The first phylogenetic and ecophysiological characterisation of *Ankistrodesmus antarcticus* CCAP 202/25, an Antarctic green alga isolated from freshwater ice

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Abstract

Glacier ice algae and snow algae are important primary producers in extreme cold environments and significantly impact global processes through their role in carbon cycling and glacier melting by reducing surface albedo. These organisms have evolved unique cold-adaptation mechanisms, making them promising candidates for biotechnological applications, yet few species are characterised in detail. Such studies are also becoming particularly urgent, as polar cryospheric habitats are rapidly changing because of the ongoing climate change. Here we describe the phylogenetic placement and ecophysiological characterisation of the strain *Ankistrodesmus antarcticus* CCAP 202/25 that was isolated from a sample of green-coloured icicle collected on Sabrina Island (Balleny Islands, Antarctica). Cells maintained the size and shape that were reported in the original description of the strain in 1968. Analysis of the 18S rDNA confirmed its placement in the Selenastraceae family and, more specifically, in the highly supported and ecologically diversified *Monoraphidium* V clade, suggesting that a revision of its taxonomic assignment to the genus *Ankistrodesmus* will be necessary. Comparison of secondary structures of ITS2 rDNA uncovered several closely related strains with diverse or unknown origin that should be regarded as conspecific with *Ankistrodesmus antarcticus* CCAP 202/25. Therefore, more data are necessary to get a detailed insight into the phylogenetic and ecological diversification within this group. The cultivation of the strain in crossed gradients of temperature and light reflected adaptation of the species to cold Antarctic habitats. Similarly to psychrophilic species, *Ankistrodesmus antarcticus* was able to grow at 1°C, but the highest growth rates were recorded in a wide range of temperature (6–25°C) making its ecophysiological classification difficult. The ability of the species to utilise very low intensity (and simultaneously wide range) of photosynthetically active radiation also indicates its adaptation to a polar cryospheric habitat, where light availability can often represent the limiting factor, but significant fluctuations of irradiance can be common, both short-term and long-term.

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Key words: *Ankistrodesmus*, Antarctica, Chlorophyta, freshwater ice, irradiance, Selenastraceae, temperature

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Introduction

Cryospheric habitats host diverse microbial communities composed of bacteria, archaea, fungi, and protists that were able to adapt to the extreme conditions of glaciers, sea ice and snow in polar and mountain regions (Morgan-Kiss et al. 2006). Phototrophic microorganisms are present where light and liquid water are available at least for a limited period of time, playing the key role of primary producers. Whereas sea ice communities are typically dominated by diatoms, streptophyte and chlorophyte algae are dominant in the weathering crust of glaciers and in snow, respectively (Laybourn-Parry et al. 2012). These microalgae are currently gaining increased attention because of their ability to form extensive blooms that decrease the albedo of ice surface and thus accelerate ice and snow melting (Hoham and Remias 2020). Glacier surfaces worldwide are inhabited namely by species from the genus *Ancylonema* (Zygnematophyceae) with brownish cells due to characteristically pigmented vacuoles (Remias at al. 2023). Most snow algae belong to the family Chlamydomonadaceae (Chlamydomonadales, Chlorophyceae) with the genera *Sanguina* and *Chloromonas* as the most represented and forming red, orange or green blooms (Hoham and Remias 2020, Procházková et al. 2019). Recently, there is growing evidence that the diversity of snow and glacier ice algae is still underestimated, and further data are needed to understand the taxonomic structure of cryospheric communities (Remias at al. 2023).

The members of the species-rich and morphologically diverse family Selenastraceae (Sphaeropleales, Chlorophyceae) are very common components of freshwater phytoplankton worldwide (Krienitz and Bock 2012). The use of the morphological species concept for this family has resulted in an underestimation of its species diversity and may have led in many cases to incorrect identification of individual species, and thus to incorrect conclusions about their ecology and distribution (Krienitz et al. 2011, da Silva et al. 2017). The common genera *Ankistrodesmus* (*A.*) and *Monoraphidium* (*M.*) are currently regarded as collective units that are awaiting a detailed taxonomic revision (Krienitz et al. 2011, Krienitz and Bock 2012).

The species from the Selenastraceae family are also found in freshwater Antarctic lakes, where they can even form a dominant component of the phytoplankton, such as *A. falcatus* in Lake Tranquil on Signy Island (Butler et al. 2000). Other species such as *Monoraphidium contortum*, *M. minutum*, *M. griffithii* or *Monoraphidium* sp. are part of the phytoplankton of Antarctic Peninsula lakes, where they can develop to occasional maxima during favourable conditions (Izaguirre et al. 2001, 2003; Nedbalová et al. 2017). Even though they are apparently able to adapt to the extremely cold conditions (< 5°C), they are rarely reported from ice or snow, where other algal group prevail. *A. tatrae*, described as cryosestic species from snow by Kol (1926), was later transferred into the genus *Koliella* (*Raphidonema*) that belongs to the class Trebouxiophyceae (Hindák 1963).

Regarding ecophysiological characteristics, cold adapted microalgae do not form a uniform group. Bloom forming snow and glacier ice species are usually considered as psychrophilic, as they can only grow at low temperatures in a relatively narrow temperature range (~ 10°C) (Hoham and Remias 2020). However, the ability to grow at low temperature is not restricted to psychrophiles and it is often difficult to classify a given species according to the classical system of Morita (1975) that was originally established for bacterial strains. Seaburg et al. (1981) reported a marked variation in temperature – growth responses within a representative set of unialgal isolates from continental Antarctica with most taxa capable of growth in a wide temperature range up to 20°C and some of them even up to 30°C. Apart from low temperatures, light availability represents another challenge for Antarctic photosynthetic microorganisms as they must cope with several months of darkness during the winter period, when the sun does not appear above the horizon (McKnight et al. 2000). In summer, light intensity can be extremely variable even within one habitat type. Whereas surfaces of glaciers and snow fields typically receive very high light intensities in sunny weather during austral summer, the situation will change dramatically in deeper layers. The photosynthetic apparatus of microalgae must thus be tuned to particular light conditions of a site and fluctuations of light intensity (Hoham and Remias 2020).

Despite the substantial progress made possible by environmental sequencing (*e.g.* Davey et al. 2019), phycologists are still far from adequate understanding the diversity of Antarctic cryospheric microalgae, and thorough polyphasic assessment of available laboratory strains is also needed. Here, we present the first molecular and ecophysiological characterisation of the only currently available laboratory strain of a chlorophyte alga from the family Selenastraceae that was isolated from Antarctic freshwater ice (Kol and Flint 1968).

Material and Methods

Origin of the algal strain, cultivation conditions and morphological characterization

The strain used for analyses was acquired from the Culture Collection of Algae and Protozoa (CCAP) located in Dunbeg (Scotland) as *A. antarcticus* CCAP 202/25. It was isolated in 1964 by E. Flint (original designation E. Flint 64/74) from snow/ice at Balleny Islands (Antarctica) that are situated about 240 km north-west of Cape Adare, Victoria Land (extending from 66°15' to 67°35' S and 162°30' to 165°00' E). The islands are of volcanic origin and almost entirely ice capped. The field sample was collected on Sabrina Islet, which is less than kilometer long and located 1.5 km south of Buckle Island. During the visit in March 1964, icicles with green layers were observed, presumably formed by algae derived from melting ice (and maybe also snow) above the icicle part. The dominant organism forming the green coloration was later described by Kol as new species of chlamydomonadacean alga, *Chlamydomonas ballenyana* (Kol and Flint 1968). Pieces of a large icicle were collected, inoculated into various cultivation media (Detmer and Pringsheim) and used for isolation of laboratory cultures, including *A. antarcticus* CCAP 202/25. The cultures were kept at the temperature of 20°C. *Ankistrodesmus*-like cells were also present in the original sample preserved by formaldehyde. Apart from the green coloration of ice at the site of collection, *Prasiola* sp. growing on rocky outcrops was also observed (Kol and Flint 1968).

The strain obtained from CCAP was cultivated at 10°C under 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).

Microscopic observations of the strain were done using an Olympus BX43 microscope (Olympus Corporation, Japan) equipped with a 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.

For scanning and transmission electron microscopy (SEM and TEM), the cells originating from actively growing culture were fixed for 24 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and post-fixed in 2% OsO_4 in the same buffer. Fixed cells were then 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 ultramicratome (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).

Molecular analysis

Total genomic DNA was extracted following the standard protocol in DNeasy Plant Mini Kit (Qiagen, Germany), with a modification described in Nedbalová et al. (2017). The 18S small subunit ribosomal RNA gene (18S rDNA) and internal transcribed spacer 2 (ITS2 rDNA) were amplified from DNA isolates by polymerase chain reaction using existing primers; for 18S rDNA: NS1 (GTAGTCATATGCTT GTCT; White et al. 1990) and 18L (CACCTACGGAAACCTTGTTACGACT T; Hamby et al. 1988), 402-23F (GCTACCACATCCAAGGAAGGC; Katana et al. 2001) and 1308-39R (CTCGTTCGTTAACGGAATTAACC; Katana et al. 2001), for ITS2 rDNA: ITS1 (TCCGTAGGTGAACCTGCGG; White et al. 1990) and ITS4 (TCCTCCGCTTAT TGATATGC; White et al. 1990). Amplification and sequencing reactions for these markers were identical to those described by Nedbalová et al. (2017). The obtained sequences of the strain *A. antarcticus* CCAP 202/25 were submitted to the National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA) Nucleotide sequence database (18S rRNA gene sequence: PQ842547; partial ITS1 + 5.8S +ITS2+ partial 26S rDNA: PQ842548).

The 18S rDNA alignment contained dataset of 58 sequences (1 629 base positions) examined in previous studies (Krienitz et al. 2011, da Silva et al. 2017), as well as the new sequence of *A. antarcticus* CCAP 202/25; *Scenedesmus obliquus*, *Scenedesmus communis*, *Pediastrum duplex*, *Bracteacoccus minor* and *Bracteacoccus aerius* were selected as the outgroup. The multiple 18S rRNA gene sequences identical to the studied species were inserted into the tree diagram after analysis. The best-fit nucleotide substitution model was estimated by the jModeltest 2.0.1 (Posada 2008). Based on the Akaike Information Criterion, the GTR+I+G was selected as the best fit

evolutionary model. The phylogenetic tree was inferred by Bayesian inference using MrBayes version 3.2.6 (Ronquist et al. 2012) and maximum likelihood (ML) method. Two parallel Markov chain Monte Carlo runs for 3,000,000 generations with one cold and three heated chains were conducted for the alignment, with trees sampled every 100 generations. The first 25% were discarded as burn-in. Convergence of the two chains was checked by the average standard deviation of split frequencies of 0.0103. Bayesian posterior probabilities were used to assess clade support. Bootstrap analysis was performed by ML using GARLI 2.0 (Zwickl 2006). ML analysis consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100,000).

The ITS2 sequences of strain *A. antarcticus* CCAP 202/25 (this study) was compared with its closest hits using NCBI blast search (*i.e.* sequence identity >90% and query cover >98%), namely *Monoraphidium griffithii* KLL_G017 (clone A: KP726253, clone B: KP726255, clone C: KP726254), *Monoraphidium* sp. nor (MN 738562), *Monoraphidium* sp. KMMCC 1531 (JQ315786.1), *Monoraphidium pusillum* Ga024 (PP711304), *Monoraphidium "pusillum"* Ga025 (PP766950), *Monoraphidium griffithii* KEM 23-13.2 (PP495501), *Ankistrodesmus* sp. WLW01 (OR502621) and *Ankistrodesmus* sp. CCAP 202/7D (MW471046).

Secondary structure of ITS2 rDNA was predicted with 5.8S–LSU stem regions using the Mfold server accessible at [1] (Zuker 2003). A model of the secondary structure consistent with the specific features of nuclear rDNA ITS2 was selected: four helixes and U–U mismatch in helix II (Coleman 2007). The sequences were aligned using the sequence-structure analysis in 4SALE (Seibel et al. 2006, 2008) in order to find compensatory base changes (CBCs; nucleotide change at both of the positions that pair with each other in a double stranded helix). The secondary structure of nuclear rDNA ITS2 was drawn using a Java lightweight Applet VARNA version 3.9 (Darty et al. 2009).

Evaluation of growth optima

For evaluation of the growth optima, the method of cultivation in crossed gradients of temperature and light was used (Halldal and French 1958). The strain was cultivated in sterile immunological plates (FB type, 9 × 12 cm, 96 wells, polystyrene, Falcon) in a volume of 0.2 ml of BBM medium per well on a massive metal block cooled along one side and heated on the opposite side. The light was placed obliquely above the third side. The initial optical density measured at 750 nm (OD_{750}) was set to 0.05, just above the detection of the plate reader used (VarioscanTM Flash Multimode Reader, Thermo Fisher Scientific, Finland). Cultures were inoculated by stock culture in a stage of linear growth. After the inoculation, the plates were sealed with a lid and polyethylene foil to reduce evaporation and incubated in a cross-gradient (PAR and temperature) cultivation unit (Kvíderová and Lukavský 2001). The gradient of photosynthetically active radiation (PAR) 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, each of them lasted 38 days). PAR intensity was evaluated using LiCOR Li-250A light meter equipped by quantum sensor Li-190 (LiCOR, USA), temperature with digital thermometer Multi-A (Fisher Scientific, USA).

Growth rate of the culture was evaluated as optical density at 750 nm (OD_{750}) over a period of 38 days directly in

wells of immunological plates using a VarioscanTM Flash Multimode Reader (Thermo Fisher Scientific, Finland). The OD_{750} 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 PAR and temperature were evaluated. The data were visualised using GraphPad Prism 9 (GraphPad Software, USA). The growth rate data for the contour plot (created in Statistica 12, StatSoft, USA) were smoothed by a distance weighted least square method.

Results

Cell morphology and ultrastructure

Cells are spindle shape, often slightly asymmetrical, with pointed ends and a thin cell wall (Fig. 1). Average cell size is 20.5±2.5 × 3.1±0.4 μm, minimal recorded values were 15.4 and 2.0 μm, maximal 26.3 and 3.9 μm (for cell length and cell width, respectively). The alga reproduces by autospores. Daughter cells originate by perpendicular splitting and as they elongate, the plane of the division becomes oblique to the long axis of mother cell (Fig. 1C). The young cells often remain attached forming irregular groups or star-like associations of cells (Fig. 1B). Ultrastructure of cells is shown in Fig. 1D, E. No pyrenoid was observed in the single parietal chloroplast. In old cultures, numerous oil droplets were deposited in cells.

Phylogenetic position of the strain

The major clades resolved in the 18S rDNA phylogenetic tree of Selenastraceae were *Monoraphidium*, *Raphidocelis*, *Kirchneriella*, *Messastrum gracile*, *Nephrochlamys*, *Selenastrum bibraianum*, *Chlorolobion*, *Ankistrodesmus*, *Curvastrum* and *Tetranephris*. The strain *A. antarcticus* CCAP 202/25 was placed into the highly supported *Monoraphidium* V clade (Fig. 2), where it formed a subclade with sister relationship to a subclade consisted of several isolates of *Monoraphidium griffithii*, the type species of the genus *Monoraphidium*. Comparisons of the secondary structures of ITS2 rDNA showed a presence of the two CBCs between *Ankistrodesmus antarcticus* CCAP 202/25 and *Monoraphidium griffithii* KLL_G017 (one in branched part of helix I and one in the apex of the helix II; Fig. 3). This indicates that strain CCAP 202/25 is independent species. However, no CBC was found between *A. antarcticus* CCAP 202/25 and the following strains: *Monoraphidium* sp. KMMCC 1531, *Monoraphidium* sp. nor, *Monoraphidium pusillum* Ga024, *Monoraphidium pusillum* Ga025, *Monoraphidium griffithii* KEM 23-13.2, *Ankistrodesmus* sp. WLW01 and *Ankistrodesmus* sp. CCAP 202/7D. This means that all these eight strains can be ranked as one species. One of these strains shares extreme habitat with *A. antarcticus* CCAP 202/25: *Monoraphidium* sp. nor was isolated from alpine lake Cheshme Sabz in Iran (2 500 m a.s.l.). Extreme habitat was also reported for *Monoraphidium griffithii* KEM-23-13.2: the puddle formed by all-terrain vehicle tracks in the coal mine drainage in Kemerovo region in Russia (54.5984 N, 86.3437 E) (Solovchenko et al. 2024).

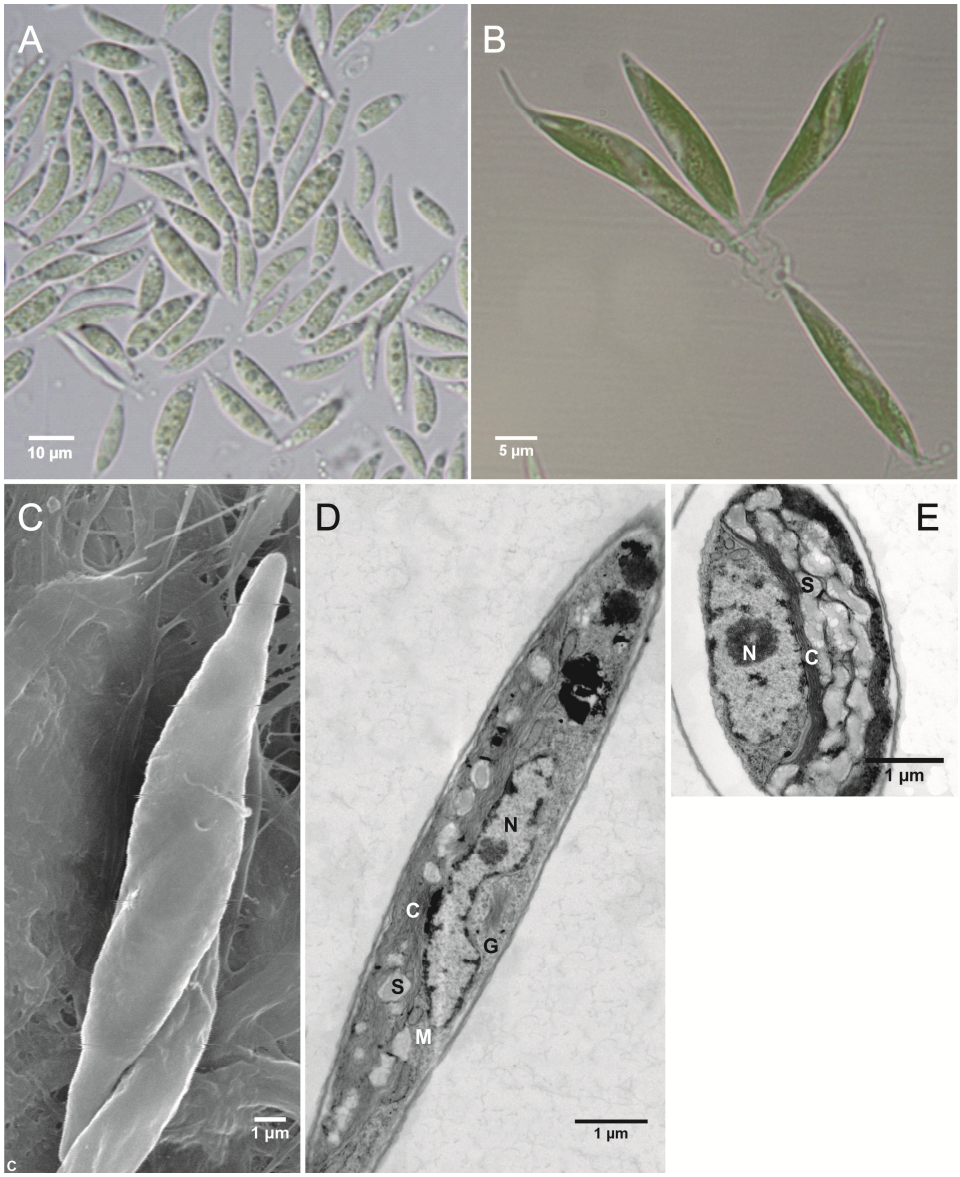

Fig. 1. Cell morphology of the strain *Ankistrodesmus antarcticus* CCAP 202/25. Light microscopy pictures showing overall cell morphology (A, note oil droplets in many cells), and star-like association of cells (B). Cell division visualized using scanning electron microscopy (C) and cell ultrastructure (D – transversal section, E – cross section) depicted by transmission electron microscopy (N – nucleus, C – chloroplast, S – starch grains, M – mitochondrion, G – Golgi apparatus).

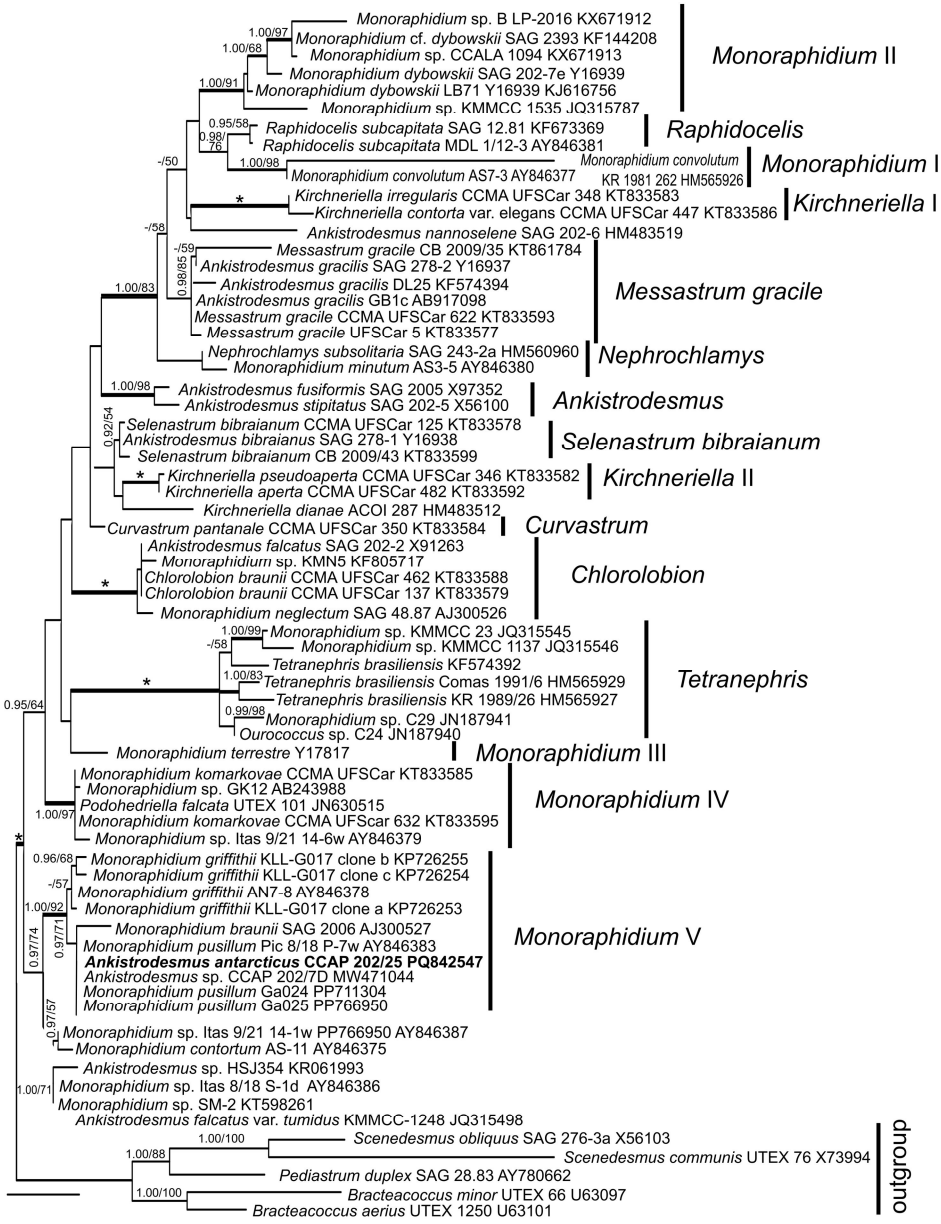

Fig. 2. 18S ribosomal RNA genes-based maximum likelihood phylogenetic tree showing phylogenetic position of some members of Selenastraceae. Posterior probabilities (0.95 or more) and bootstrap values from maximum likelihood (50 or more) are shown. Thick branches represent nodes receiving the highest posterior probability support (1.00). Full statistical support (1.00/100) is marked with an asterisk. The newly obtained sequence is in bold. Accession numbers and strain codes are indicated after each species name.

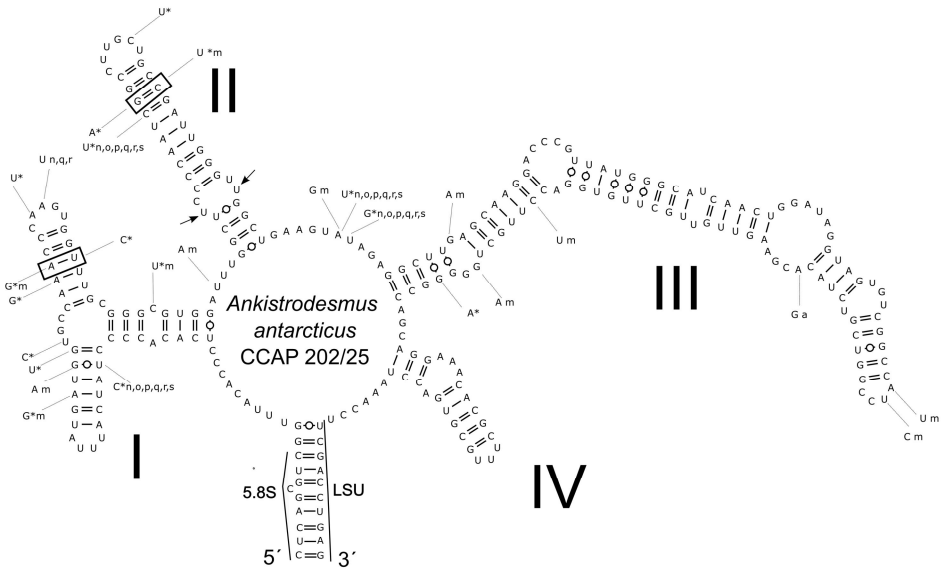

Fig. 3. Comparison of the secondary structure of ITS2 rDNA transcripts between *Ankistrodesmus antarcticus* CCAP 202/25 (PQ842548) and closely related isolates: *Monoraphidium griffithii* strain KLL_G017 (clone A: KP726253 – 93.53% identity, 260 out of 278 bp, clone B: KP726255 – 93.88% identity, 261 out of 278 bp, clone C: KP726254 – 93.88% identity, 261 out of 278 bp), *Monoraphidium* sp. KMMCC 1531 (JQ315786; 94.62% identity, 264 out of 279 bp) and *Monoraphidium* sp. nor (MN738562; 97.76% identity, 218 out of 223 bp). Helices are labelled with Latin numbers. Nucleotide differences are outside the structure and linked by dotted lines. One asterisk means that the difference was detected in all above clones of *Monoraphidium griffithii* strain KLL_G017 while letter ʻaʼ is a for single additional difference in the clone A. Letter ʻmʼ indicates difference in *Monoraphidium* sp. KMMCC 1531. Letter ʻnʼ implies a difference in *Monoraphidium* sp. nor. Letter ʻoʼ is a for the difference in *Monoraphidium pusillum* strain Ga025 (PP766950; 98.58% identity, 277 out of 281 bp), ʻpʼ for *Monoraphidium pusillum* strain Ga024 (PP711304; 98.58% identity, 98.58% identity), ʻqʼ for *Ankistrodesmus* sp. CCAP 202/7D (MW471044; 98.22% identity, 277 out of 281 bp), "r" for *Monoraphidium griffithii* strain KEM-23-13.2 (PP495501; 98.56% identity, 276 out of 281 bp), "s" for *Ankistrodesmus* sp. isolate WLW01 (OR502621; 98.50% identity). Two compensatory base changes (indicated by rectangles) between the *Ankistrodesmus antarcticus* CCAP 202/25 strain and *Monoraphidium griffithii* strain KLL_G017 were found. Note the U–U mismatch in helix II (arrows).

Temperature and light growth optima

The dependence of growth rate on temperature and light intensity was estimated using cultivation unit for crossed gradients. The response of the strain to the varying cultivation conditions significantly differed across both gradients. Maximal relative growth rate reached the value of 0.55 day^{-1}. The strain was able to grow in a wide temperature range (1–25°C) and the highest growth rates were detected in the range 6–25°C. However, the growth at 25°C was much slower (~0.25 day^{-1}), when the highest light intensity was applied. Lower growth rates compared to 6–25°C were observed at 1°C (0.1–0.3 day^{-1}). At temperatures above 25°C, the growth rate steeply decreased and virtually no growth was observed at 30°C (Figs. 4, 5).

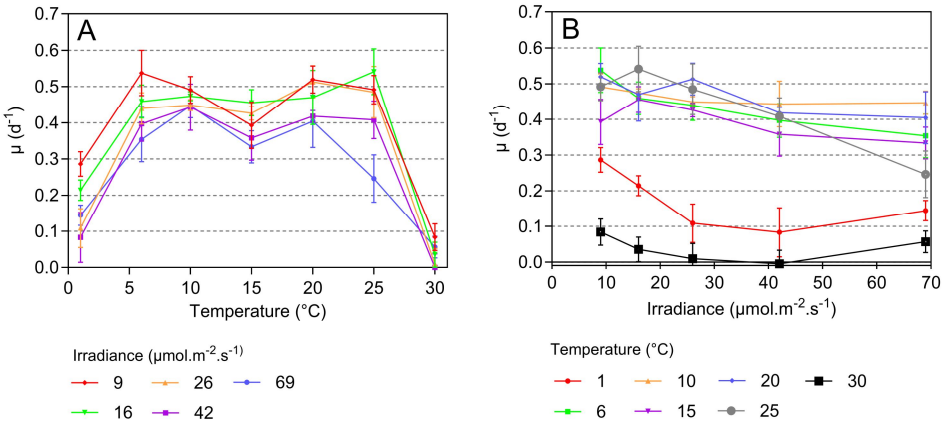

Fig. 4. Dependence of growth rate of *Ankistrodesmus antarcticus* CCAP 202/25 on temperature at different irradiance levels (A) and dependence of growth rate on irradiance at different temperatures (B), mean + SD, μ – growth rate, T – temperature, I – irradiance.

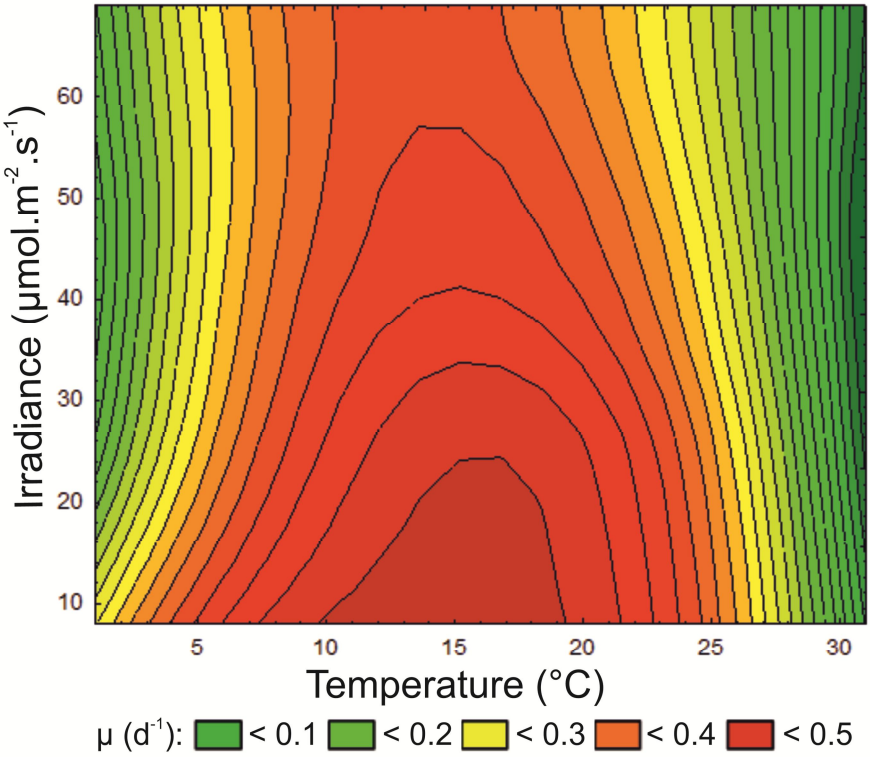

Fig. 5. Contour plot showing the growth rate (μ) of *Ankistrodesmus antarcticus* CCAP 202/25 with respect to temperature and irradiance during cultivation in crossed gradients. The data were smoothed using the weighted least squares distance method.

The strain grew at all the PAR intensities that were used in the growth experiment. With increasing PAR, growth rate decreased. The highest growth rate was detected at lowest irradiances (9–16 μmol m^{-2} 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 strain to PAR (Figs. 4, 5). The statistical evaluation of the variability in growth rates at different temperatures and irradiance levels are given in Tables S1 and S2.

Discussion

Strain origin, cultivation and morphology

In their description of the samples from which the strain *A. antarcticus* CCAP 202/25 was isolated, Kol and Flint (1968) raised some doubts regarding the original habitat of the species and suggested that snow or melting ice could be the source of green coloration of the icicles. The dominant alga forming the bloom was described as *Chlamydomonas ballenyana*, however, no culture of this organism is available. Therefore, molecular analysis of the species enabling its taxonomic characterisation in the context of currently available data on Antarctic cryosestic communities (Davey et al. 2019, Soto et al. 2020) is not possible as well. Chlamydomonadacean algae with flagellated life cycle stages are often causing the coloration of snow (Hoham and Remias 2020). *Chlamydomonas ballenyana* (as var. *minor*) was also observed as dominant component of green cryosestic community in samples from Terre Adélie suggesting its wide geographic distribution in Antarctica (Kol 1971).

Kol and Flint (1968) observed the cells of *A. antarcticus* in the original sample, suggesting that the species was an important component of the microbial community and not only occasionally present. Also, a combined origin of the icicle community cannot be excluded. Except for the Sabrina Island samples, microalgae from Selenastraceae family were not reported from snow and glacier ice blooms, making this community unique in a wider context. However, cold adapted species from the genera *Ankistrodesmus* and *Monoraphidium* are known to prevail in the plankton of some Antarctic lakes, which demonstrates a large ecological plasticity within this group (*e.g.*, Butler et al. 2000, Nedbalová et al. 2017).

The strain *A. antarcticus* CCAP 202/25 (originally E. Flint 64/74) was isolated in 1964, which makes it one of the oldest (and probably the oldest) strains isolated from a cryospheric habitat that is kept in a culture collection until today. This also further highlights the importance of culture collections for biodiversity conservation (Giudice and Rizzo 2020).

Upon arrival to the laboratory from the CCAP culture collection, the strain was kept in the *Euglena gracilis* medium containing tryptone and yeast extract as indicated on the CCAP webpage. A lower cultivation temperature compared to the culture collection conditions was used, as we assumed that the strain would be cold adapted given its geographic origin (Kol and Flint 1968). However, these conditions resulted in a poor growth of the microalgae and strong fungal and bacterial contamination. The problems with cultivation were finally overcome by switching to mineral medium (BBM) combined with serial dilution of the culture.

Despite the long-term laboratory cultivation, cells of *A. antarcticus* CCAP 202/25 retained the same morphological characteristics that are given in the original description of the species, including the formation of autospores (Kol and Flint 1968). The members of the Selenastraceae family are known for their large variability of cell shapes and sizes that facilitated the description of many species based solely on morphological data (Komárková-Legnerová 1969). Cells were mostly solitary, but star-shaped clusters were occasionally observed, formed by the connection of several cells by the mucus at their ends (Fig. 1B). Colony formation can be a very variable feature and changes in environmental conditions can lead to their disintegration (Krienitz and Bock 2012). Krienitz et al. (2001) observed the disintegration of *Ankistrodesmus* colonies into single cells in rapidly growing cultures. Regarding cell ultrastructure, the absence of pyrenoid is also in line with the original description (Kol and Flint 1968). The abundance of oil droplets observed in old cultures indicates a possible biotechnological potential of the strain. Recently, numerous strains from the Selenastraceae family have been shown to be suitable candidates for the production of oils for various applications, *e.g.* food supplements or biodiesel (*e.g.*, Yee et al. 2016). The cold adapted strains can be of particular interest as they produce high amounts of polyunsaturated fatty acids (PUFAs) and maintain relatively high growth rates at low temperatures which potentially enables the expansion of outdoor large-scale cultivations to cold seasons and/or higher latitudes (Řezanka et al. 2017).

Phylogenetic relationships and ecology

As shown by previous phylogenetic studies, Selenastraceae family is a very diversified group, and morphological characteristics traditionally used for taxonomic delimitation of genera are only of limited relevance (Krienitz et al. 2001, Fawley et al. 2005, Krienitz and Bock 2012). While a formal revision was already performed for some genera (*e.g.*, *Selenastrum*, da Silva et al. 2017), it is still lacking for the members of the species-rich genera *Ankistrodesmus* and *Monoraphidium*. The analysis of 18S rDNA placed the strain *A. antarcticus* CCAP 202/25 in the *Monoraphidium* V clade, which also contains several strains of *Monoraphidium griffithii*, the type species of the genus. On the other hand, the type species of the genus *Ankistrodesmus* (*A. fusiformis*) forms a separate clade together with a strain of *A. stipitatus* that is distant from the *Monoraphidium* V clade (Fig. 2), suggesting that the CCAP 202/25 should be transferred into different genus in future. Its closest relatives with a 100% identity in the 18S rDNA phylogeny (*M. pusillum* Pic 8/18 P-7w, *M. braunii* SAG 2006, *Ankistrodesmus* sp. CCAP 202/7D) do not originate from cryospheric habitats (eutrophic pond in Minessota, USA, a lowland humic lake in Germany, unspecified site between Croby and Uppingham in United Kingdom) pointing to diversified ecological requirements of the members of the clade. The origin of further closest relatives (*M. pussilum* Ga024 and *M. pussilum* Ga025) is unknown. However, one of these closest relatives of *A. antarcticus* CCAP 202/25, *M. pusillum* Pic 8/18 P-7w, has a similar morphology (Fawley et al. 2005).

The number of available cold adapted strains from the Selenastraceae family is very limited compared to mesophilic ones. Apart from *A. antarcticus* CCAP 202/25 that originated from melting ice or snow, two strains of *Monoraphidium* sp. were isolated from plankton of Antarctic lakes on James Ross Island (Nedbalová et al. 2017, one of them shown in Fig. 2 as *Monoraphidium* sp. CCALA 1094). Ac-

cording to 18S rDNA phylogeny, they belong to a different clade than *A. antarcticus* CCAP 202/25 (*Monoraphidium* II), indicating that adaptation to low temperature environments has probably evolved several times within Selenastraceae. Identification of other species of *Monoraphidium* and *Ankistrodesmus* found in Antarctic lakes was done based on morphological data (Butler et al. 2000, Izaguirre et al. 2001, 2003).

The comparison of the ITS2 secondary structures confirmed that *Monoraphidium griffithii* represents a separate species but revealed five strains of *Monoraphidium* and two strains of *Ankistrodesmus* that should be regarded as very closely related (conspecific according to the CBC species concept, Wolf et al. 2013) to *A. antarcticus* CCAP 202/25. Interestingly, one of these *Monoraphidium* strains is probably cold adapted, as it originates from an alpine lake. Another strain was isolated from a river in South Korea without any further specification of temperature range. To conclude, more data are apparently needed to get a better understanding of phylogenetic and ecological diversification in the Selenastraceae family in general and also among the close relatives of the strain *A. antarcticus* CCAP 202/25.

Ecophysiological characteristics

The response of the strain to the gradient of temperature revealed that even after long-term cultivation in artificial conditions of a culture collection, it maintained the ability to grow at temperatures close to zero (1°C) reflecting its original cryospheric habitat (Kol and Flint 1968). However, the highest growth rates were detected in the range from 6 to 25°C (Figs. 4, 5), where they reached values comparable to unlimited growth of some mesophilic microalgae (Reynolds 2006). Simultaneously, the rapid slowing down of growth at temperatures above 25°C indicated a specific ecophysiology of the strain.

It is not possible to assign the studied strain unambiguously into a category established by Morita (1975). The ability to tolerate relatively high temperature has also been reported in several other polar algae from the genera *Navicula*, *Chlorella* or *Chlamydomonas* (Seaburg et al. 1981, Teoh et al. 2004, Cao et al. 2016). The ability of phototrophic microorganisms to grow in a wide range of temperatures has been associated mainly with environments with large temperature fluctuations (Seaburg et al. 1981). However, a very similar growth response as in *A. antarcticus* CCAP 202/25 was recorded for the abovementioned strains of *Monoraphidium* sp. dominating in plankton of Antarctic lakes with stable temperature conditions, suggesting that not only psychrophilic microorganisms can be successful in such environmental conditions (Nedbalová et al. 2017). To our best knowledge, no psychrophilic species from the Selenastraceae family has been described so far in contrast to some other chlorophyte groups (Chlamydomonadaceae) or streptophyte algae from cryospheric habitats (Hoham and Remias 2020).

The growth response of *A. antarcticus* CCAP 202/25 to the gradient of light indicated the ability of the strain to utilise very low intensities of PAR and a relatively wide tolerance to light conditions. This is in line with the fact that light conditions in ice and snow can dramatically change from the surface to deeper layers. Generally, photosynthetic microorganisms, living under or in ice, are well adapted to very low solar radiation intensities. Diatoms living in sea ice have been reported to be photosynthetically active at irradiances ~ 0.5 μmol m^{-2} s^{-1}, which is equivalent to 0.03% of the surface solar radiation (Cota 1985).

In contrast to bloom forming snow algae from Chlamydomonadaceae, the members of Selenastraceae including *Ankistrodesmus* do not have any flagellated life cycle stage, and cannot actively move to preferred environmental conditions, including light intensity. As in the case of temperature preferences, the response to light was similar to the Antarctic strains of *Monoraphidium* sp. from lake plankton. Their adaptation to low light is reflecting the conditions in the water column of ice-covered polar lakes (Nedbalová et al. 2017).

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Web sources / Other sources

[1] http://mfold.rna.albany.edu/?q5mfold

Table S1, S2. Statistical evaluation of the dependence of the growth rate of the studied strains on temperature (Table S1) and irradiance (Table S2) using analysis of variance (ANOVA) and Tukey's multiple comparison method. The tested null hypothesis was that temperature and radiation intensity do not affect the growth rate. The results were considered significant for $p < 0.05$. Temperature (°C), Irradiance ($\mu mol \cdot m^{-2} \cdot s^{-1}$).

Table S1.

Temperature/ Irradiance	**69**	**42**	**26**	**16**	**9**
ANOVA	Yes	Yes	Yes	Yes	Yes
30 vs 25	Yes	Yes	Yes	Yes	Yes
30 vs 20	Yes	Yes	Yes	Yes	Yes
30 vs 15	Yes	Yes	Yes	Yes	Yes
30 vs 10	Yes	Yes	Yes	Yes	Yes
30 vs 6	Yes	Yes	Yes	Yes	Yes
30 vs 1	Yes	Yes	Yes	Yes	Yes
25 vs 20	Yes	No	No	Yes	No
25 vs 15	Yes	No	Yes	Yes	Yes
25 vs 10	Yes	No	No	Yes	No
25 vs 6	Yes	No	No	Yes	No
25 vs 1	Yes	Yes	Yes	Yes	Yes
20 vs 15	Yes	Yes	Yes	No	Yes
20 vs 10	No	No	Yes	No	No
20 vs 6	No	No	Yes	No	No
20 vs 1	Yes	Yes	Yes	Yes	Yes
15 vs 10	Yes	Yes	No	No	Yes
15 vs 6	No	No	No	No	Yes
15 vs 1	Yes	Yes	Yes	Yes	Yes
10 vs 6	Yes	No	No	No	No
10 vs 1	Yes	Yes	Yes	Yes	Yes
6 vs 1	Yes	Yes	Yes	Yes	Yes

Table S2.

Irradiance/ Temperature	30	25	20	15	10	6	1
ANOVA	Yes	Yes	Yes	Yes	Yes	Yes	Yes
69 vs 42	Yes	Yes	No	No	No	No	Yes
69 vs 26	Yes	Yes	Yes	Yes	No	Yes	No
69 vs 16	No	Yes	Yes	Yes	No	Yes	Yes
69 vs 9	No	Yes	Yes	Yes	Yes	Yes	Yes
42 vs 26	No	Yes	Yes	Yes	No	No	No
42 vs 16	Yes	Yes	No	Yes	No	Yes	Yes
42 vs 9	Yes	Yes	Yes	No	Yes	Yes	Yes
26 vs 16	No	No	No	No	No	No	Yes
26 vs 9	Yes	No	No	No	Yes	Yes	Yes
16 vs 9	Yes	No	No	Yes	No	Yes	Yes