CHAMAESIPHON FONTINALIS SP. NOV., A NEW SPECIES OF UNICELLULAR ASYMMETRICALLY DIVIDING CYANOBACTERIA

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Extensive brownish biofilms of algal-bacterial communities were found on stones in streams in the Valdaysky National Park (Russia). The dominant unicellular cyanobacteria in biofilms was determined as *Chamaesiphon* sp. Although species of this genus often occur in the periphyton of fast-flowing waters, many of them have a geographically limited distribution and occur mainly in mountain streams and rivers in Europe. The strain *Chamaesiphon* sp. CALU 1864 was isolated from epilithic biofilms in the spring Tekunok. We found that it differs from previously described species not only by its unusual purple pigmentation, but also by morphological features. Molecular-genetic analysis of the primary 16S rRNA sequence and secondary structures of the internal transcribed spacer of the ribosomal operon also confirmed its uniqueness. Phylogenetically, the strain represents an independent lineage on the evolutionary tree. Based on the differences found, which provide strong support for its delineation, we propose to consider strain CALU 1864 as a new species, *Chamaesiphon fontinalis* sp. nov., first discovered in Russia and described in accordance with the current rules of the International Code of Nomenclature for Algae, Fungi and Plants.

Key words: Chamaesiphonaceae, morphotype, molecular-genetic features, polyphasic taxonomy, Protected Area

Introduction

Cyanobacteria form a morphologically heterogeneous group of photosynthetic bacteria, distributed in a variety of ecotopes (Komárek & Anagnostidis, 1998; Castenholz, 2015). Unicellular, asymmetrically dividing cyanobacteria, belonging to the genus *Chamaesiphon* Braun, are known as biofoulers, usually found epilithically (forming spots on stones) or epiphytically (as single cells or groups of cells attached to aquatic plants and filamentous algae) in freshwater springs, streams and ponds (Rabenhorst, 1865; Komárek & Anagnostidis, 1998; Hoffmann et al., 2005; Casamatta & Hašler, 2016). *Chamaesiphon* spp., together with the representatives of the genera *Geitleribactron*, *Clastidium*, and *Cyanophanon* (the last two have not yet been studied by molecular-genetic methods), constitute the family Chamaesiphonaceae Borzi (phylum Cyanobacteria Stanier ex Cavalier-Smith, class Cyanophyceae Schaffner, order Gomontiellales Strunecký & Mareš) (Hoffmann et al., 2005; Komárek et al., 2014; Guiry & Guiry, 2022; Strunecký et al., 2023). Species of the family Chamaesiphonaceae differ by cell shape and size, as well as in the type of colonies. In addition, cells of the same species have various morphotypes, which alternate depending on the stage of development: mature cells are heteropolar (have a distinct apical and basal parts) and form small rounded daughter cells (exospores) by means of asymmetric binary fission, also called budding (Herdman et al., 2015). Thus, the cells of *Chamaesiphon* spp. can vary from round to ovoid or pear-shaped (cylindrical) during the life cycle (Komárek & Anagnostidis, 1998).

In order to expand our knowledge about the biodiversity of algae in water bodies of the Protected Areas of the Northwest Russia, several field expeditions have been carried out in the Valdaysky National Park since 2012 (Smirnova, 2014, 2015, 2017; Smirnova & Beljakova, 2016). This Protected Area (total area is about 1585 km^2) is located on the conditional border of the subzones of southern taiga and coniferous-deciduous forests. In the Valdaysky National Park, there are 257 lakes (five are relatively large and the others are small) and many rivers belonging to the basins of the River Msta, River Volga, and River Western Dvina (Zapadnaya Dvina). The density of the river network is estimated at 0.80–0.98 km/km^2. Most of these rivers are small, either fast- or slow-flowing (with varying degrees of the trophic state), with abundant aquatic vegetation. The majority of the water bodies are slightly saline, hydrocarbonate (salinity does not exceed 200 mg/l), with a pH range of 6.8–8.3 (Efimova & Frolova, 2013). Rivers in the Valdaysky National Park are mainly fed by springs, in which we often found stones, cov-

ered by algal-bacterial communities in the form of brownish biofilms on their surface. In various samples collected in streams near the villages of Novotroitsy, Moiseevichi, and Russkie Novinki, we observed *Chamaesiphon* species that predominate among unicellular cyanobacteria, whereas *Heteroleibleinia* sp. (Geitler) Hoffmann, *Leptolyngbya crassior (*Skuja) Anagnostidis, *Microcoleus fonticola* (Kirchner) Strunecky, Komárek & Johansen, *Phormidium favosum* Gomont, and *Pseudanabaena minima* Anagnostidis are abundant among filamentous cyanobacteria, along with some eukaryotic microalgae (Smirnova, 2021).

Chamaesiphon species lead a benthic or substrate-attached lifestyle and prefer siliceous or carbonate running waters of mountain streams (e.g. in the Alps and Carpathians) (Rott, 2008; Sant'Anna et al., 2011; Cantonati et al., 2015; Gutowski et al., 2015; Casamatta & Hašler, 2016; Loza et al., 2013a, 2018; Kurmayer et al., 2018). They are often settled on wet rocky surfaces along river banks (Rott & Wehr, 2016; Aigner et al., 2018). Analysis of *Chamaesiphon* spp. records on 429 sampling sites over a 6-year observation period has led to the conclusion that *C. confervicola* Braun, *C. fuscus* (Rostafinski) Hansgirg, *C. incrustans* Grunow, *C. polymorphus* Geitler, and *C. starmachii* Kann can be considered as common species in periphyton of clear, fast-flowing waters in Germany (Gutowski et al., 2015). Therefore, *Chamaesiphon* species have been proposed for biomonitoring the ecological quality of streams and rivers in ac-

cordance with the European Water Framework Directive 2000/60/EC (Loza et al., 2013b; Gutowski et al., 2015). Representatives of the genus *Chamaesiphon* (about 40 species) are rare in Russia, and so far no new species have been discovered (Hollerbach et al., 1953; Melekhin et al., 2019). Moreover, only eight laboratory-cultivated strains (*C. subglobosus* PCC 7430, *C. minutus* PCC 6605 *C. polymorphus* CCALA 037, *C. polonicus* SAG 32.87, *C. geitleri* №1023, *C.* sp. PCC 8308, *C. investiens* UAM 386, and *C.* cf. *incrustans* №1036) are known to date (Herdman et al., 2015; Kurmayer et al., 2018).

The aim of this study is to describe a new species of *Chamaesiphon* found in the Valdaysky National Park by the polyphasic taxonomic approach (Komárek, 2016). For this purpose, a laboratory strain was obtained from a natural specimen; its morphology, physiology, pigment composition, and the molecular-genetic features (16S rRNA and secondary structures of the internal transcribed spacer sequences) were determined.

Material and Methods
Sampling site location
The *Chamaesiphon* strain CALU 1864 was isolated from epilithic biofilm samples collected from stones in the forest spring Tekunok, flowing into Lake Golova in the Valdaysky National Park (58.114919° N, 33.290215° E) (Fig. 1). The water temperature in spring depends on the season and ranges from 4.0°C to 9.5°C; the pH value is around 7.0.

Fig. 1. The location of Valdaysky National Park (a), spring Tekunok (b), and a view of brownish biofilms on stones in the sampling site (c). Scale bars: a – 100 km, b – 5 km, c – 25 cm.

Strain isolation

Biofilm samples were scraped off the stones and placed in 25-ml glass tubes with BG11 medium (Stanier et al., 1971), modified by replacing FeSO$_4$ for ferrous citrate and 5-fold reduction in NaNO$_3$. It also contained 250 mg × ml^{-1} cycloheximide (Oxoid, UK) to suppress eukaryotic microalgae growth. Samples were incubated under continuous illumination (10–15 µmol photons m^{-2} × s^{-1}) with a cool-white light lamp, at 12°C. Strain isolation was performed by transferring cells with the *Chamaesiphon* morphotype to Petri dishes containing 1% agarised BG11, using standard microbiological methods (Rippka et al., 1979). Cultivation was carried out by periodic re-inoculations from agarised to liquid BG11 medium in 25-ml glass tubes or in 50-ml Erlenmeyer flasks. After undergoing the axenisation procedure, the obtained strain *Chamaesiphon* was deposited in the collection of microorganisms as CALU 1864 (Saint Petersburg State University, Russia, https://researchpark.spbu.ru/en/collection-ccem-eng/1930-ccem-kollekciya-calu-eng), and was placed in the algal herbarium of Komarov Botanical Institute RAS (LE) as dry material.

To learn more about the strain physiology, cell growth curves were evaluated in batch liquid cultures incubated at various temperatures (7°C, 15°C, 25°C). Cells were mechanically suspended using a glass tube homogeniser and their density measured at 750 nm by BioSpectrometer (Eppendorf).

Morphological studies

Basic morphological features were analysed under a Leica DM 2500 microscope, equipped with a digital camera with the help of differential interference contrast. The average cell size was calculated by measuring about 100 cells of each morphotype. Transmission electron microscopy (TEM) was used to study the ultrastructure. Cell material was prepared as described by Mareš et al. (2019). Briefly, cells were fixed in 0.1-M Na-phosphate buffer (pH 7.0), with 2.5% glutaraldehyde for 2 h at 10°C and then post-fixed with 1.0% OsO$_4$ (at 10°C, 12 h). Fixed cells were washed with 0.15-M Na-phosphate buffer, then dehydrated in a series of ethanol (50–100%) and embedded in LR White resin according to the manufacturer's protocol (Sigma-Aldrich). Ultrathin sections of cells were made by Leica EM UC7 microtome. Sec-

tions were post-stained with 1% uranyl acetate and lead citrate according to Reynolds (1963). TEM microphotographs were taken with a JEM 1400 microscope at 80 kV. Cell structures and inclusions were determined as described by Stanier & Cohen-Bazire (1977).

Pigment analysis and chromatic adaptability

Cells were cultured in liquid BG11 under the previously described conditions for one month, followed by harvesting by centrifugation (5000 g, 10 min). Pigments were extracted from ~ 100 mg (wet weight) of cell pellets with 100% methanol at 10°C, 60 min. To remove large particles, the lysate was centrifuged (10 000 g, 20 min). The absorption spectra of the extracted pigments were recorded at 300–700 nm on a BioSpectrometer (Eppendorf).

Chromatic adaptation was studied by incubating liquid cultures under red or green Lee 108 and 124 light filters at maximum transmissions above 575 nm and at 475–560 nm, respectively. After two weeks, the cells were harvested by centrifugation (5000 g, 10 min). The phycobiliprotein (PBP) fraction was obtained from ~ 100 mg (wet weight) of pellet suspended in 500 µl of 0.1 M Na-phosphate buffer (pH = 7.0) by 3–4 repeated freeze/thaw cycles (from -18°C to 45°C). The resulting lysate was centrifuged (10 000 g, 20 min) to remove cell debris. The absorbance of the extracted PBPs was recorded at 300–700 nm on a BioSpectrometer (Eppendorf).

DNA isolation, PCR and sequencing conditions

Genomic DNA was obtained from harvested cell material (~ 200 mg of wet weight), washed twice with 10 mM TE buffer (Tris-HCl buffer, pH = 8.0 containing 1 mM Na$_2$-EDTA). DNA was extracted by CTAB (cetyltrimethylammonium bromide) according to the protocol of Doyle & Doyle (1990), and then treated with RNAse A (Thermo Fisher Scientific, Germany) at 37°C, 15 min.

PCR amplification of the nearly full-length 16S rRNA gene was performed using 27F/1492R primers (Lane, 1991) and the 16S-23S rRNA internal transcribed spacer (ITS) was amplified with 322F/340R primers (Iteman et al., 2000). PCR mixture (25 µl) contained 2.5 µl DreamTaq buffer (Thermo Fisher Scientific, Germany; 1 × commercial buffer with 20 mM MgCl$_2$), 0.75 µl dNTPs (10 mM each), 0.75 µl of each primer (10 µM each), 0.15 µl of DreamTaq polymerase, 15.35 µl sterile water, and 2.5 µl of DNA template (30 ng).

PCR-fragments were amplified on a T100 thermocycler (Bio-Rad, USA). The protocol included an initial denaturation at 95°C (3 min), followed by 35 cycles: 95°C, 30 s, annealing at 55°C, 30 s and elongation at 72°C, 1.3 min. PCR products and DNA sample were checked by gel electrophoresis on 1.0% (w/v) RA agarose (VWR Life Science, Amresco) in standard TBE (Tris-borate/EDTA) electrophoresis buffer, and visualised by staining with 0.05% ethidium bromide. PCR products were sequenced with the Big Dye Terminator v3.1 Cycle Sequencing kit on an ABI Prism 310 Genetic Analyser (Applied BioSystems) according to the manufacturer's instructions, at the Research Centre «Molecular and Cell Technologies» (Saint Petersburg State University, Russia).

16S rRNA and 16S-23S ITS analysis

A pairwise sequence similarity search performed using the BLASTn tool (https://blast.ncbi.nlm.nih.gov). The p-distance of 16S rRNA was determined using Mega 5.0 (Tamura et al., 2011), and calculated as follows:

$$Similarity = 100\% \times (1-p)$$

Multiple sequence alignment (ClustalW) and phylogeny reconstruction were obtained using the CIPRES Science Gateway ver. 3.3 (Miller et al., 2010). For 16S rRNA phylogeny, the GTR + I + G model of nucleotide substitution with the lowest Bayesian Information Criterion (BIC) score was selected by jModeltest 2 (Darriba et al., 2012). For the Bayesian Inference (BI) tree, two runs of four Markov chains were used until the standard deviation of the splitting frequency was less than 0.01 (diagnosis was calculated every 1000 generations). The initial 25% of the sampled trees were discarded as burn-in and the rest was used to calculate the posterior probabilities (Ronquist et al., 2012). A maximum likelihood (ML) tree was calculated in IQ-TREE v. 6.1, generating 1000 samples for ultrafast bootstrap and 1000 bootstrap replications (Trifinopoulos et al., 2016). The resulting trees were visualised in FigTree v.1.4. Evolutionary distances in the ML/BI tree were calculated, and bootstrap values below 50 and probabilities below 95 were omitted. ML and BI phylogenetic trees with similar topology involved 52 sequences, with a total of 1067 positions in the final dataset. The robustness of our phylogenetic findings was achieved through different tree reconstruction approaches.

The positions of the D1–D1′, B-box, V2, V3, and putative D5 regions of 16S-23S ITS were determined as previously described (Iteman et al., 2000; Johansen et al., 2011; Mühlsteinová et al., 2014). The Mfold web server was used to fold the helices with default settings (Zuker, 2003). tRNAs were found by the tRNAscan-SE 2.0 web server (Lowe & Chan, 2016).

Results and Discussion

A new microorganism belonging to the genus *Chamaesiphon* was discovered as a result of field research in the Valdaysky National Park. The strain differs from the currently described species by morphological features, such as the size and shape of pseudosporangia and an unusual crimson-brown colour of the cells. The evolutionary divergence of the strains was verified by phylogenetic analysis. The relatively low similarity of the 16S rRNA gene with other *Chamaesiphon* species and the specific organisation of secondary structures 16S-23S ITS regions confirm its uniqueness. On this basis, we propose that strain CALU 1864 to be considered as a new species, described in accordance with the rules of the International Code of Nomenclature for algae, fungi, and plants (Turland et al., 2018).

Taxonomic treatment

Phylum Cyanobacteria Stanier ex Cavalier-Smith, 2002

Class Cyanophyceae Schaffner, 1909

Order Gomontiellales Strunecký & Mareš, 2023

Genus: *Chamaesiphon* Braun, 1864

Description of the new species Chamaesiphon fontinalis Velichko, Makeeva, Averina & Smirnova

Diagnosis. In nature it occurs in extensive brownish biofilms on the upper surface of stones in a cold stream (spring). The laboratory strain in liquid medium forms a dense dark-crimson or reddish-brown biofilm on the walls and bottom of glassware, or grows as convex, round-lobed, brown colonies on solidified medium (Fig. 2).

Cell size and shape depend on the stage of the life cycle: unequal binary fission usually occurs near the apical pole of the cell and results in 1–2 (rarely 3) small rounded exospores. The cells can vary from round or ovoid to pear-shaped or cylindrical. Cells are 6.6–16.7(25.2) × 5.5–8.2(11.1) μm in size with a length-to-width ratio close to 2 : 1. They have a thick (up to 2 μm), colourless U-shaped pseudovagina (a multilayered extracellular sheath). After cell division, the pseudovagina delaminates at the apex, releasing exospores.
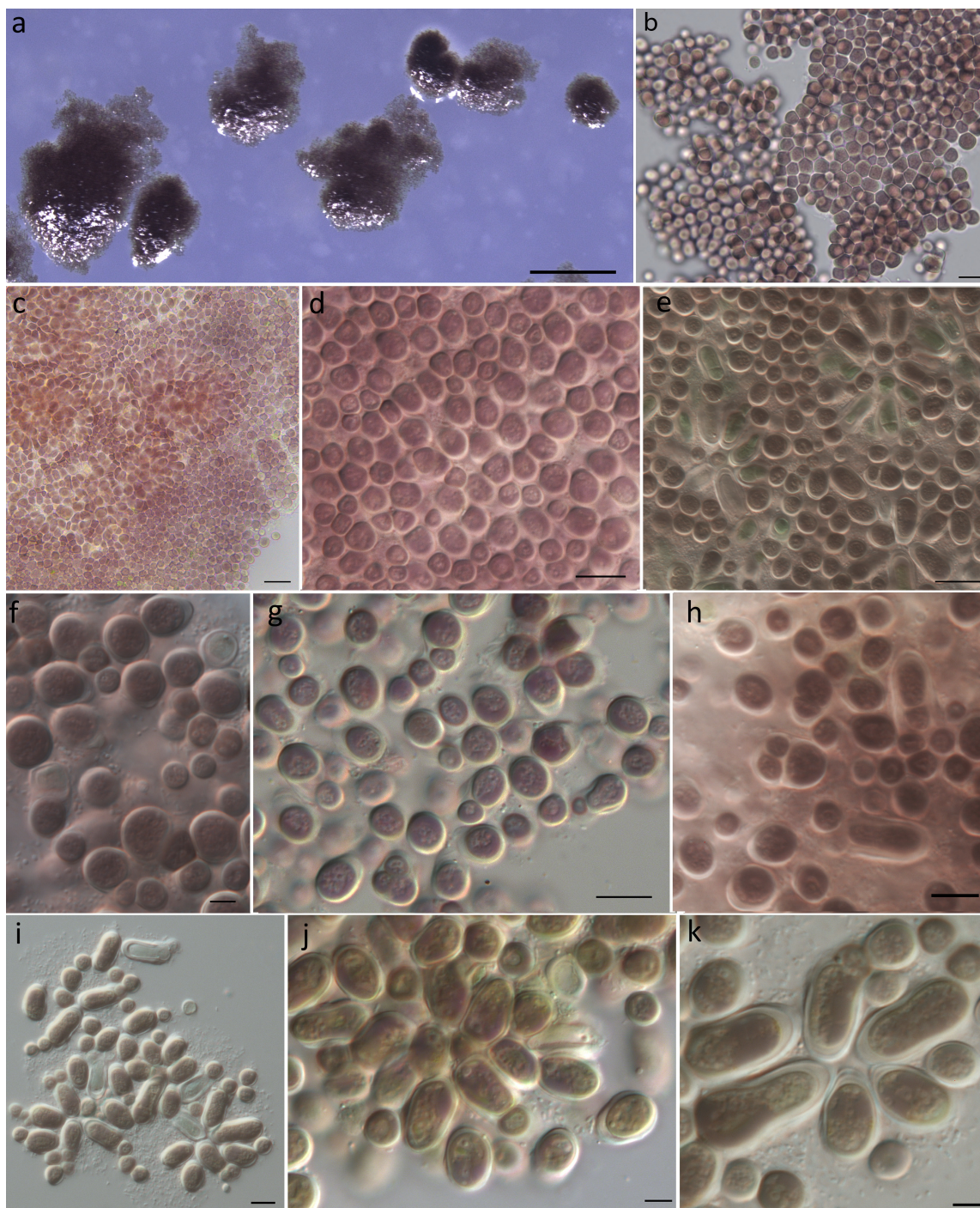
Fig. 2. Morphology of *Chamaesiphon fontinalis* sp. nov. (light micrographs). Scale bars: 500 μm (a), 20 μm (c), 10 μm (b, d, e, g, i), 5 μm (f, h, j, k).

Cell contents are heterogeneous, brownish-crimson in colour, with prominent cytoplasmic granules. Exospores are about 3 μm in diameter; in the case of a large mother cell, they can reach 6 μm. The cells showed parietal arrangement of thylakoids (3–5 layers) and the presence of carboxysomes, polyphosphates, and glycogen inclusions.

Type material. Holotype: herbarium material of *Chamaesiphon* strain CALU 1864 (dry biomass) is stored in the Herbarium of Komarov Botanical Institute RAS (LE), Saint Petersburg, Russia (LE A0002381).

Type locality. Biofilms occur on the surface of the upper part of stones in spring Tekunok, located in the Valdaysky National Park (58.114919° N, 33.290215° E), Novgorod Region, Russia.

Reference strain. *Chamaesiphon fontinalis* strain CALU 1864 was deposited in the collection of cyanobacteria and algae of the Saint Petersburg State University, Saint Petersburg, Russia. GenBank accession numbers: OP415410 for 16S rRNA and OP429223 for 16S-23S ITS.

Isotypes. These are LE A0003534, LE A0003535 and LE A0003536 herbarium material.

Etymology. The choice of the specific epithet for *C. fontinalis* sp. nov. is based on the fact that the species was found in a cold stream (spring) (L. masc. adj. fon.ti.na′lis, L. masc. nom. *fons*, originating from spring).

Ecology. It was found only in the type habitat in epilithic periphyton. The water temperature in the spring on the sampling day was 9.0°C, and the pH was about 7.0.

Differential diagnosis

The new species can be clearly distinguished from the type species, *C. confervicola*, by cell morphology and habitat type. *Chamaesiphon confervicola* is an epiphyte of aquatic plants and green filamentous algae *Cladophora* Kützing and *Oedogonium* Link ex Hirn. It usually grows as single cells or cells in groups, and has large elongated cells up to 200 µm in length (Komárek & Anagnostidis, 1998). Chamaesiphon confervicola has been described only morphologically so far, and we have no data on its successful cultivation.

Chamaesiphon fontinalis sp. nov. has similar morphology with *C. geitleri* Luther and *C. fuscus* (Rostafinski) Hansgirg: oval, oval-elongated or cylindrical pseudosporangium, with a length to width ratio of 2 : 1. But the new species differs from them in the type of colonies (not bushy-like), the size of the pseudosporangium (it is on average smaller), and the shape (rarely club-shaped cell), the cell colour (brownish-red, rather than pale) and the colour of the pseudovagina (colourless, in contrast to brownish or blackish). The morphospecies *C. starmachii* is the most similar to *C. fontinalis* sp. nov. It also has a pear-shaped or ellipsoidal type of pseudosporangium, similar cell size (6–18 × 4.0–7.2 µm), a reddish-violet or purple colour of the cell content and prominent granules in the cytoplasm. The exospores are more or less rounded-polygonal, may remain attached 1–3 to the mother cell when the sheath is closed (Komárek & Anagnostidis, 1998). However, *C. starmachii* differs from *C. fontinalis* sp. nov. by a thicker black pseudovagina (Kurmayer et al., 2018). Notably, the dark pigmented coatings of *C. geitleri* and *C. starmachii* are thought to be due to the presence of UV-protectors such as scytonemin (Aigner et al., 2018).

Morphology and culture properties

Chamaesiphon fontinalis sp. nov. forms convex, round-lobed, slightly slimy, brown colonies of 1–2 mm in diameter that exhibit the ability to grow towards a light source under laboratory conditions on solidified medium (Fig. 2a). In a liquid unstirred culture, strain CALU 1864 grows on the walls of glassware and forms a brownish or dark crimson biofilm, in which cells are arranged irregularly in 1–3 layers (Fig. 2b,c,d) or can form rosette-like structures (Fig. 2e). They are tightly pressed together in the biofilm and have a polygonal shape when viewed from above (Fig. 2b). It is noteworthy that strains of *C. subglobosus* PCC 7430 and *C. polonicus* SAG 32.87 grow in liquid cultures in irregular groups, and are unable to form colonies, as in nature (Waterbury & Stanier, 1977; Komárek & Anagnostidis, 1998; Kurmayer et al., 2018).

Morphological features such as cell size and shape are very important for distinguishing species of the genus *Chamaesiphon*, but they change during the life cycle. In this regard, the laboratory cultivation is therefore of great importance. Our long-term observations have shown that the shape and size of pseudosporangia of *C. fontinalis* sp. nov. depend on the age of the studied culture. In young cultures (up to 4–6 months), cells are round or almost ovoid (6.6–8.6 µm in length and 5.5–7.1 µm in width; the average length to width ratio is 1.4 : 1.0) and covered with a colourless sheath (Fig. 2f,g,h). It is noteworthy that, unlike novel strain, *C. subglobosus* PCC 7430, *C. minutus* PCC 6605, and *C.* cf. *incrustans* №1036 lost their sheaths under laboratory conditions (Herdman et al., 2015; Kurmayer et al., 2018). In old cultures (over six months), most cells of *C. fontinalis* sp. nov. are pear-shaped, club-shaped or elongated (8.0–16.7 µm in length, 6.0–8.2 µm in width; average length to width ratio is 2 : 1) and have an extracellular coating (Fig. 2i,j,k). In mature cells, the sheath laminates at the apical pole after budding to release exospores. Unequal cell division leads to the formation of 1–2 (rarely 3) small, rounded exospores, which can be arranged in short rows (Fig. 2i) or lie in pairs inside a closed pseudovagina (Fig. 2g,h).

The ultrastructure of *C. fontinalis* sp. nov. is generally similar to other studied *Chamaesiphon* species (Waterbury & Stanier, 1977; Gromov & Mamkaeva, 1980; Komárek & Ludvik, 1982; Wujek & Gretz, 1984; Kurmayer et al., 2018) (Fig. 3). The cell wall on TEM micrographs consists of three layers, typical for cyanobacteria: the cytoplasmic membrane (CM), the outer membrane (OM) and the thick (up to 30–40 nm) peptidoglycan layer (PG) (Fig. 3i).
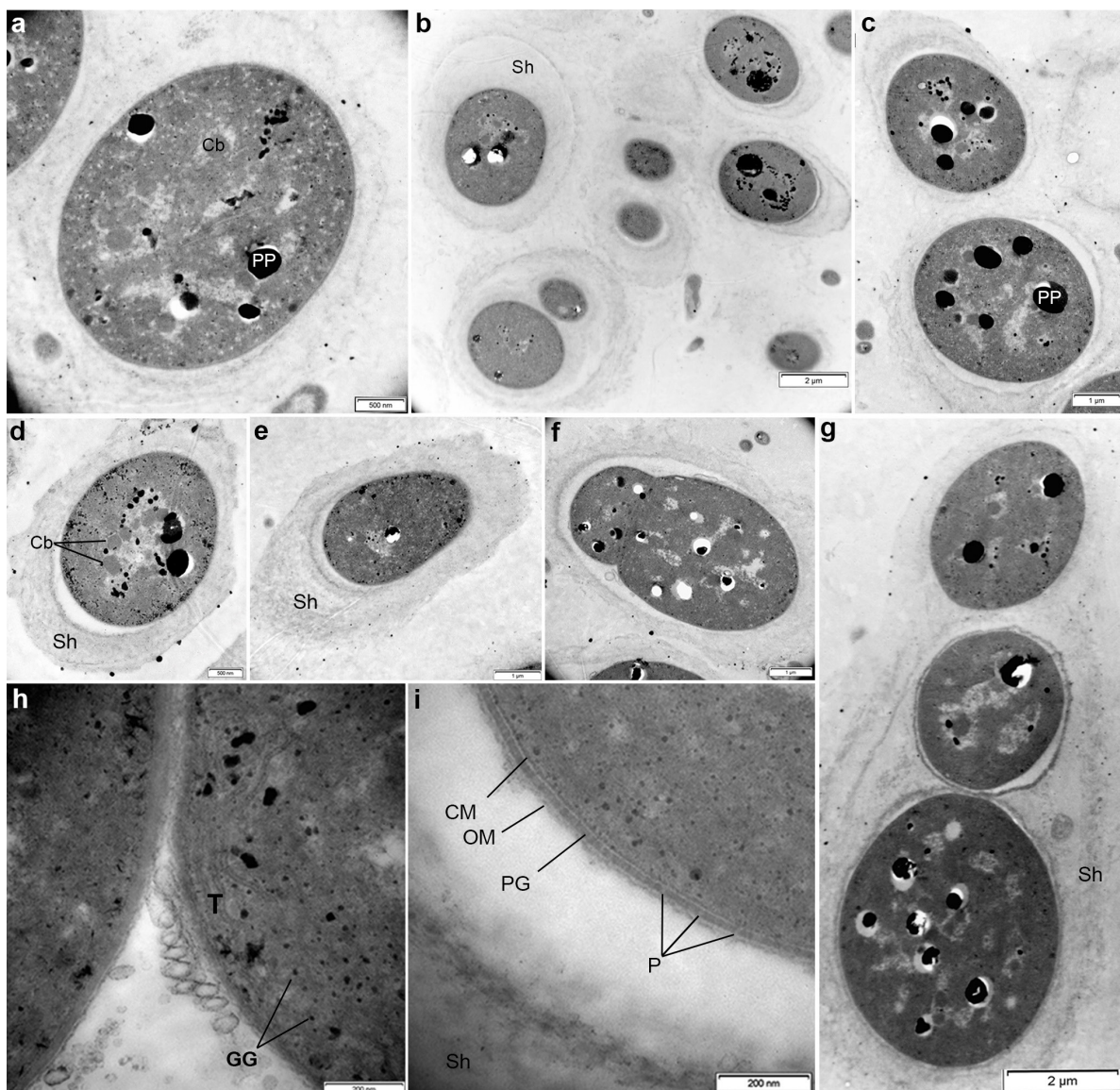
Fig. 3. Ultrastructure of *Chamaesiphon fontinalis* sp. nov. Designations: Cb – carboxysomes, CM – cytoplasmic membrane, GG – glycogen granules, OM – outer membrane, P – pores, PG – peptidoglycan layer, PP – polyphosphate granule, Sh – multilayered sheath, T – thylakoid. Scale bars: 2 µm (b, g), 1 µm (c, e, f), 500 nm (a, d), 200 nm (h, i).

Numerous pores penetrating the cell wall through the peptidoglycan layer were found in the basal part of the cell and in the area of cell division (Fig 3i). Mucilaginous sheath (pseudovagina) has a clearly defined layered structure (Fig. 3). Cells have 3–5 layers of thylakoids arranged at the periphery parallel to the cytoplasmic membrane, but 1–2 layers may invaginate into the central cytoplasmic zone. Cells usually contain 3–6 polyhedral bodies (carboxysomes) about 200 nm in size (Fig. 3a,d,g), small glycogen granules (10–20 nm, repeatedly occurring among thylakoids) (Fig. 3h,i), and 5–10 rounded polyphosphate inclusions (large black spots, up to 500 µm in diameter) (Fig. 3a,b,c,d,f,g).

In strain CALU 1864 cell division occurs, as in other *Chamaesiphon* spp. (Waterbury & Stanier, 1977; Komárek & Ludvik, 1982; Wujek & Gretz,

1984). Septate and constrictive binary fission proceeds asymmetrically: it initiates at the apical pole of the mother cell and begins with ingrowth from the cell wall and build-up of the peptidoglycan septa (Fig. 3f,h). This leads to an unequal division and the formation of exospores (Fig. 3g).

Chamaesiphon fontinalis sp. nov. grows very slowly under laboratory conditions and has a delayed initial growth phase (Fig. 4). Intensive growth was observed after 30–35 days of cultivation in liquid medium at 7°C and 15°C, while at 25°C cells do not remain viable for a long time. This indicates that the strain has a temperature optimum of growth below 15°C, as observed in psychrophilic forms of bacteria (Morita, 1975; Nadeau & Castenholz, 2000). Psychrophilic cyanobacteria are known to be much less common than psychrotolerant ones, possibly due to the poor nutritional adaptability or specificity of

regulatory processes (Morita, 1975; Wynn-Williams, 1990; Nadeau & Castenholz, 2000).

Pigment composition and complementary chromatic adaptation

The pigment composition of *C. fontinalis* sp. nov. is similar to cyanobacteria in general (Herdman et al., 2015; Aigner et al., 2018). Spectrometric characterisations of whole cells and methanol extracts are shown in Fig. 5a. In the absorption spectrum of whole cells, the peaks at 450 nm and 675 nm correspond to chlorophyll *a*, and there are also peaks of some carotenoids (475–525 nm). Among phycobiliproteins (PBPs), C-phycoerythrin (C-PE, peak 525–560 nm) and C-phycocyanin (PC, peak 625–630 nm) were identified. An additional shoulder in the region of 570–575 nm may probably belong to a special spectral form of PE previously identified in *Gloeothece* sp. PCC 6909 and *Oscillatoria* sp. PCC 7823 or it can be considered as some phycoerythrocyanine (PEC, peak 570–595) (Bryant, 1982). Under low light conditions (10–15 µmol m^{-2} × s^{-1}, cool-white lamp), strain CALU 1864 has a purple to dark crimson colour and is relatively rich in PEC (data are not shown). At high levels of irradiation (30–40 µmol m^{-2} × s^{-1}, cool-white lamp), the strain exhibited photosensitivity, which led to a colour change from intense red-violet to yellowish-green.

Cyanobacteria are known to be able to change the composition of PBPs in light-harvesting antennae (phycobilisomes) in response to changes of light spectral composition (green or red light). This phenomenon is called complementary chromatic adaptation (CA) (Tandeau de Marsac, 1977). Water-soluble cells extracts of *C. fontinalis* sp. nov., grown under green or red light, were analysed to determine capability to CA. We have established that *Chamaesiphon fontinalis* sp. nov. can only regulate PE content (decreases PE, but does not increase PC, when exposed to red light) (Fig. 5b). This fact indicates the CA type 2 (Tandeau de Marsac, 1977; Hirose at al., 2015). In contrast, the reference strains *Chamaesiphon subglobosus* PCC 7430 and *Chamaesiphon minutus* PCC 6605 exhibit type 1 and type 3 of chromatic adaptation, respectively (Herdman et al., 2015).

Molecular phylogeny assessment

Phylogenetic relationships were reconstructed using the nearly full-length (1403 nt) 16S rRNA gene sequence of *C. fontinalis* sp. nov., all cultured strains of *Chamaesiphon* spp. and reference strains of cyanobacteria (Fig. 6). *Chamaesiphon fontinalis* sp. nov. represents the separate phylogenetic lineage within the cluster «*Chamaesiphon*» (see Fig. 6). Members of the family Gomontiellaceae Elenkin ex Geitler, 1942, in particular, *Hormoscilla pringsheimii* Anagnostidis, Komárek and *Crinalium epipsammum* de Winder, Stal & Mur are the closest neighbours of this cluster (Kurmayer et al., 2018).
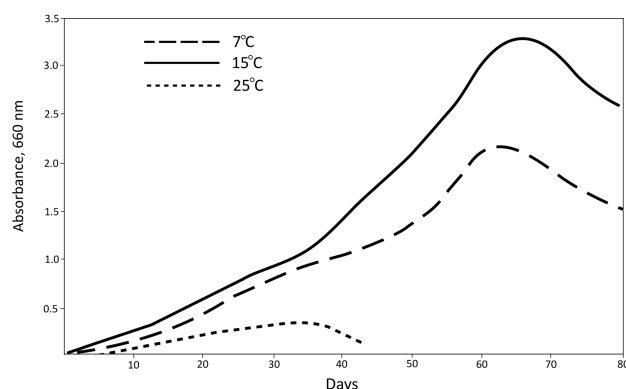

Fig. 4. Culture growth curves of *Chamaesiphon fontinalis* sp. nov.
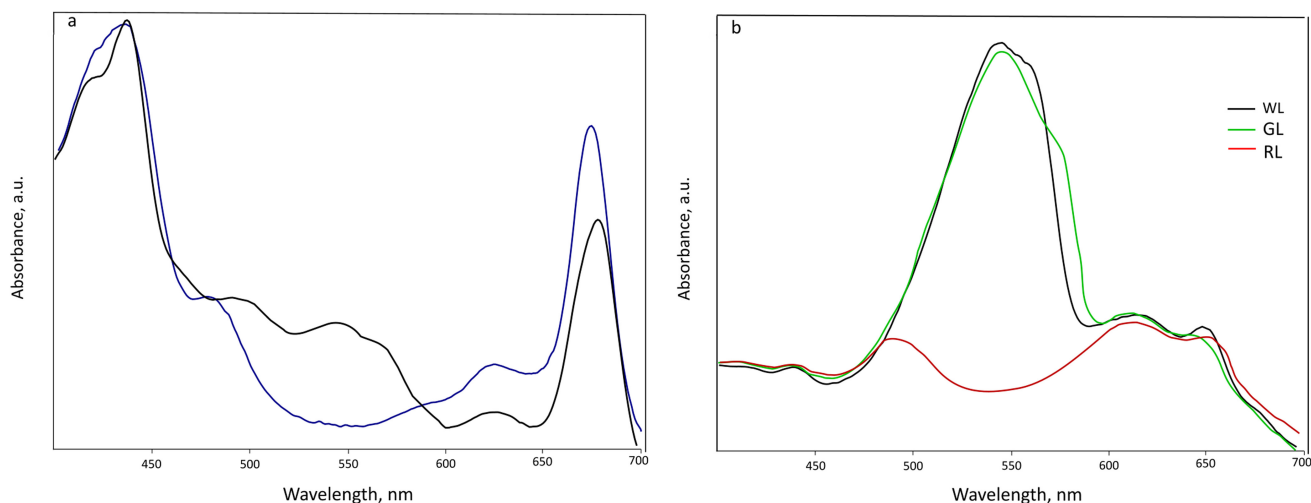

Fig. 5. Absorption spectra of whole cells (black line) and methanol extract (blue line) of *Chamaesiphon fontinalis* sp. nov., grown under white light (WL) conditions (a). Absorption spectra of the water-soluble pigment fraction of the strain grown under white light (WL, black line), green light (GL, green line), and red light (RL, red line) (b).
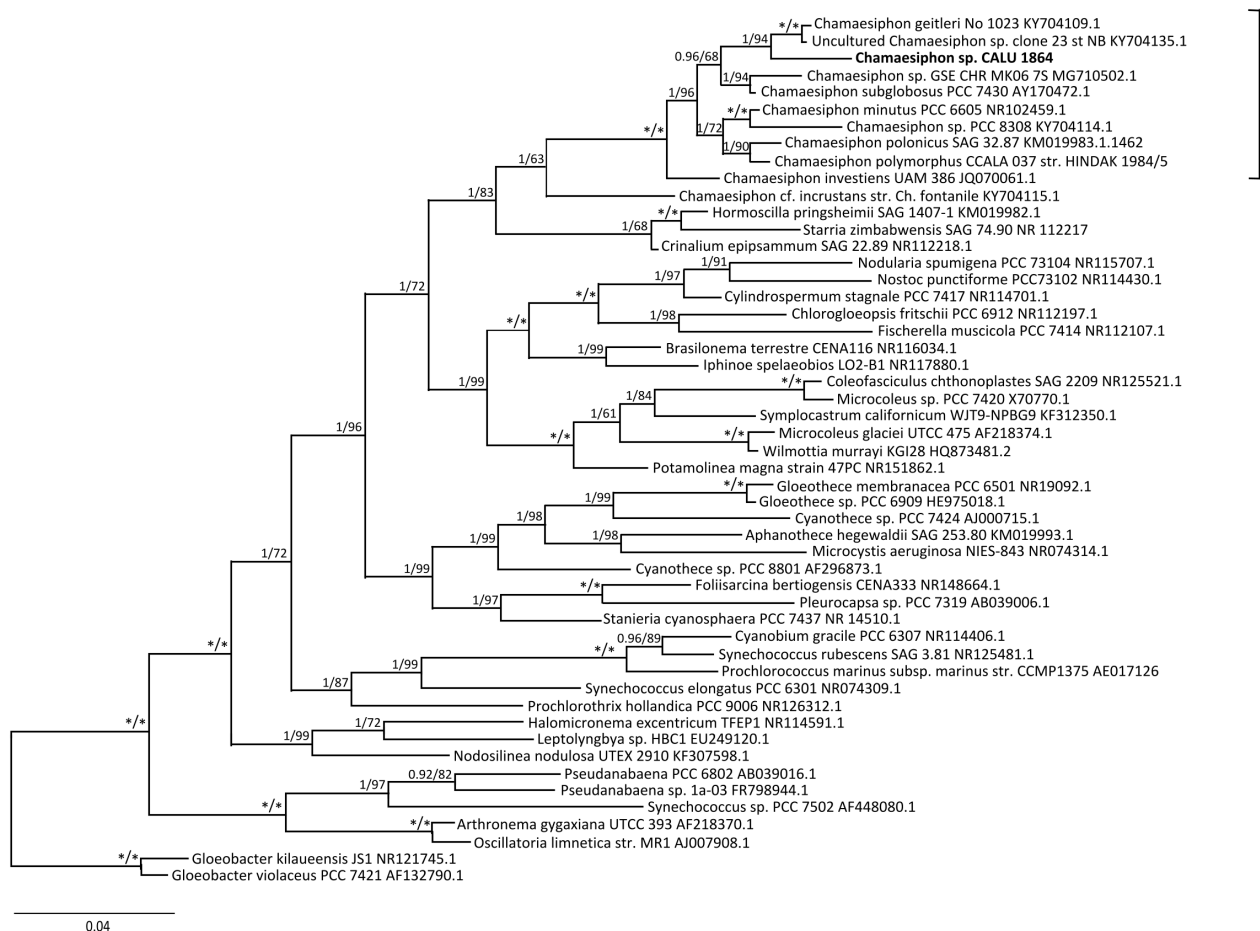
Fig. 6. Maximum likelihood tree based on 16S rRNA gene sequences showing the phylogenetic position of strain CALU 1864. Numbers at nodes indicate posterior probability (> 0.50) and bootstrap values (> 50%) obtained from Bayesian Inference (BI) and maximum likelihood (ML) analyses, respectively. Support values are given in brackets after each clade in the following order: BI/ML at the same node. Asterisks (*) indicate maximum support values (posterior probability of 1.0 and bootstrap values of 100%).

The BLASTn search results that *C. fontinalis* sp. nov. has the highest similarity (98.0–98.2%) with the 16S rRNA sequences of uncultured cyanobacteria found in various geographic regions, including the Antarctica. The greatest resemblance (98.2%) has the uncultured PCR clone st_23 NB (KY704135.1), derived from a single colony isolated from epilithic biofilm found in the stream Nederbach, Austrian Alps, and attributed to *C. starmachii*, based on morphological features (Kurmayer et al., 2018). The 16S rRNA similarity of *C. fontinalis* sp. nov. with other *Chamaesiphon* spp. ranges from ~ 93.0% to 98.2% (Table 1). In accordance with the established level of 16S rRNA similarity of 98.65% as a criterion for separating a new bacterial species (Kim et al., 2014), and taking into account the distinct phylogenetic position of the strain CALU 1864, we define it as a new *Chamaesiphon* species.

Intraspecific analysis of p-distances of 16S rRNAs within the *Chamaesiphon* spp. showed that *C. fontinalis* sp. nov. has the highest similarity with *C. geitleri* №1023 (98.2%), and the least with *Chamaesiphon* cf. *incrustans* №1036 (92.5%). The last one groups separately from all sequences of *Chamaesiphon* spp. and probably belongs to a different genus. In this case, maximum level of 16S rRNA species dissimilarity for the remaining strains of the monophyletic cluster «*Chamaesiphon*» is 7.2%. The *Geitleribactron purpureum* Tovel-4 strain was also taken for 16S rRNAs pairwise comparison to show the genetic distance (mean 10.65%) between genera of Chamaesiphonaceae. *Geitleribactron purpureum* was previously found to cluster distantly from all *Chamaesiphon* spp. (Mareš & Cantonati, 2016).

16S-23S ITS secondary structure evaluation
The secondary structures of 16S-23S ITS regions are very useful for comparative taxonomy at the intrageneric level and can provide insight

into whether a taxon belongs to a new species (Iteman et al., 2000; Johansen et al., 2011; Perona et al., 2022). We compared the secondary structures of conserved and variable regions of 16S-23S ITS in *Chamaesiphon* strains. The total length of the ITS region was found to be the shortest for *C. fontinalis* sp. nov. (567 nt) and the longest for *C. polymorphus* CCALA 037 (632 nt) (Table 2).

Nucleotide sequences and secondary structures of 16S-23S ITS (domains D1–D1′, B-box, and V2) of *C. fontinalis* sp. nov. differ considerably from other species (Fig. 7). It should be noted that all 16S-23S ITS of *Chamaesiphon* spp., including the strain CALU 1864, had alternative conserved sequences of D2 (CTTTCAAAC<u>TT</u> instead of CTTTCAAAC<u>TA</u>) and A-box (G<u>C</u>ACCTTGA<u>C</u>AA instead of G<u>A</u>ACCTTGA<u>A</u>AA), previously described in Iteman et al. (2000). The V2 domain and pre-B-box spacer differ in length in *Chamaesiphon* strains. The V2 region located between tRNA^{Ile} and tRNA^{A1A} varied from 57 nt to 71 nt in length, while the pre-B box spacer varied from 49 nt to 62 nt in length (Table 2). The B-box domain has common structure, but different nucleotide composition in the terminal and basal internal loops in all studied *Chamaesiphon* species (Fig. 7). *Chamaesiphon fontinalis* sp. nov. and PCR clone 23_st_NB have similar V2 structures (except for CUU/UUC inversion, marked by circles in Fig. 7), but quite different in helices from other species (Fig. 7). In all *Chamaesiphon* spp. we analysed, tRNA^{Ala} have a common structure, despite some substitutions in the T-loop and T-stem. At the same time, the tRNA^{Ile} in *C. fontinalis* sp. nov., PCR clone 23_st_NB and *C. geitleri* №1023 is two nucleotides shorter (72 nt) than in other strains (74 nt) (Table 2).

Table 1. The percentage of similarity (p-distance) matrix of *Chamaesiphon* and *Geitleribactron* strains based on partial 16S rRNA sequences (1207 nt)

	Strain	1	2	3	4	5	6	7	8	9	10
1	*Chamaesiphon fontinalis* sp. nov. strain CALU 1864 (OP415410)	100									
2	*Chamaesiphon* cf. *incrustans* No 1036 (KY704115.1)	92.5	100								
3	*Chamaesiphon geitleri* No 1023 (KY704109.1)	98.2	93.7	100							
4	*Chamaesiphon investiens* UAM 386 (JQ070061.1)	96.8	93.2	97.2	100						
5	*Chamaesiphon minutus* PCC 6605 (KY704112.1)	96.9	92.8	97.4	98.3	100					
6	*Chamaesiphon polonicus* SAG 32.87 (KY704111.1)	96.9	92.8	97.5	97.8	98.9	100				
7	*Chamaesiphon polymorphus* CCALA 037 (KY704110.1)	97.1	93.1	97.5	97.8	93.0	98.9	100			
8	*Chamaesiphon* sp. PCC 8308 (KY704110.1)	93.0	98.9	93.4	94.1	95.9	94.7	95.2	100		
9	*Chamaesiphon subglobosus* PCC 7430 (KY704113.1)	97.9	93.1	98.3	97.7	98.0	97.8	98.3	93.9	100	
10	*Geitleribactron purpureum* Tovel–4 (KT819293.1)	89.4	88.8	90.6	90.6	90.6	90.8	90.6	87.9	90.2	100

Table 2. Comparison of the 16S-23S ITS conserved domains lengths (nt) of *Chamaesiphon* strains

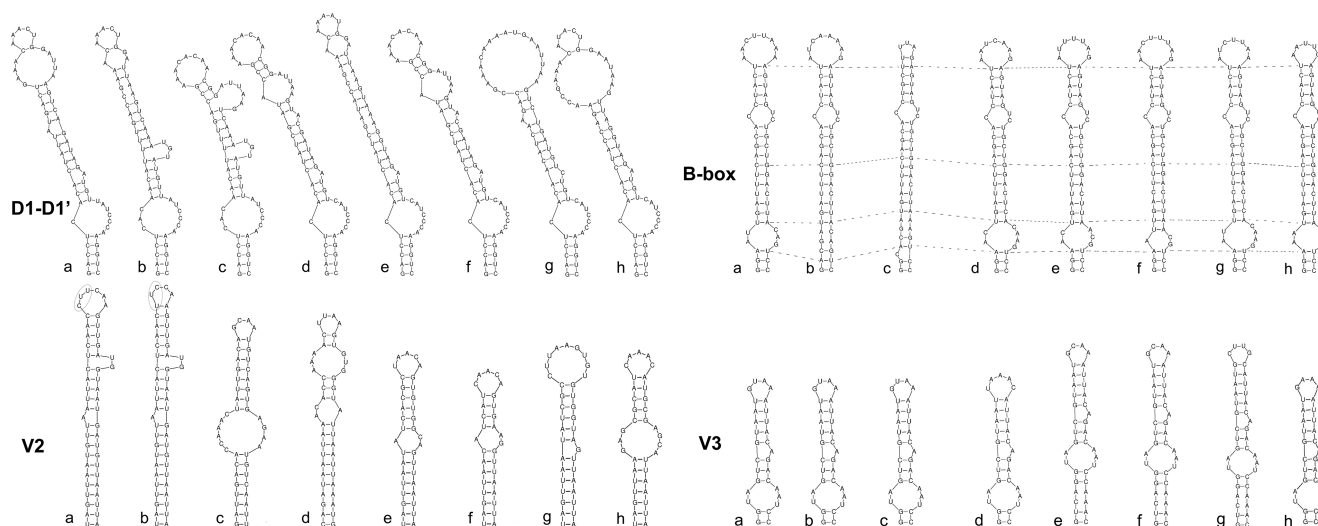| Strain ⟍ ITS region	*C. fontinalis* sp. nov.	PCR clone st_23_NB	*C. geitleri* №1023	*C. minutus* PCC 6605	*C. subglobosus* PCC 7430	*C.* sp. PCC 8308	*C. polonicus* SAG 32.87	*C. polymorphus* CCALA 037
Complete	567	600	591	587	592	574	594	632
Leader	8	8	8	8	8	8	8	8
D1–D1′	62	62	62	62	63	62	62	62
D2 + pre-D2 spacer	33	33	33	33	33	32	33	33
Spacer + D3 + spacer	23	23	23	22	24	20	24	22
tRNA^{Ile} gene	**72**	**72**	**72**	74	74	74	74	74
V2	71	71	68	62	61	57	61	61
tRNA^{Ala} gene	73	73	73	73	73	73	73	73
Pre-B-box spacer	62	56	52	50	54	51	59	49
B-box	54	53	53	54	53	54	54	51
Post-B-box spacer	5	6	5	5	5	5	5	5
A-box	12	12	12	12	12	12	12	12
D4	7	7	7	7	7	7	7	7
Spacer	14	14	14	14	10	10	10	14
V3	30	30	30	31	38	38	40	29
Spacer + D5 + spacer	41	80	79	80	77	71	72	132

Fig. 7. Secondary structures of the 16S-23S ITS regions. *Chamaesiphon fontinalis* sp. nov. (a), PCR clone 23_st_NB (b), *C. geitleri* №1023 (c), *C. minutus* PCC 6605 (d), *C. subglobosus* PCC 7430 (e), *Chamaesiphon* sp. PCC 8308 (f), *C. polonicus* SAG 32.87 (g), *C. polymorphus* CCALA 037 (h).

Conclusions

Chamaesiphon species are predominantly epiphytes of aquatic plants (in particular, *Cladophora* and *Oedogonium*) and biofoulers of stones in periphyton. They are widely distributed in freshwater bodies and streams in Europe and Russia (Hollerbach et al., 1953; Komárek & Anagnostidis, 1998; Rott, 2008; Sant'Anna et al., 2011; Loza et al., 2013a, 2018; Cantonati et al., 2015; Gutowski et al., 2015; Casamatta & Hašler, 2016; Kurmayer et al., 2018). It is worth to mention that the type species of this genus, *C. confervicola*, like the vast majority of about 40 species, have only a morphological description (Guiry & Guiry, 2022). They still have to be verified in accordance with the current taxonomic requirements used for new cyanobacterial taxa, including morphological, physiological and genetic methods (Vandamme et al., 1996; Iteman et al., 2000; Johansen & Casamatta, 2005; Ramasamy et al., 2014; Dvořák et al., 2015; Komárek, 2016; Raina et al., 2019; Perona et al., 2022).

In this paper, a new species of *Chamaesiphon* found in the Valdaysky National Park (Russia) is investigated, using a polyphasic taxonomic approach. *Chamaesiphon fontinalis* sp. nov. was identified based on the morphology, type of chromaic adaptation, physiology and molecular-genetic features of strain CALU 1864. It was found to be unique in the specific organisation of secondary structures of 16S-23S ITS regions. Strain divergence is supported phylogenetically, by a relatively low 16S rRNA gene similarity with other *Chamaesiphon* spp.

Our findings extend the present understanding on the diversity of the genus *Chamaesiphon* and represent a novel species, discovered for the first time in Russia.

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CHAMAESIPHON FONTINALIS SP. NOV., НОВЫЙ ВИД ОДНОКЛЕТОЧНЫХ АССИММЕТРИЧНО ДЕЛЯЩИХСЯ ЦИАНОБАКТЕРИЙ

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На камнях ручьев и родников национального парка «Валдайский» (Россия) были обнаружены обширные коричневатые биопленки альго-бактериальных сообществ. Доминирующими одноклеточными цианобактериями в исследованных биопленках являлись морфотипы *Chamaesiphon* sp. Хотя виды этого рода часто встречаются в перифитоне проточных вод, многие из них имеют географически ограниченное распространение и часто встречаются в горных ручьях и реках Европы. Штамм *Chamaesiphon* CALU 1864 был выделен из эпилитных биопленок родника Текунок. Мы обнаружили, что он отличается от ранее описанных видов не только необычной малиновой окраской, но и некоторыми морфологическими особенностями. Молекулярно-генетический анализ первичной последовательности 16S рРНК и вторичных структур внутреннего транскрибируемого спейсера рибосомального оперона подтвердил его уникальность. Филогенетически штамм представляет собой независимую линию на эволюционном древе. На основании выявленных отличий мы предлагаем рассматривать штамм CALU 1864 как новый вид, *Chamaesiphon fontinalis* sp. nov., впервые обнаруженный в России и описанный в соответствии с действующими правилами Международного кодекса номенклатуры водорослей, грибов и растений.

Ключевые слова: Chamaesiphonaceae, молекулярно-генетические признаки, морфотип, особо охраняемая природная территория, полифазная таксономия