Deuterostichococcus alpinus sp. nov. (Chlorophyta; Trebouxiophyceae) from Antarctica

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Abstract

Lichens are the most dominant components in flora of Antarctica. A lichen epiphyte green alga FACHB-2327, isolated from Antarctic lichen *Stereocaulon alpinum*, was identified as a new species belonging to the genus *Deuterostichococcus*. The specimen of lichen *Stereocaulon alpinum* was collected from King George Island, Antarctica. A comprehensive analysis, including morphology, ultrastructure, habitat, phylogeny and secondary structure of SSU rDNA V9 region, was carried out. The green alga FACHB-2327 was described and named as *Deuterostichococcus alpinus* sp. nov. to recall the continent where it was discovered. The alga FACHB-2327 forms single celled thalli or pseudofilaments up to three cells; cells are cylindrical with mean size 7-18 (20) × 3-5 µm and rounded ends. It can be distinguished from the other species in genus *Deuterostichococcus* by morphological characters, including cell size, length/width ratio and phylogenetic position using SSU rDNA and ITS rDNA sequences.

Key words: Antarctica, lichen, microalgae, Prasiolales, phylogeny, King George Island

Abbreviations: BBM: Bold's Basal Medium, FACHB: Freshwater Algae Culture Collection at the Institute of Hydrobiology, ITS: Internal Transcribed Spacer, PCR: Polymerase Chain Reaction, PDA: Potato Dextrose Agar, SSU: Nuclear Small Subunit, TEM: Transmission Electron Microscopy

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Introduction

The *Stichococcus*-like green algae are worldwide distribution and could be found in diverse habitats including the freshwater and marine localities, snow and soil (Kol 1968, Ettl and Gärtner 2014, Pröschold and Darienko 2020). *Stichococcus*-like green algae, reproducing vegetatively, are characterized by a short cylindrical and elongate or spherical to slightly oval shaped cells containing chloroplast without pyrenoid. Among *Stichococcus*-like green algae, three species were reported to be different from the others: (1) *Deuterostichococcus deasonii* (Neustupa, Eliáš and Šejnohová) Pröschold and Darienko, (2) *Tetratostichococcus jenerensis* (Neustupa, Eliáš and Šejnohová) Pröschold and Darienko, a species that has pyrenoid (Neustupa et al. 2007, Pröschold and Darienko 2020, Pröschold et al. 2020); and (3) *Pseudostichococcus monallantoides* L. Moewus, a species with zoospore formation (Moewus 1951).

The morphology of *Stichococcus*-like algae is simple, and the traditional species concepts were based solely on morphological features, such as length/width ratio or presence of pyrenoids visible in the light microscope (Moewus 1951, Printz 1964, Beck et al. 2019). Since the application of phylogenetic in the classification of *Stichococcus*-like algae, these traditional species concepts were largely found incompatible with phylogenetic structure of the group, which pointed out to a much higher diversity than previously estimated. Recently, the genera *Stichococcus* Nägeli, *Pseudostichococcus* L. Moewus, *Diplosphaera* Bialosuknia and *Desmococcus* F. Brand had been revised using 18S and ITS rDNA sequences from 34 strains assigned as the above genera (Pröschold and Darienko 2020). Four new genera together with four new species were proposed that many described species of *Stichococcus*-like algae were transferred to new genera and synonymized by polyphasic studies (Pröschold and Darienko 2020).

Deuterostichococcus Pröschold and Darienko is one of the four new genera. This genus has been recorded from Europe, North America, Asia, Antarctica with diverse habitat such as freshwater, soil, photobiont, epiphytic, ice (Pröschold and Darienko 2020). The genus is characterized by the following characteristics: cells short cylindrical to cylindrical, length/width ratio among 1.9-2.9, with rounded ends; chloroplast single, parietal, with or without pyrenoid; cells with single central nucleus; reproduction by fragmentation of filaments and by vegetative cell division. It differs from other genera on V9 region of the SSU sequences. Four species have been recorded within this genus: the type species is *D. deasonii* Pröschold and Darienko; the other species are *D. allas* Pröschold and Darienko, *D. epilithicus* Pröschold and Darienko, *D. lewinii* Pröschold and Darienko (Pröschold and Darienko 2020).

The limited morphology and ecology information enhanced the systematic study gap of the *Stichococcus*-like algae. In current study, a lichen epiphytic green alga was isolated from lichen *Stereocaulon alpinum* Laur., which was collected from Fildes Peninsula, King George Island, Antarctica. A single cell green alga is demonstrated as a new species within the genus *Deuterostichococcus* based on the comprehensive analysis approach, including morphology, ultrastructure, ecology and phylogeny and second structure of SSU sequence (V9 region).

Material and Methods

Study area

The samples of lichen *Stereocaulon alpinum* Laur. were collected at the Fildes Peninsula. It is the second largest ice-free area of the South Shetland Islands, located in the southwest of King George Island. The Fildes Peninsula has been reported with a yearly mean temperature lower than 1°C, sunshine duration shorter than 600 h, rainfall/snow less than 600 mm (Yang et al. 2013). There has been observed at least 120 lichen species and only one vascular plant hairgrass (*Deschampsia antarctica* Desv.)[1].

Isolation and culture

The lichen specimen *S. alpinum* (d-B1) was collected from King George (62° 12.69' S, 58°55.70' W) on 14th Feb. 2014 and was incubated at 4°C till the isolation was processed. The *S. alpinum* specimen (d-B1) was used to isolate the green alga, which was kept in the Resource-sharing Platform of Polar Samples which includes samples of Biology, Ice-snow, Rock and Sediment. A strain of green alga (Freshwater Algae Culture Collection at the Institute of Hydrobiology, FACHB-2327) was isolated by an improved tissue culture procedure (Cao et al. 2018) and then cultured on PDA and BBM petri-dish medium. All the operations were taken out under aseptic conditions. The isolations were incubated in an illumination incubator (4°C, 12/12 light/dark photoperiod, natural lighting). The algal cultures were maintained in both PDA and BBM Petri-dish medium at 4°C.

Light and electron microscopy

The compound microscopes Nikon Eclipse 80i and Nikon ACT-1 V2.70 were used for observing and photographing the algal cultures.

The cultured algal cells were used for transmission electron microscopy (TEM), after fixing by 2.5% glutaraldehyde buffer. The procedures and reagents (including 2.5% glutaraldehyde buffer) followed the method presented earlier (Cao et al. 2018). A Jeol JEM1230 transmission electron microscope at 80–120 kV A was used to observe the 70 nm cell sections and the sections were cut by a Leica EM UC6 ultramicrotome and stained with 3% uranyl acetate and lead citrate.

An Olympus SIS VELETA CCD camera was used to capture the micrographs using iTEM software (Solingen, Germany).

DNA extraction, amplification, sequencing and analysis

The total genomic DNA was extract using the method from (Cao et al. 2015). The following primer pairs NS1, NS4; NS3, NS6; NS5, NS8 (White et al. 1990) and ITS5, O2 (Cao et al. 2015) were used to amplify the regions of SSU rDNA and ITS rDNA, respectively. A 50 μl volume PCR reaction was selected, PCR application and products verification followed the earlier described procedure (Cao et al. 2015). The double-stranded PCR products were sequenced on an ABI3730XL sequencer (ThermoFisher).

SEQMAN program within Lasergene v.7.1 software (DNASTAR Inc.) was selected to check the double-directional ITS rDNA and SSU rDNA sequences. The introns within ITS rDNA and SSU rDNA sequences were trimmed off, respectively. The sequences representing the new species were submitted to GenBank (MW023948, MW023947).

ClustalW algorithm within MEGA X (Kumar et al. 2018) was performed to align the sequences with default parameters (Thompson et al. 1994) and then adjusted manually. The Maximum Likelihood (ML) was selected to calculate the ITS phylogenetic structures, as well as Minimum Evolution (ME) method for SSU sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X using the evolution model GTR+I+gamma (Kumar et al. 2018). A 1000 resampling bootstrap was tested for the reliability of the inferred trees.

In total, 27 sequences were retrieved from GenBank (Table 1). These sequences were just the sequences used in the study of Pröschold and Darienko (2020).

The secondary structure of SSU rDNA was calculated using MARNA (Siebert and Backofen 2005), and compared with the structure of the genus *Deuterostichococcus* presented by Pröschold and Darienko (2020).

Results

Deuterostichococcus alpinus Shunan Cao & Qiming Zhou, sp. nov.
FACHB-2327 (Figs 1-4)

Holotype: Strain FACHB-2327, Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-Collection) Fig. 2B

Type locality: Antarctica, Fildes Peninsula, on stone (62°12.69' S, 58°55.70' W), 36 m a. s. l.; isolated from the Antarctic lichen *Stereocaulon alpinum* (collection No. 274 d-B1) on 14th Feb. 2014.

Habitat: Epiphytic green alga, living with lichen *Stereocaulon alpinum* Sub-Antarctic climate.

Description: Algae are single cell or unbranched up to three cells, cells are cylindrical with rounded ends, 7-18 (20) x 3-5 μm with the length/width ratio 2.3-3.6 (4). Cell wall smooth, double in ultrastructure. Single nucleus adherent to cell wall. Chloroplast single, parietal, with pyrenoid. Reproductive cells were not observed.

Diagnosis: Differs from the closely related species *D. epilichicus* in cell size, length/width ratio, SSU rDNA and ITS rDNA sequences. Morphologically, the isolate FACHB-2327 distinguished from the phylogenetically close species *D. epilichicus* (5.1-7.6 × 2.9-3.8 μm, length/width ratio 1.7-2.7, without pyrenoid) by its larger cell size when both species are cultured in freshwater medium, and with pyrenoid in chloroplast. In addition, *D. epilichicus* was isolated from Europe freshwater whereas FACHB-2327 was from Antarctica as lichen epiphytic.

Etymology: The species is named for the lichen species which the algae strain was isolated from.

Species	Collection No.	Origin	Habitat	GenBank No.	
				SSU rDNA	ITS rDNA
Desmococcus olivaceus	SAG 1.94	Antarctica	epilithic	MT078159	MT078159
D. olivaceus	SAG 63.90	—[†]	—	MT078160	MT078160
Deuterostichococcus allas	AB15.001B5	Antarctica	photobiont	—	MH670339
D. allas	AB15.003B3	Antarctica		—	MH670344
D. alpinus [‡]	FACHB-2327	Antarctica	epilithic	MW023948	MW023947
D. deasonii	SAG 2139	North America	soil	MT078164	MT078164
D. epilithicus	SAG 10.97	Europe	freshwater	MT078169	MT078169
D. lewinii	SAG 107.80	North America	unknown	MT078162	MT078162
	SAG 108.80	—	—	—	MT078163
Deuterostichococcus sp1.	MT27.1	Titulcia, Madrid, Spain	lichen *Buellia zoharyi*	—	OK636223
Deuterostichococcus sp1.	MT27.3	Titulcia, Madrid, Spain	lichen *Buellia zoharyi*	—	OK636227
Diplosphaera chodatii	SAG 2049	Asia	freshwater	MT078180	MT078180
D. chodatii	SAG 49.86	Europe	freshwater	MT078182	—
D. chodatii var. mucosa	SAG 48.86	Antarctica	soil	MT078178	MT078178
Protostichococcus edaphicus	SAG 2481	Europe	soil	MT078161	MT078161
Pseudostichococcus monallantoides	SAG 380-1	Europe	freshwater	KM020066	KM020066
P. monallantoides var. exiguus	SAG 379-4	North America	freshwater	MT078184	MT078184
Stichococcus bacillaris	SAG 379-1b	Europe	freshwater	AJ416107	AJ431678
S. bacillaris	SAG 379-2		freshwater	HE610125	HE610125
Tetratostichococcus jenerensis	SAG 2138	Asia	soil	MT078183	MT078183
Tritostichocuccus coniocybes	ST-8	Europe	epilithic	—	MT078175
T. coniocybes				—	MT078172
T. solitus	SAG 2406	Europe	freshwater	MT078171	—

Table 1. Sequences used in the present study. *Note*: [†]: "—" information unknown; [‡]: script in bold indicates the new species illustrated in current study.

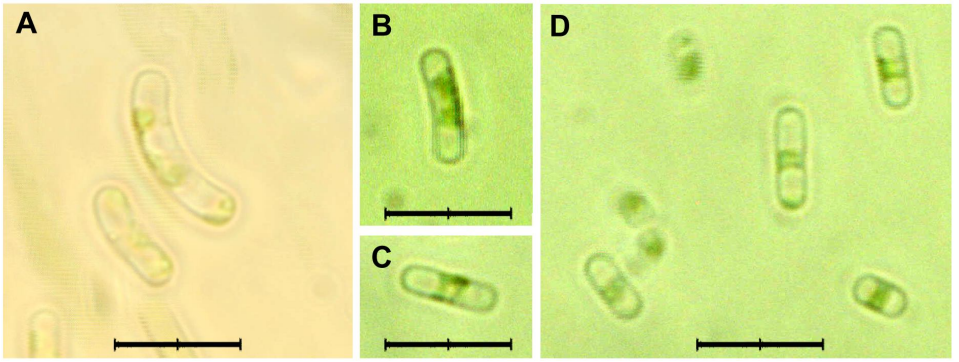

Fig. 1. *Deuterostichococcus alpinus* Shunan Cao & Qiming Zhou sp. nov., light microphotographs. Cells were cultured in BBM medium. *Scale bar*: 10 µm.

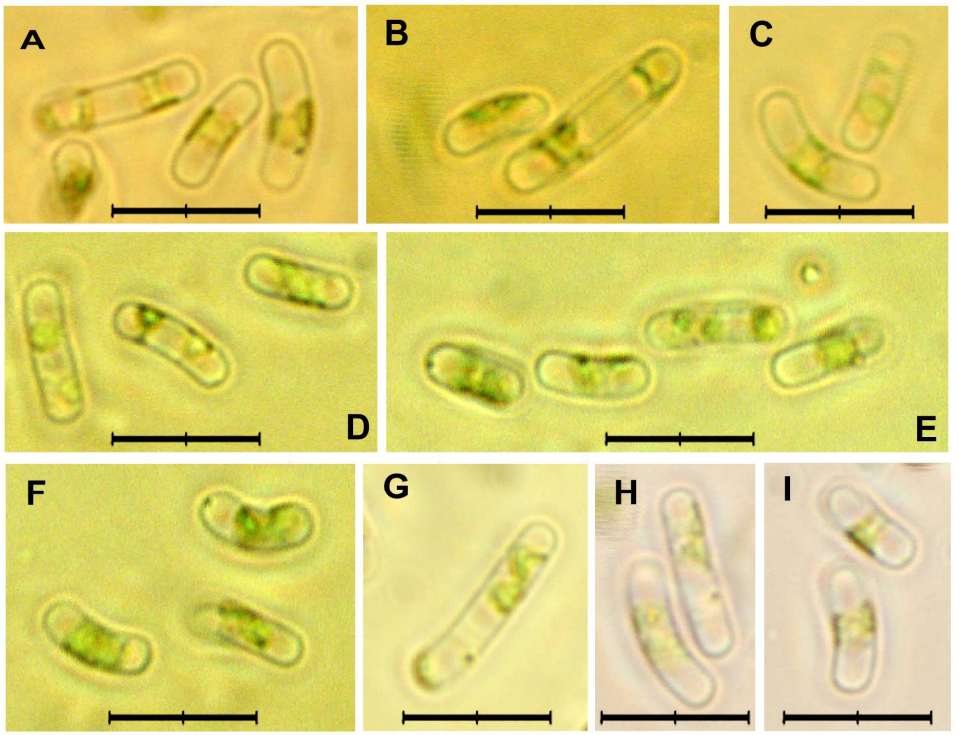

Fig. 2. *Deuterostichococcus alpinus* Shunan Cao & Qiming Zhou sp. nov. light microphotographs. Cells were cultured in PDA medium. *Scale bar*: 10 µm.

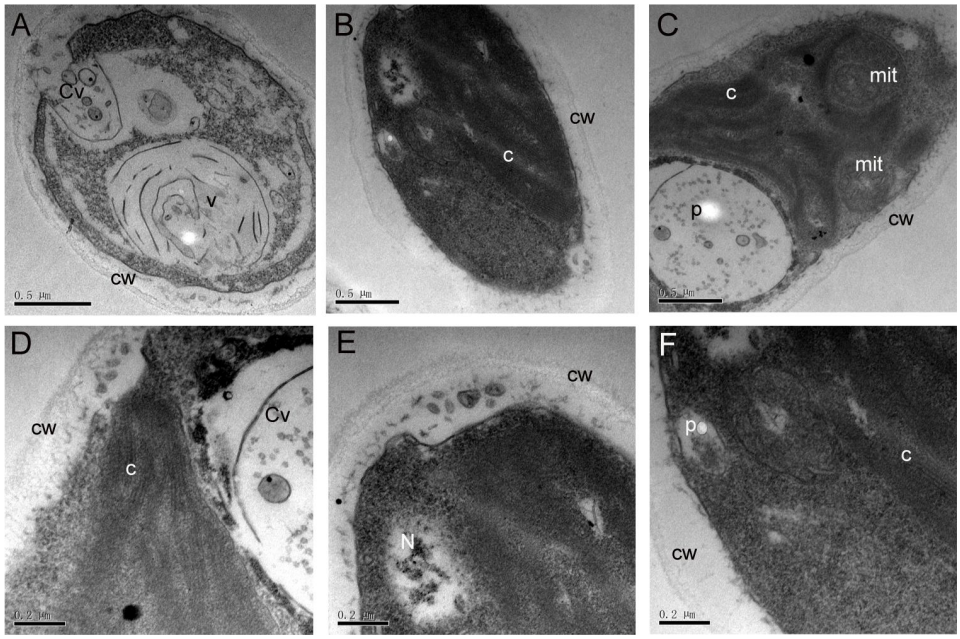

Fig. 3. Ultrastructure of *Deuterostichococcus alpinus* Shunan Cao & Qiming Zhou sp. nov. in PDA medium. *Abbreviations*: chloroplast (c); cell wall (cw); contractile vacuole (Cv); mitochondria (mit); nucleus (N); pyrienoid (p); vacuole (v)

Molecular analyses

The phylogenetic analysis of both ITS rDNA and SSU rDNA supported that the isolated alga FACHB-2327 was a species of the genus *Deuterostichococcus* new to science. In the ITS rDNA phylogenetic result, the alga FACHB-2327 clustered with the *Deuterostichococcus* species as a single group. Though the sequences of FACHB-2327 and *D. epilithicus* were closely clustered as one branch (Fig. 4A). For the SSU rDNA sequences, the sequences of *Deuterostichococcus* species clustered into a clade supported by bootstrap value 92. The alga FACHB-2327 and *D. epilithicus* was clustered into a subgroup but supported by low bootstrap value 54 (Fig. 4B), which indicated that these two species were insufficiently supported statistically.

In addition, the secondary structure of FACHB-2327 SSU rDNA V9 region in current study showed the same structure as D3 type of *Deuterostichococcus* which was calculated by Pröschold and Darienko (2020). This result confirmed that the isolated strain FACHB-2327 is one of the genus *Deuterostichococcus*.

Molecular and morphological analyses done within our study indicate that algal isolation FACHB-2327 represents a *Deuterostichococcus* species new to science, and we named this isolate as *D. alpinus* Shunan Cao & Qiming Zhou *sp. nov*.

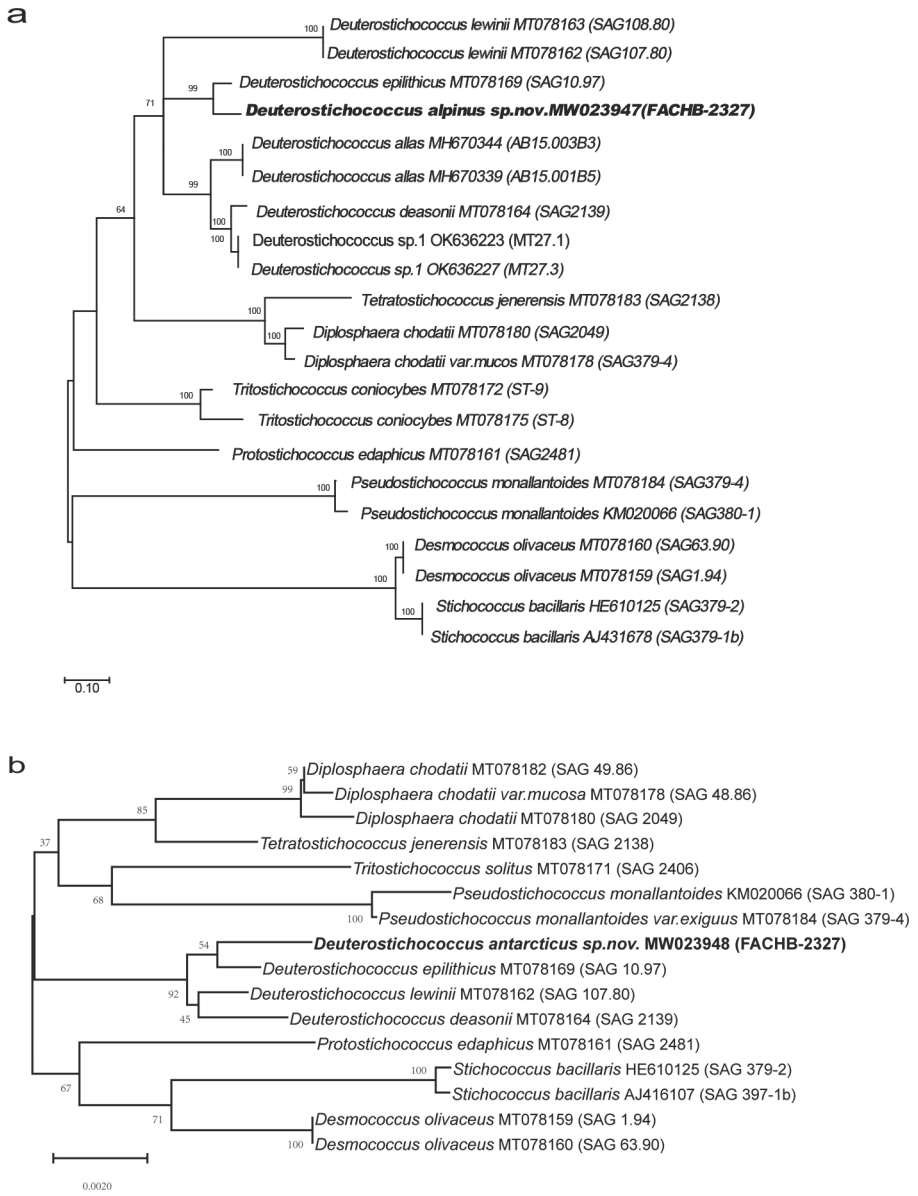

Fig. 4. The phylogenetic analyses of *Sticococcus*-like algae related to the genus *Deuterostichococcus*. **4A**: The ML tree based on ITS rDNA sequences; and **4B**: The ME tree based on SSU rDNA sequences. The sequences obtained from the current study were in bold. Bootstrap <50 was not shown.

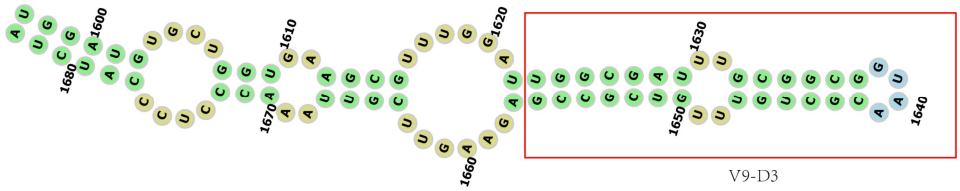

Fig. 5. Secondary structure of the V9 region (Helix49) of the SSU rDNA. The variable region within the V9 is highlighted in red square. The number indicated the deoxyribonucleotide location.

Discussion

The *Stichococcus*-like algae are common, and several classifications criteria including morphology traits and molecular features have been used to make progress in *Stichococcus*-like algae study single or combined. However, there are difficulties in classification and systematic study due to their simple and similar morphology. Furthermore, most of the recorded species are poorly described although more than 100 species names were listed in the literatures. Information on their morphology and ecology are rather limited (Guiry and Guiry 2019), INA[2] and rare cultural strains were obtained; and introns are common within the SSU rDNA sequences. This leads to higher difficulty in PCR analysis because more additional primer reactions are needed.

Recently, Pröschold and Darienko (2020) provided a clearl delimitation among the *Stichococcus*-like algae using a comprehensive analysis, and established a new genus *Deuterostichococcus* Pröschold and Darienko. There are four species originally included: *D. deasonii*, *D. allas*, *D. epilithicus* and *D. lewinii*. In the same year, the nomenclatural corrections were made that *D. deasonii* was the basionym of *D. marinus* (Deason) Pröschold and Darienko, *D. allas* was the heterotypic synonyms of *D. tetrallantoideus* (Kol) Pröschold and Darienko (Pröschold et al. 2020). Thus, the corrected names for the above reported species are *D. marinus* (which is the type species), *D. tetrallantoideus*, *D. epilithicus* and *D. lewinii*. They were reported for soil, snow, marine and fresh water, distributed in Europe, America, Canada *etc*. Furthermore, only *D. epilithicus* was recorded from red snow in King George Island, Antarctica.

The green alga FACHB-2327 isolated from Antarctic lichen *S. alpinum* is a newly described species of the genus *Deuterostichococcus*. Both the ITS rDNA and SSU rDNA phylogenetic analysis supported all the sequences of *Deuterostichococcus* as a monophyletic clade with boot strap value 71 and 92, respectively. The strain FACHB-2327 is most closely with *D. epilithicus* in phylogeny, whereas the supporting value is 99 of the ITS rDNA and 54 of the SSU rDNA phylogenetic analysis. The morphology of the strain FACHB-2327 differs from *D. epilithicus* in having pyrenoid in chloroplast, larger cell size and relatively higher length/width ratio (FACHB-2327: 7.0-18.0 × 3.0-5.0 µm, 2.3-3.6(4) *vs*. *D. deasonii*: 5.0-7.9 × 2.8-3.3 µm, data absent (Beck et al. 2019). Contrastingly, the strain FACHB-2327 differs from the other species in larger cell size and the relatively higher length/width ratio (*D. allas*: 3.5-12.0 × 1.5-3.0 µm, 1.0-4.0; *D. deasonii*: 5.0-7.9 × 2.8-3.3 µm, data absent; *D. lewinii*: 4.1-7.9 × 2.1-3.4 µm, 2.5) (Beck et al. 2019, Pröschold and Darienko 2020). Also, the second structure of SSU rDNA V9 region confirms it is the species of *Deuterostichococcus*, even though a spe-

cies *Deuterostichocuccus* sp.1 isolated from lichen *Buellia zoharyi*, significantly differed from the reported *Deuterostichocuccus* species (Chiva et al. 2022). We compared ITS rDNA sequences of *Deuterostichocuccus* sp.1 and the current algae species *D. alpinus*. The phylogenetic result confirmed that these are two species. The comprehensive study revealed that the strain FACHB-2327 is a newly described species of *Deuterostichococcus*.

Lichens are the typical symbiotic organisms mainly composed of fungi partner, the mycobiont, and algae/cyanobacteria partner, the photobiont. Moreover, there are algal species living with the lichen thalli, the epiphytic algae. It is well known that the photobionts in *Sterocaulon* Hoffm. are the species of green algal genus *Asterochloris* Tschermak-Woess (Kosecka et al. 2021, Vančurová et al. 2020) from Trebouxiaphyaece, and the most frequently observed photobiont is *A. phycobiontica* Tschermak-Woess (Vančurová et al. 2020). Thus, the current described algal species is the epiphytic algae of the lichen *S. alpinum*.

Conclusions

The green alga FACHB-2327 isolated from Antarctic lichen *S. alpinum* is new to science based on the comprehensive analysis. The green alga FACHB-2327 was described and named as *Deuterostichococcus alpinus* sp. nov. after the analyzing of morphology, ultrastructure, habitat, phylogeny and secondary structure of SSU rDNA V9 region.

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