**Coccomyxa greatwallensis** sp. nov. (Trebouxiophyceae, Chlorophyta), a lichen epiphytic alga from Fildes Peninsula, Antarctica

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**Abstract**

A single-celled green alga *Coccomyxa greatwallensis* Shunan Cao & Qiming Zhou, sp. nov., isolated from a specimen of Antarctic lichen *Poroma hypnorum* (Vahl) Gray, is described and illustrated based on a comprehensive investigation of morphology, ultrastructure, ecology and phylogeny. The cells of *C. greatwallensis* are ovoid to long ellipsoidal and measured 3–5 µm × 6–12 µm. The new species has distinct ITS rDNA and SSU rDNA sequences and differs from the phylogenetic closely related species *C. antarctica*, *C. arvernensis* and *C. viridis* in cell size, distribution and habitat.

**Keywords**

Lichen epiphyte, Morphology, TEM, Phylogeny
Introduction

The coccoid green algal genus *Coccomyxa* Schmidle (1901) is well known for its diversified ecological habitats and worldwide distribution. Algae of this genus has been reported as free living (Blanc et al. 2012), endophytic (Tremouillaux-Guiller et al. 2002; Zuykov et al. 2014) and lichen photobionts (Zoller and Lutzoni 2003; Muggi et al. 2010). *Coccomyxa* can survive under extremely harsh environments, such as in the spent fuel cooling pond of a nuclear reactor (Rivasseau et al. 2016), in a highly acidic lake (pH~2.6) (Hrdinka et al. 2013), as well as in polar regions (as low as -88 °C) (Blanc et al. 2012).

Based on the mucilaginous colonies’ structure, cell length and width variability details, Jaag (1933) delimited 33 species of this genus, including 14 free-living, 13 lichenised and 6 lichen epiphytic species. Subsequently, only seven species were well recognised based on some morphological characters, such as cell shape and size, chloroplasts numbers and mucilage properties (Ettl and Gärtner 1995). However, the morphological characters of *Coccomyxa* depend on the culture conditions, for example, salinity influenced the phenotypic plasticity significantly (Darienko et al. 2015) and nutrient availability influenced the presence of mucilaginous sheaths (Malavasi et al. 2016). As the instability of morphological features led to a problematic morphological delineation of the genus *Coccomyxa*, a DNA-barcode based method has been developed and seven distinct species were subdivided (Darienko et al. 2015). Malavasi et al. (2016), combining morphological characters, ecological features and DNA sequences of *Coccomyxa*, recognised 27 species scenarios. Subsequently, *Coccomyxa antarctica* Shunan Cao & Qiming Zhou, 2018 was described as a new epiphytic species living with lichen *Usnea aurantiacoatra* on King George Island (Cao et al. 2018).

Currently, 28 species scenarios have been accepted, amongst which *C. actinabiotos* Rivasseau, Farhi & Couté, 2016, *C. antarctica*, *C. avernensis* Jaag, 1933, *C. polymorpha* T. Darienko & T. Pröschold, 2015, *C. subellipsoidea* E. Acton, 1909 and undescribed *Coccomyxa* spp., belonging to Clade I, Clade KL and Clade N according Malavasi et al. (2016), are the eight epiphytic species scenarios. Meanwhile, the species *C. avernensis* and *C. subellipsoidea* are also reported as lichen photobionts. The other lichenised species scenarios include *C. dispar* Schmidle, 1901, *C. solorinae* Chodat, 1909, *C. viridis* Chodat, 1913 and *Coccomyxa* Clades A, D and F (Malavasi et al. 2016). The species *C. antarctica*, *C. dispar*, *C. subellipsoidea* and *C. simplex* Mainx, 1928 show the Antarctic distribution, amongst which *C. simplex* is the only free living one (Holm-Hansen 1964; Darienko et al. 2015; Borchhard et al. 2017; Cao et al. 2018).

The Fildes Peninsula undergoes a typical sub-Antarctic oceanic climate with relatively high precipitation (89%) with 56–64 mm rainfall, wind blowing from west through northwest with a speed of 6.8–7.4 m/s and the average temperature ranging from 0.5–1.8 °C in summer (Yang et al. 2013). About 127 lichen species have been recorded in Fildes Peninsula (http://www.aari.aq/KGI/Vegetation/lst_lichens.html). The lichen *Psoroma hypnorum* (Vahl) Gary, one of the four *Psoroma* spp. found in this region, is characterised by its squamulose thallus without secondary products, dull brown discs, apothecia margin without or with very short hairs (Øvstedal and Smith 2001). Both cyanobacteria and green algae have been reported as photosynthetic part-
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In the current study, a lichenicolous single cell green alga was isolated from *P. hypnorum*. Based on the comprehensive analysis approach, including morphology, ultrastructure, ecology and phylogeny, the green alga is demonstrated to be new to science.

**Material and methods**

**Isolation and culture**

The lichen specimen (collection No. 274) of *Psoroma hypnorum* was collected from Fildes Peninsula, King George Island, Antarctica (62°12.69’S, 58°55.70’W) during the 30th Chinese National Antarctic Research Expeditions in summertime (1 February 2014–15 March 2014). The specimen was kept in the Resource-sharing Platform of Polar Samples which includes samples of Biology, Ice-snow, Rock, Deep-space and Sediment (BIRDS ID 2131C0001ASBM100076) at 4 °C till the isolation was processed. A single algal cell was obtained following a modified aseptic isolation procedure (Cao et al. 2018). The isolations, cultured on a petri-dish with PDA and BBM medium in an illumination incubator (4 °C, 12 hr light/12 hr dark), were deposited in the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB) as an open collection (FACHB-2139).

**Light and electron microscopy**

For observing and photographing the algal cultures, compound microscopes Nikon Eclipse 80i and Nikon ACT-1 V2.70 were used. After fixing with 2.5% glutaraldehyde buffer, the algal cells were used for transmission electron microscopy (TEM). The procedures and reagents (including 2.5% glutaraldehyde buffer) used followed Cao et al. (2018). The 70 nm cell sections, cut by a Leica EM UC6 ultramicrotome and stained with 3% uranyl acetate and lead citrate, were observed using a Jeol JEM1230 transmission electron microscope at 80–120 kV. The micrographs were captured using iTEM software by an Olympus SIS VELETA CCD camera.

**DNA extraction, amplification, sequencing and analysis**

A modified CTAB method (Cao et al. 2015) was used to extract the alga genomic DNA. Primer pairs NS1, NS4; NS3, NS6; NS5, NS8 (White et al. 1990) and primer pair ITS5, O2 (Cao et al. 2015) were used to amplify the SSU rDNA and ITS rDNA, respectively. A 50 µl volume PCR reaction was selected, PCR application and products verification followed Cao et al. (2015) and double-stranded PCR products were sequenced by an ABI3730XL sequencer.
SEQMAN programme within Lasergene v.7.1 software (DNASTAR Inc.) was selected to check the double-directional ITS rDNA and SSU rDNA sequences. These two regions were overlapped into one single contig and the flanking regions were trimmed off. The sequence representing the new species was submitted to GenBank (MF465899).

ClustalW algorithm, including in MEGA 7 (Kumar et al. 2016), was performed to align the sequences with default parameters (Higgins et al. 1994) and then adjusted manually. The Neighbour-Joining (NJ) was selected to calculate the ITS phylogenetic structures.

Table 1. Coccomyxa spp. sequences used in the present study.

| Species                                      | Collection No. | ITS rDNA  | SSU rDNA |
|----------------------------------------------|----------------|-----------|-----------|
| Clade B* Coccomyxa sp.                      | GA5a           | AB917140  | AB917140  |
| Clade D* Coccomyxa sp.                      | CCAP 216/24    | FN298927  | FN298927  |
|                                              | CCAP 812/2A    | HG972992  | HG972992  |
| Clade E* Coccomyxa sp.                      | IB-GF-12       | –         | KM020052  |
| Clade E* Coccomyxa subellipsoidea           | CCAP 812/3     | HG972972  | HG972972  |
| Clade H* Coccomyxa sp.                      | KN-2011-U5     | HE856557  | –         |
| Clade I* Coccomyxa sp.                      | KN-2011-T3     | HE856515  | HE856515  |
|                                              | KN-2011-T1     | HE856550  | –         |
| Clade K* Coccomyxa sp.                      | KN-2011-C4     | HE856508  | HE856508  |
| Clade L* Monodus sp.                        | UTEx B SNO83   | –         | HE856506  |
| Clade M* Monodus sp.                        | CR2-4          | HE856519  | HE856519  |
| Clade N* Coccomyxa viridis 3                | CAUP H5103     | HG973007  | HG973007  |
|                                              | SAG 2040       | HG973004  | HG973004  |
| Coccomyxa actinabiots                       | 216-25         | FR850476  | FR850476  |
|                                              | KN-2011-T4     | HE856516  | HE856516  |
| Coccomyxa antarctica                       | FACHB-2140     | MF465900  | MF465900  |
| Coccomyxa arvernensis                       | SAG 216-1      | –         | HG972999  |
|                                              | Wien C19       | HG973000  | HG973000  |
| Coccomyxa dispar                            | SAG 49.84      | HG972998  | HG972998  |
| Coccomyxa elongata                          | CAUP H5107     | HG972981  | HG972981  |
|                                              | SAG 216-3b     | HG972980  | HG972980  |
| Coccomyxa galuniae                          | CCAP 211/97    | FN298928  | FN298928  |
|                                              | SAG 2253       | HG972996  | HG972996  |
| Coccomyxa greatwallensis sp. nov.           | FACHB-2139     | MF465899  | MF465899  |
| Coccomyxa melkonianii                       | SCCA048        | KU696488  | KU696488  |
| Coccomyxa onubensis                         | ACCV1          | HE617183  | HE617183  |
| Coccomyxa polymorpha                        | CAUP H5101     | HG972979  | HG972979  |
|                                              | KN-2011-T2     | HE856514  | HE856514  |
| Coccomyxa simplex                           | CAUP H 102     | HE856504  | HE856504  |
|                                              | SAG 216-2      | HG972989  | HG972989  |
| Coccomyxa solonraine                        | SAG 216-12     | HG972987  | HG972987  |
|                                              | SAG 216-6      | HG972988  | HG972988  |
| Coccomyxa subellipsoidea                    | SAG 216-7      | HG972976  | HG972976  |
|                                              | Wien C20       | HG972975  | HG972975  |
|                                              | CAUP H5105     | HG972974  | –         |
| Coccomyxa vinatzeri                         | ASIB V16       | HG972994  | HG972994  |
| Coccomyxa viridis                           | SAG 216-14     | HG973002  | HG973002  |
|                                              | SAG 216-4      | HG973001  | HG973001  |
| Elliptochloris bilobata                     | SAG 245.80     | HG972969  | HG972969  |
| Hemichloris antarctica                      | SAG 62.90      | HG972970  | HG972970  |

* Clades referred after Malavasi et al. (2016).
as well as Maximum Likelihood (ML) method for SSU sequences. Pairwise distances of ITS rDNA and SSU rDNA sequences were calculated using MEGA 7. A 1000 resamplings bootstrap was tested for the reliability of the inferred trees. In total, 42 sequences, which have been confirmed by Malavasi et al. (2016), were retrieved from GenBank (Table 1).

**Results**

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Figures 1, 2

**Holotype.** Strain FACHB-2139, Freshwater Algae Culture Collection, the Institute of Hydrobiology (FACHB-Collection) (Fig. 1a).

**Type locality.** Antarctic, Fildes Peninsula, on soil (62°12.69'S, 58°557.70'W), 40 m a.s.l.; isolated from the Antarctic lichen *Psoroma hypnorum* (collection No. 274, BIRDS ID: 2131C0001ASBM100076) on 14 February 2014.

**Habitat.** Epiphytic green alga, living with lichen *Psoroma hypnorum* in Sub-Antarctic climate.

**Description.** Single-celled green alga, ovoid to long ellipsoidal, asymmetrical, measured 3–5 µm × 6–12 µm, some cells nearly rounded in nutrient-rich PDA medi-

![Figure 1](image-url). *Coccomyxa greatwallensis* Shunan Cao & Qiming Zhou, sp. nov., light microphotographs. Cells cultured in BBM medium (**a, b**) and in PDA medium (**c, d**). Scale bar: 10 µm.
um; cells without mucilaginous sheath (Fig. 1). Cell wall smooth, three layers in ultrastructures. Protoplast filled with lipid droplets. Chloroplast parietal, without pyrenoid and with starch granules in the inter thylakoidal spaces. One nucleus in the central part of the cell present. Reproductive process not observed (Fig. 2).

**Molecular analyses**

The pairwise distance analysis of ITS rDNA sequences shows that the overall mean distance is $0.171 \pm 0.015$. The pairwise distance between our algal strain FACHB-2139 and the other species of *Coccomyxa* ranged from 0.253 to 0.022, of which *C. arvern-*
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Coccomyxa greatwallensis shows the minimum distance with our isolate of 0.022 followed by Coccomyxa sp. Clade N of 0.030 (Suppl. material 1: Table S1). The pairwise distance analysis of SSU rDNA sequences shows that the overall mean distance is 0.017±0.002. In addition, the pairwise distance between alga strain FACHB-2139 and the other species of Coccomyxa ranged from 0.025 to 0.001, amongst which both C. arvernensis and C. viridis show the minimum distance of 0.001 with our sample (Suppl. material 1: Table S1). That indicated that alga FACHB-2139 is closely related to C. arvernensis and C. viridis.

For the ITS rDNA, all the Coccomyxa sequences clustered into one group supported with bootstrap value 100 and within Coccomyxa, six subgroups have been clustered. The alga FACHB-2139 together with C. antarctica, C. arvernensis, C. viridis, Coccomyxa spp. of clade KL, Clade M and Clade N clustered as a subgroup, were supported with a bootstrap value 100; but the newly isolated strain FACHB-2139 differs from the other species clearly, no well supported clade for FACHB-2139 and species mentioned above were formed (Fig. 3a). In the SSU rDNA phylogenetic result, the sequences of Coccomyxa clustered into five subgroups and the alga FACHB-2139, C. antarctica, C. arvernensis, C. viridis, Coccomyxa spp. Clade K, Clade L, Clade M and Clade N lay in the same subgroup whose bootstrap value was 97. Though alga strain FACHB-2139 and C. arvernensis formed a monophyletic group, this clade was supported by a low bootstrap value 53, which indicated that these two species were insufficiently supported statistically (Fig. 3b).

**Figure 3.** The NJ tree based on ITS rDNA (a) and the ML tree based on SSU rDNA (b) sequences phylogenetic analyses. The sequences marked with Coccomyxa clade A–N referred after Malavasi et al. (2016).
Diagnosis

Morphologically, our sample FACHB-2139 can be distinguished from its phylogenetically close congeners *C. viridis* (1.8–3.6 µm × 4.7–8.4 µm) and *C. arvernensis* (3–4 µm × 6–8 µm) (Müller 2005, Hodač 2015) by its larger cells and from *C. antarctica* (4–7 µm × 8–12 µm) by its smaller cells (Cao et al. 2018). The cell sizes of the above species were recorded when cultured in BBM medium. In addition, both *C. viridis* and *C. arvernensis* are lichenised or are epiphytic species and have not been recorded as an Antarctica distribution.

Our molecular and morphological analyses indicate that algal isolate FACHB-2139 represents a new *Coccomyxa* species which we named *Coccomyxa greatwallensis* Shunan Cao & Qiming Zhou sp. nov.

Discussion

*Coccomyxa greatwallensis* Shunan Cao & Qiming Zhou sp. nov., isolated from Antarctic squamulose lichen *P. hypnorum*, is one of the *Coccomyxa* species, which is characterised by ovoid to ellipsoidal single cells. The usage of molecular barcode provides an effective and stable tool to identify and classify the species of *Coccomyxa* (Darienko et al. 2015; Malavasi et al. 2016). In the current study, both ITS and SSU rDNA were used and a comparison with the closely related species had been listed in Suppl. material 3:Table S3. The minimum pairwise distance was calculated between *C. arvernensis* and *C. greatwallensis* using ITS rDNA sequences, but the bootstrap value, which was lower than 50, did not support these two species as a monophyletic group. A similar result was also obtained using SSU rDNA sequences.

Though some *Coccomyxa* species could be the photosynthetic partner of lichens (Honegger and Brunner 1981), due to the lichen mycobiont’s selectivity to its photobiont partner, one photobiont group occurs within relative stable lichen groups (Tschermak-Woess 1988; Cao et al. 2015); for example, *Coccomyxa* is known as the photobiont of lichenised ascomycots belonging to Peltigerales (i.e. *Nephroma* Müll. Arg., *Peltigera* Willd. and *Solorina* Ach.), Baeomycetales (*Baeomyces* Pers., *Dibaeis* Clem., *Orceolina* Hertel and *Placynthiella* Gyeln.), Pertusariales (*Icmadophila* Trevis.), Agaricales (*Lichenomphalia* Redhead, Lutzoni, Moncalvo & Vilgalys), Lecanorales (*Micarea* Fr.) and Cantharellales (*Multiclavula* R.H. Petersen) (Poulsen et al. 2001; Smith et al. 2009; Wirth et al. 2013), as well as the basidiomycots belonging to Agaricales (*Omphalina* Quél.) (Jaag 1933; Zoller and Lutzoni 2003). In addition, *Coccomyxa* is optionally lichenised with the fungus *Schizoxylon albescens* Gildenstam, H. Döring & Wedin (Ostropales) (Muggi et al. 2010). Furthermore, there is also evidence to support the photosynthetic partner of Antarctic lichen *P. hypnorum* is cyanobacteria or the green algae *Myrmecia* Printz (Brodo et al. 2001; Øvstedal and Smith 2001; Wirtz et al. 2003; Ekman et al. 2014) but not the species of *Coccomyxa*. We therefore conclude that the newly described green alga *C. greatwallensis* is an epiphytic alga of lichen *P. hypnorum*. 
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Supplementary material I

Table S1. Pairwise distance calculated using ITS rDNA sequences
Authors: Shunan Cao, Fang Zhang, Hongyuan Zheng, Fang Peng, Chuanpeng Liu, Qiming Zhou
Data type: molecular data
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Link: https://doi.org/10.3897/phytokeys.110.26961.suppl1
Supplementary material 2

Table S2. Pairwise distance calculated using SSU rDNA sequences
Authors: Shunan Cao, Fang Zhang, Hongyuan Zheng, Fang Peng, Chuanpeng Liu, Qiming Zhou
Data type: molecular data
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Link: https://doi.org/10.3897/phytokeys.110.26961.suppl2

Supplementary material 3

Table S3. Comparison of the closely related species
Authors: Shunan Cao, Fang Zhang, Hongyuan Zheng, Fang Peng, Chuanpeng Liu, Qiming Zhou
Data type: species data
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Link: https://doi.org/10.3897/phytokeys.110.26961.suppl3