closest strain (*M. cf. dybowskii* SAG 2393) was not

Fig. 4 Comparison of secondary structure of ITS2 rDNA transcript between the strain A (accession number: KX671911) and B (accession number KX671910). Note the U-U mismatch in helix II (*arrow-heads*). Differences characteristic for the strain B are shown by nucleotides outside the secondary structure



available for ITS2 sequencing at the time of preparation of this manuscript.

Morphology and ultrastructure

Morphology of the newly isolated strains was observed by light and electron microscopy using natural samples and liquid cultures in exponential growth phase. Cells were spindle shaped, always significantly tapering toward the apices, arcuate with fine rounded ends. The ends of the cells were in the same plane. Cells of the strain A were slightly more arcuate in comparison with the strain B (Fig. 5). Cell dimensions (length and width) of both strains were nearly the same (Table 2). The cells were solitary and their appearance was very similar in natural samples and under laboratory conditions. Reproduction proceeds by the formation of autospores, what is typical for the genus *Monoraphidium* (Fig. 5c, d).

One parietal chloroplast with starch grains, nucleus, mitochondria, Golgi complex and vacuoles were observed in the cells. A spherical naked pyrenoid was a prominent structure of the chloroplast. Thylakoids were not traversing the pyrenoid (Fig. 5e, f). There were no differences in cell ultrastructure between the strains A and B.

Temperature and light growth requirements

The dependence of growth rate on temperature and light intensity was estimated using cultivation unit for crossed

gradients. The response of the two strains to the experimental conditions was similar and the effects of conditions on growth rate were significant. Maximal relative growth rates reached the values of 0.5 day^{-1} (strain A) and 0.4 day^{-1} (strain B). Both strains were able to grow in a wide temperature gradient ($1\text{--}20 \text{ }^\circ\text{C}$) and the highest growth rates were detected in the range $6\text{--}20 \text{ }^\circ\text{C}$. Lower growth rates were observed at $1 \text{ }^\circ\text{C}$ ($0.1\text{--}0.3 \text{ day}^{-1}$). At temperatures higher than $20 \text{ }^\circ\text{C}$, the growth rates steeply decreased and virtually no growth was observed above $25 \text{ }^\circ\text{C}$ (Fig. 6).

The strains grew at all light intensities that were tested in this experiment. With increasing irradiance, growth rate of both strains decreased. The highest growth rate was detected at lowest irradiances ($9\text{--}16 \mu\text{mol m}^{-2} \text{ s}^{-1}$). A limiting low light intensity level was thus not reached. However, the differences in growth rate across the irradiance gradient were rather low, indicating a relatively broad tolerance of the studied strains (Fig. 6).

Fatty acid composition

The relative content of fatty acids (as % of total fatty acids) in the biomass of the strains A and B cultivated at $3 \text{ }^\circ\text{C}$ is given in Table 3. The table gives only those fatty acids that have abundance greater than 0.1% of the total. Only fatty acids with 16 or 18 atoms in the carbon chain were detected. The analysis showed very high levels of PUFA (around 70% of total fatty acids in both strains), whereas

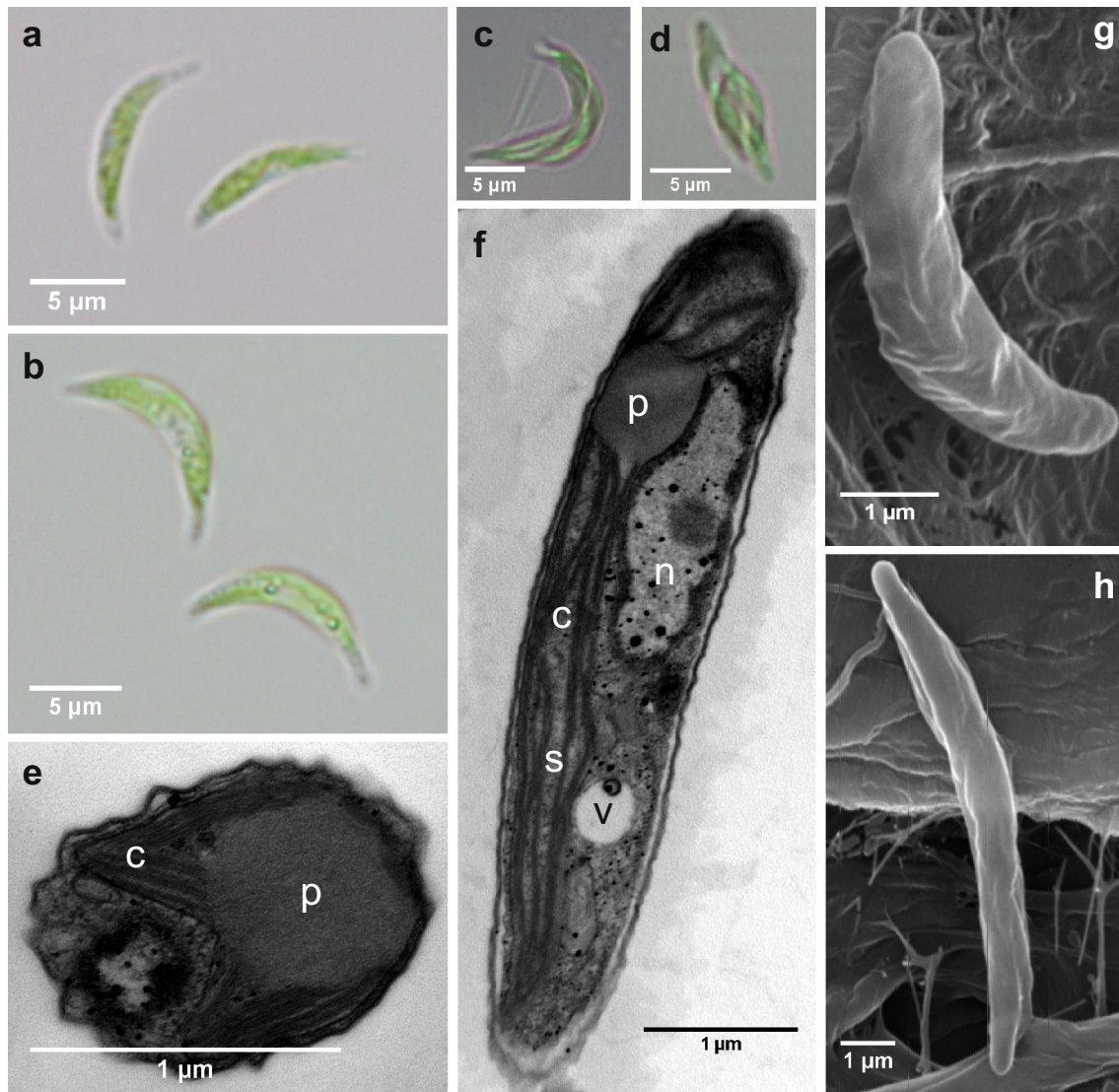


Fig. 5 Light, scanning and transmission electron micrographs of the strains A (**a**, **c**, **e**, **g**) and B (**b**, **d**, **f**, **h**). The formation of autospores is shown (**c**, **d**). On the transmission electron micrographs, the follow-

ing structures are marked: pyrenoid (*p*), nucleus (*n*), chloroplast (*c*), starch grains (*s*), vacuole (*v*)

the content of saturated fatty acids did not surpass 20% (being formed mainly by palmitic acid, 16:0). The major PUFA were stearidonic acid (6,9,12,15–18:4), together with hexadecatetraenoic acid (4,7,10,13–16:4). There were no significant differences in the fatty acid composition between the two strains.

Discussion

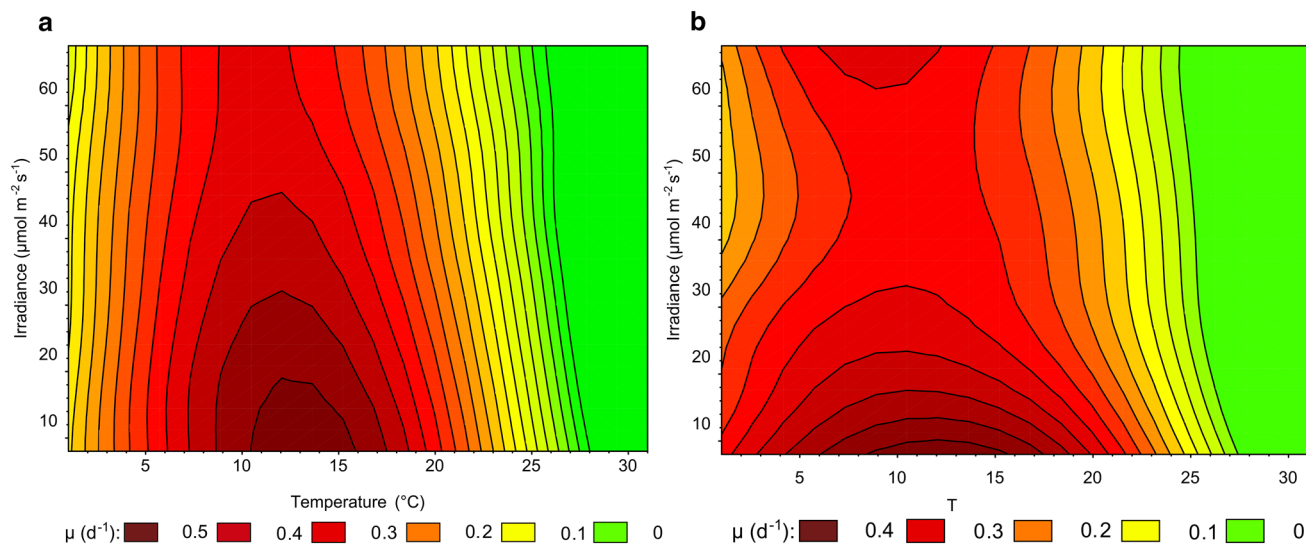
Phylogeny and morphology

Phylogenetic evidence on the basis of 18S rDNA sequences confirmed the tentative determination of the strains as

Monoraphidium sp. that was based on microscopic observation of field samples. This genus belongs to the family Selenastraceae (Chlorophyceae) and together with the genus *Ankistrodesmus* it is currently considered as polyphyletic (Krienitz et al. 2011). The newly isolated strains A and B were found to be closely related to the strains assigned as *M. dybowskii*. We consider them as members of the clade *Monoraphidium*-like-II that was established by Krienitz et al. (2011) and originally contained only two strains (*M. dybowskii* SAG 202-7e a *M. dybowskii* CB 2009/27). Since the type species of the genus, *M. griffithii* (Berkley) Komárková-Legnerová, belongs to another clade within the Selenastraceae, the members of the *Monoraphidium*-like-II clade should be probably described as

Table 2 Cell dimensions of the strains A and B

Strain	Length (μm)				Width (μm)			
	Average	SD	Min	Max	Average	SD	Min	Max
A	7.7	1.4	5.3	10.4	1.5	0.2	1.1	2.1
B	8.2	1.1	5.6	10.4	1.4	0.2	0.8	1.8

**Fig. 6** Growth rates of the strain A (a) and B (b) in the crossed gradients of temperature and irradiance

new genus in future. The closest relative of the two Antarctic strains, *M. cf. dybowskii* SAG 2393, was isolated from a calcifying biofilm of a karst creek in Germany (Hodač et al. 2015). This is a further indication that many coccoid green algae could have a wide geographic distribution as it was recently demonstrated for the genera *Chlorella* and *Stichococcus* (Hodač et al. 2016). The two Antarctic strains and *M. cf. dybowskii* SAG 2393 form a highly supported clade within the *Monoraphidium*-like-II group. However, a detailed comparison of the German and Antarctic strains would be necessary for the possible establishment of a new genus and species. Unfortunately, the strain SAG 2393 is currently not available for further study that would allow such comparison.

Secondary structure of ITS2 rDNA is a variable marker used for the definition of species according to the CBC concept which states: Two organisms/strains whose ITS2 sequences differ by even a single CBC in conserved regions of the ITS2 represent two different biological species (Coleman 2000). CBC species concept was recently subjected to large-scale testing, indicating that if there is no CBC then the strains represent the same species with

Table 3 Fatty acid composition (% of total fatty acids) in the biomass of the strains A and B

Fatty acid	% Total fatty acids	
	Strain A	Strain B
16:0	15.1	19.2
7–16:1	0.3	0.3
9–16:1	0.6	0.5
7,10,13–16:3	3.3	3.9
4,7,10,13–16:4	18.5	23.1
18:0	1.1	0.5
9–18:1	10.7	9.8
9,12–18:2	0.9	1.4
6,9,12–18:3	1.3	0.9
9,12,15–18:3	13.7	10.4
6,9,12,15–18:4	34.5	30.0
Σ PUFA _s	72.2	69.7

a probability of ~ 0.76 (Wolf et al. 2013). Hemi-CBC describes the situation when one base from base-pair in a helix changed but pairing in the secondary structure remain

preserved. We observed the change from G-U to G-C in the terminal part of helix II when the strain A was compared to B. Such evolutionary change from G-U to G-C in secondary ITS2 structure is classified as one of common hCBC (Caisová et al. 2011), nevertheless it depends on exact position of such change in the ITS2 secondary structure. A consensus secondary structure model of ITS2 molecule showing conserved and variable positions was recently created for Chlorophyceae (Caisová et al. 2013). The observed change is located in variable nonhomologous part of the structure and should not be assigned as hCBC. The other two detected nucleotide changes in secondary structure were also found in variable regions. Therefore, we infer that based on the secondary structure of ITS2 rDNA, the strains A and B represent one biological species.

Morphological analysis of the Antarctic isolates based on light microscopy indicated that the two strains can hardly be assigned with certainty as any species from the Selenastraceae described so far. Their morphology does not correspond to the original description of *M. dybowskii* that was shown to be their closest relative based on 18S rDNA phylogeny. The cells of *M. dybowskii* differ by being larger, nearly straight or only slightly arcuate and with only slight tapering towards the apices (Komárková-Legnerová 1969). When looking at the traditional characteristics used to identify species within the Selenastraceae, the new isolates would rather fit to the description of *M. convolutum* (Corda) Komárková-Legnerová or *M. subclavatum* Nygaard (Komárek and Fott 1983). However, there are significant discrepancies that does not allow us to assign our strains as any of the mentioned species. In their polyphasic study of isolates from the Selenastraceae, Fawley et al. (2006) clearly showed that the diversity of this group is probably significantly underestimated due to the application of a broad species concept. The morphological characters used to identify species were inconsistent with the molecular results in some cases. Using such polyphasic approach, the new strains should be probably described as new species in future.

Even though the genus *Monoraphidium* was traditionally characterised by the absence of pyrenoid (Komárková-Legnerová 1969), it is often observed in cells when using TEM. This was also the case of the Antarctic strains having a similar type of pyrenoid that was observed by Krienitz et al. (2001) in cells of *M. dybowskii*. However, the presence or absence of a pyrenoid cannot be regarded as useful for delimiting species within the Selenastraceae (Fawley et al. 2006; Krienitz and Bock 2012).

Ecology and ecophysiology

In Antarctic lakes that are permanently ice-covered or lose their ice only for short periods in summer, wind-driven

turbulence in the water column is absent or significantly limited. In such environment, it is advantageous for phytoplankton to be motile to maintain the position in the euphotic zone. Flagellates from different taxonomic groups thus mostly form the dominant component of phytoplankton in such lakes (Laybourn-Parry and Wadham 2014). Here, we report the dominance of green microalga *Monoraphidium* sp. in deep lakes on James Ross Island that remain at least partly ice-covered in summer. The success of these immotile algae is probably possible because of their relatively high growth rates, small cell size that prevents rapid sinking outside the euphotic layer combined with isothermal vertical profile that enables some mixing of water layers even under the ice cover. Phytoplankton abundances reached rather high values for such extreme environment, enabling the existence of a significant grazer population (*Boeckella poppei*) in the lakes. Chlorophyll-*a* concentrations were the same or higher when compared, e.g., to an oligotrophic lake on Signy Island that lies in a region with significantly milder climatic conditions (Butler et al. 2000). On the other hand, extremely low chlorophyll-*a* values are characteristic for permanently ice-covered lakes in continental Antarctica (Laybourn-Parry and Bayliss 1996).

Members of the Selenastraceae were already reported from maritime Antarctic lakes that can better support non-motile species due to the absence of ice cover in summer months. *M. minutum* and *M. griffithii* were observed in the plankton of the hypereutrophic Pingüi Pond at Hope Bay (Antarctic Peninsula) by Izaguirre et al. (2003). *Ankistrodesmus falcatus* and other *Ankistrodesmus* spp. were even found to be dominant in some lakes on Signy Island (Butler et al. 2000). As the identification of these species was based on light microscopy, it would be very interesting to assess their relationship to the strains from James Ross Island using molecular markers.

The temperature requirements for growth of the two newly isolated *Monoraphidium* strains does not fit easily into the categories established by Morita (1975). The strains did not grow above 25 °C, however, the highest growth rates were reached in a broad range from 6 to 20 °C, which is not in agreement with the definition of a psychrophile. They also showed a good performance at very low temperatures that were close to their original habitat, which is apparently crucial for their success in the James Ross Island lakes. The ability to grow in a broad temperature range is believed to be connected with high temperature fluctuations in the original habitat (Seaburg et al. 1981). However, Dolhi et al. (2013) demonstrated that microorganisms with temperature requirements similar to our *Monoraphidium* strains occur also in the extreme but stable environment of lakes in McMurdo Dry Valleys. In fact, a surprisingly low number of “true psychrophilic”

algae were isolated so far (Pocock et al. 2004; Morgan-Kiss et al. 2008). This applies also for cyanobacteria, because polar strains were found to be mostly psychrotrophic (Tang et al. 1997) and psychrophiles were only rarely observed (Nadeau and Castenholz 2000).

Beside the ability to grow at low temperatures the two Antarctic strains of *Monoraphidium* sp. were apparently well adapted to low irradiances that are characteristic for their original habitat. Due to the presence of a thick layered ice cover, the amount of light reaching the open water is significantly limited in the four lakes on James Ross Island, where *Monoraphidium* sp. was dominant. Light is probably the main factor limiting primary production in the open water of the lakes, because concentrations of dissolved nutrients were found to be surprisingly high (Nedbalová et al. 2013). For example, in the lake Naděje, from where the strain A was isolated, the intensity of photosynthetically active radiation just under ice in February was $8 \mu\text{mol m}^{-2} \text{s}^{-1}$ during a cloudy day and $97 \mu\text{mol m}^{-2} \text{s}^{-1}$ during a sunny day (Nedbalová, unpublished data). There are no experimental data available for other Antarctic species from Selenastraceae, however, a shade-adapted strain of *Monoraphidium contortum* was isolated from mountain Lake Tahoe (California-Nevada). This alga was light saturated at very low irradiance levels that were comparable with values obtained within this study (Vincent 1982).

Fatty acid composition and biotechnological potential

The changes in the content of fatty acids are regarded as one of the strategies that are necessary for successful colonization of low-temperature environments. In particular, the unsaturation of membrane lipids is considered to play a major role in maintaining of membrane fluidity (Morgan-Kiss et al. 2006). High proportion of PUFA have been reported for algae originating from cold habitat (Morgan-Kiss et al. 2002) as well as for mesophilic ones (Krienitz and Wirth 2006). However, the relative content of PUFA in the two Antarctic strains of *Monoraphidium* is unusual and comparable only with a few reports, e.g., with ~75% PUFA in total fatty acids in a field sample of snow algae (Řezanka et al. 2008). Such high relative content of PUFA indicates that they are an important element ensuring survival in extreme cold environments.

The major PUFA in the Antarctic *Monoraphidium* strains were stearidonic acid (6,9,12,15–18:4, 30–34%), together with hexadecatetraenoic acid (4,7,10,13–16:4, 19–23%). Stearidonic acid is occasionally found as a minor component in plants (Lísa et al. 2009), but it occurs mainly in algae and fish oils. The content of this acid in *Monoraphidium* strains from the SAG culture collection never surpassed 20% and it was below 10% in most strains

(Lang et al. 2011). Algae are the only significant source of hexadecatetraenoic acid. The content of this acid in the newly isolated strains was again significantly higher, when compared to other analyzed strains from the same genus (always below 10%). Higher levels of these two acids were detected only in several strains from other algal genera, mostly *Chlamydomonas* (Lang et al. 2011). Both acids have significant pharmacological effects (Guichardant et al. 1993).

Algae from the genus *Monoraphidium* started recently to be tested for the use in biotechnologies. Some strains of this genus were identified as a promising source of lipids for biofuel production (Yee 2016). However, the growth characteristics and lipid composition of these strains correspond to the fact that they were isolated from mesophilic habitats. Based on their unprecedentedly high PUFA content, relatively high growth rates in a broad temperature range and low light requirements, the two newly isolated Antarctic strains can be considered as good candidates for low-temperature biotechnological applications.

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