

***Mychonastes frigidus* sp. nov. (Sphaeropleales/Chlorophyceae), a new species described from a mountain stream in the Subpolar Urals (Russia)**

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Abstract: This paper describes a new species from the Class Chlorophyceae, *Mychonastes frigidus* sp. nov., isolated from a cold-water mountain stream in the north of Russia (Subpolar Ural). The taxon is described using morphological and molecular methods. *Mychonastes frigidus* sp. nov. belongs to the group of species of the genus *Mychonastes* with spherical single cells. Comparison of ITS2 rDNA sequences and its secondary structures combined with the compensatory base changes approach confirms the separation between *Mychonastes frigidus* sp. nov. and other species of the genus. *Mychonastes frigidus* sp. nov. represents a cryptic species that can only be reliably identified using molecular data.

Key words: *Mychonastes*, new species, SSU rDNA, ITS2 rDNA secondary structure, CBC approach, Subpolar Urals

INTRODUCTION

The genus *Mychonastes* P.D. Simpson & Van Valkenburg 1978 comprises autosporic small-celled organisms that live alone, in small groups or organized in colonies, surrounded by hyaline, mucilaginous envelopes without distinctive structures. Species of the genus are common in phytoplankton in stagnant and running fresh and brackish waters. It is difficult to differentiate most taxa of the genus *Mychonastes* from other single small-celled, spherical or ellipsoidal chlorophytes such as *Choricystis* (Skuja) Fott, *Meyerella* Fawley et K. Fawley and *Nannochloris* Naumann (KRIENITZ et al. 1996, 1999; FAWLEY et al. 2005) under light microscopy.

Like many other green algae, morphological convergence and high levels of morphological plasticity complicate the application of traditional classification methods in this genus. Molecular data have demonstrated that the *Mychonastes* clade represents a monophyletic lineage within the Chlorophyceae, which is clearly differentiated from other evolutionary lineages containing small-celled coccoid green algae (KRIENITZ et al. 2011). Notably, KRIENITZ et al. (2011) stated that only molecular data can provide conclusive evidence for the characterization and separation of species of the genus *Mychonastes*.

Species of this genus have previously been

repeatedly noted in terrestrial habitats in the northern regions of the Urals (PATOVA & NOVAKOVSKAYA 2018). The Subpolar Urals comprises the northernmost part of the Ural Mountains in Russia. It is the highest part of the Ural mountain system, which is a complex folded structure of the Upper Paleozoic age. Unlike other parts of the Urals, it is characterized by a peculiar landscape, orography and climate (TRESHNIKOV 1985). The border position between the European continent and Siberia (or Europe and Asia) and the complex natural processes of the last millennia associated with glaciation have led to the region's richest diversity of flora and fauna, which combine tundra and forest species and elements of western and eastern species. The flora of vascular plants in the region comprises more than 600 species, including a number of endemic taxa (DEGTEVA 2016). The algal flora of the Subpolar Urals is represented by 125 species of cyanoprokaryotes and 704 eukaryotic algae taxa, including 57 chlorophytes species (PATOVA 2010). Soil algal flora include 75 taxa of cyanoprokaryotes and 133 taxa of eukaryotic algae, including 68 species of chlorophytes (PATOVA & NOVAKOVSKAYA 2018). Data on the diversity of algae in this territory is actively increasing through the study of previously inaccessible areas, as well as through the application of new molecular methods to the study of algae.

Here, we use molecular data to describe a new

species of the genus *Mychonastes* from a cold–water stream in the Subpolar Urals.

MATERIAL AND METHODS

Study area. Sample was collected from an unnamed mountain stream in the Subpolar Urals on August 8, 2015 at GPS coordinates 65°12'48"N, 60°16'04"E, 676 m above sea level (Fig. 1). The research area is located in the northeast of the European part of Russia, in the Intinsky administrative region of the Komi Republic, and is included in the borders of the “Yugyd va” national park (TRESHNIKOV 1985). The climate is severe and sharply continental, which is due to the geographical location of the region and the considerable height of the mountain ridges (KOLESNIK 1964). The Subpolar Urals have the highest precipitation of the Urals; in the western slopes where this stream is located the annual precipitation reaches 1500 mm. The average annual air temperature is –4.4 °C. The area has a well–developed river network; all rivers originate on the western slope of the Ural ridge and often start from small but deep lakes. Rivers accounts for 50–60% of the snow supply, up to 30% of the rain supply, and 15–20% of the underground water supply (KOLESNIK 1964).

Samples and collection. Samples were collected from a rocky stream bottom consisting of white quartz. A scraping of yellow–green plaque was collected from the stones. The water temperature at the time of collection was 12 °C, pH = 6.0, and the specific conductance was 21 μS.cm⁻¹. Other environmental parameters of the water are presented in Table. Water mineralization and temperature measurements were performed using the HI98129 Combo (Hanna Instruments, Inc., Woonsocket, RI, USA). Hydrochemical analysis was conducted at the eco–analytical laboratory of the Institute of Biology FRC Komi SC UB RAS. The strain described here was isolated by E. Patova and I. Novakovskaya, and cultures were transferred to the Collection of microalgae strains in the Institute of Biology FRC Komi SC UB RAS under the number SYKOA Ch–111–18.

Culturing. The sample was placed immediately in the field in a sterile Waris–H medium (McFADDEN & MELKONIAN 1986). Monoclonal strain was established by examination of micropipetted single cells under an inverted microscope. Non–axenic unialgal cultures were maintained in Waris–H liquid medium at 10°C in a growth chamber with a 12:12 h light:dark photoperiod. Morphological features were investigated after cultivation on 3N BBM, WC, BG–11 liquid and agar mediums (ANDREYEVA 1998; ANDERSEN 2005).

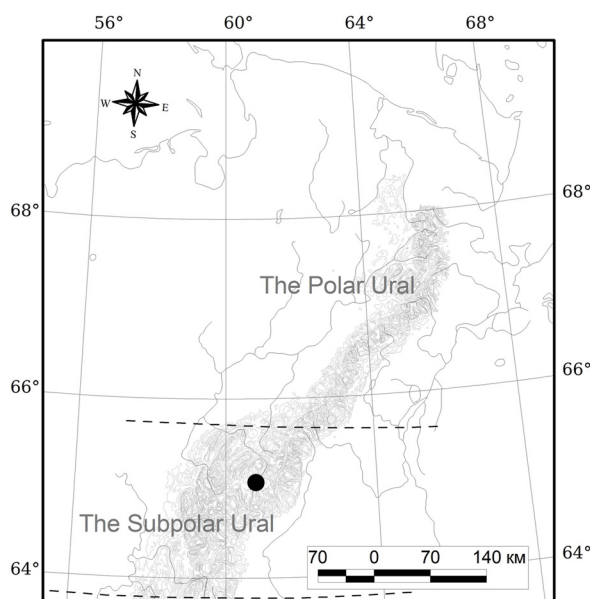


Fig 1. Map of the study area.

Light microscopy. Light microscopy observations and photography were carried out using a Zeiss Scope A1 microscope (Carl Zeiss AG, Oberkochen, Germany), equipped with an oil immersion objective (Plan–Apochromat 100×/1.40 Oil DIC M27) and Nikon Eclipse80i (Nikon, Japan) equipped with an oil immersion objective (Plan–Fluor 100×/1.30 Oil DIC D–ND 6) at 1000–fold magnification. Cells were stained with 0.1% methylene blue solution and 1.0% ink solution for the determination of the mucilage structure. Cultures in exponent and stationary growth phases.

Light micrographs were taken with an AxioCam ERc 5s Rev.2 and Nikon Digital Sight Ds – 2Mv cameras. Nikon Eclipse80i (Nikon, Japan) with optics of Nomarskiy (DIC).

Extraction of DNA and amplification. The total DNA of the monoclonal culture was extracted using InstaGene™ Matrix according to the manufacturer’s protocol. Fragments of SSU rRNA (1718 bp) and ITS2 rRNA (382 bp) were amplified using primers EukA and EukB from MEDLIN et al. (1988) for SSU rRNA fragments and ITS1–ITS4 from WHITE et al. (1990) for ITS2 rRNA fragments. Amplification of all studied fragments was carried out using the premade mix ScreenMix (Evrogen, Russia) for the polymerase chain reaction (PCR). The conditions of amplification for both SSU rRNA and ITS2 rRNA fragments were: an initial denaturation of 5 min at 95 °C, followed by 35 cycles at 94 °C for denaturation (30 s), 52 °C for annealing (30 s) and 72°C for extension (50–80 s), and a final

Table 1. Environmental parameters of the studied stream.

Major Ions (mg.l ⁻¹)							Major biogenic elements (mg.l ⁻¹)				
Cl ⁻	SO ₄ ²⁻	HCO ₃ ⁻	Ca ²⁺	Mg ²⁺	Na ⁺	K ⁺	P _{mineral.}	P _{total.}	N _{total.}	Si	Fe _{total}
0.27	not detected	5.1	1.62	0.153	0.83	0.058	0.004	0.009	0.56	5.0	not detected

extension of 10 min at 72 °C. The resulting amplicons were visualized by horizontal agarose gel electrophoresis (1.5%), colored with SYBR Safe (Life Technologies, Carlsbad, CA, USA). Purification of DNA fragments was performed with the ExoSAP-IT kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. SSU rRNA and ITS2 rRNA fragments were decoded from two sides using forward and reverse PCR primers and the Big Dye system (Applied Biosystems, Foster City, CA, USA), followed by electrophoresis using a Genetic Analyzer 3500 sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis of SSU rDNA data. Sequences were edited manually and assembled using BioEdit v7.1.3, and MegaX (KUMAR et al. 2018). Newly determined sequences and GenBank sequences of 42 other chlorophytes from different morphological groups were included in the alignment. Also, two species (*Ulothrix zonata* (F.Weber et D.Mohr) Kütz. and *Gloeotilopsis planctonica* M.O.P.Iyengar et Philipose) were chosen as outgroup taxa. Sequences were aligned using global SILVA alignment for rRNA genes in the SINA v1.2.11 (PRUESSE et al. 2012). jModelTest ver. 2.1.1 indicated that the GTR model of nucleotide substitution, with Gamma (G) distributed rates across sites and a proportion of invariable sites (I), was the most appropriate evolutionary model for the SSU rDNA alignment (POSADA 2006). Maximum likelihood phylogeny (ML) was constructed using MegaX (KUMAR et al. 2018) with the Subtree-Pruning-Regrafting tree rearrangements algorithm (SPR). The bootstrap analysis with 1,000 replicas was used. Bayesian Interference (BI) analysis was conducted with MrBayes-3.2.5 (RONQUIST & HUELSENBECK 2003). Three "hot" and one "cold" Markov chains were run for 1×10^6 cycles in two repetitions with the selection of each 200th generated tree. Phylogenetic tree and posterior branching probabilities were obtained after discarding the first 25% to produce estimate parameter models of nucleotide substitutions and likelihood. Viewing and editing of trees were carried out in the programs FigTree (ver 1.4.2) and Adobe Photoshop CC (19.0).

Internal transcribed spacer 2 annotation, phylogeny, and secondary structure modeling. For the annotation ITS2 sequence, was used tool ITS2-Annotation (ANKENBRAND et al. 2015). This tool uses HMMer (EDDY 1998) to annotate ITS2 sequences with Hidden Markov Models (HMMs) (KELLER et al. 2009). It returns by definition of the ITS2 the sequence between the conserved 5.8S and 28S (or equivalent) rRNA coding region. The secondary structure of the ITS2 rDNA region was modeled using the Mfold version 2.5 program (ZUKER 2003). The construction of the model took into account the presence of the pyrimidine-pyrimidine unpaired section in the second helix at seventh position (CAISOVÁ et al. 2013), as well as the length and nucleotide composition of the spacers in the core of the model defining the helix boundaries (SCHULTZ et al. 2005). Detail features of the ITS2 secondary structures of studying species, for example, presence of helices Ib and IIIb, were also checked using secondary structures of others *Mychonastes* species (KRIENITZ et al. 2011). The resulting secondary structure was visualized in the program PseudoViewer3 (BYUN & HAN 2006). The ITS2 sequences were aligned according to the secondary structure by ClustalW algorithm (THOMPSON et al. 1994) in tool 4SALE (SEIBEL et al. 2006). For delimitations species secondary structures of *Mychonastes* strains were checked for compensatory base changes (CBCs) according to COLEMAN (2000, 2009); MÜLLER et al. (2007) in comparison with species described by KRIENITZ

et al. (2011). For the phylogenetic analysis the sequence part from the sequence-structure alignment (as obtained by 4SALE) was further processed, using the same programs and methods as described above for the 18S data, except for the model of nucleotide substitution – Kimura-2-parameter+I.

RESULTS

Cells of the strain SYKOA Ch-111-18 are small, spherical, commonly solitary, and sometimes found in small groups after sporulation (Figs 2–5). Staining with 0.1% methylene blue solution and 1.0% ink solution did not reveal any stalks between cells. Analysis of the SSU rDNA gene sequences revealed that the strain SYKOA Ch-111-18 belongs in the *Mychonastes* clade on the phylogenetic tree of Chlorophyceae (Fig. 6). Thus, morphologically, the strain SYKOA Ch-111-18 belongs in a group with species *M. homosphaera*, *M. pusillus*, *M. rotundus* and *M. huancayensis*, which possess solitary spherical cells (see Supplementary table). Species in this group can be distinguished only by molecular criteria like ITS2 sequences and a presence of CBC in the conserved regions of ITS2 due to their small size, lack of sexual reproduction and high interspecies similarity (KRIENITZ et al. 2011). Analysis of the ITS2 rDNA sequences of the strain SYKOA Ch-111-18 allowed the recognition of the new lineage on the ITS2 rDNA tree referable to a new species (Fig. 7).

Mychonastes frigidus sp. nov. Patova, Novakovskaya, Martynenko, Gusev, Kulikovskiy (Figs 2–5)

Description: cells single or rarely in small groups (colonies), adult cells spherical, usually 2–5 µm, very rarely up to 10 µm in old cultures, without mucilaginous envelope. Colonies usually four-celled, 9–15 µm in diameter, cells connected via mucilaginous mother cell wall remnants. The cell wall is thin, in the light microscope appears smooth. Chloroplast single, parietal, cup-shaped, without pyrenoid. Cytoplasm with oil droplets. Propagation by two or four (eight) autospores. Species differs from other species of the genus by the order of the nucleotides in ITS rDNA gene sequences.

Holotype: a large drop of unfixed dried cells of the strain SYKOA Ch-111-18 on watercolor paper (hic designatus), deposited at SYKO Herbarium, Komi Scientific Centre, Russian Academy of Sciences, Syktyvkar, Russia under the designation SYKO №1001-A. Representative strain and DNA sample deposited at the SYKOA Collection of microalgae strains in the Institute of Biology FRC Komi SC UB RAS, Russian Academy of Sciences under the designation SYKOA Ch-111-18. The strain is also conserved as a formaldehyde-fixed sample.

Authentic strain: SYKOA Ch-111-18.

Representative DNA sequences: GenBank accession numbers MT192073 (nuclear ITS2 rDNA), and MT187587

(nuclear SSU rDNA).

Etymology: the specific epithet (*frigidus*) reflects the occurrence of this species in cold waters.

Type locality: an unnamed mountain stream in the Subpolar Urals of Russia. Latitude/Longitude: 65°12'48"N, 60°16'04"E, elevation 676 m above sea level. Collected August 8, 2015 by E. Patova.

Distribution: To date, *Mychonastes frigidus* has only been observed in the type locality. At the time of collection, the pH was 6.0, temperature 12 °C and specific conductance 21 $\mu\text{S}\cdot\text{cm}^{-1}$.

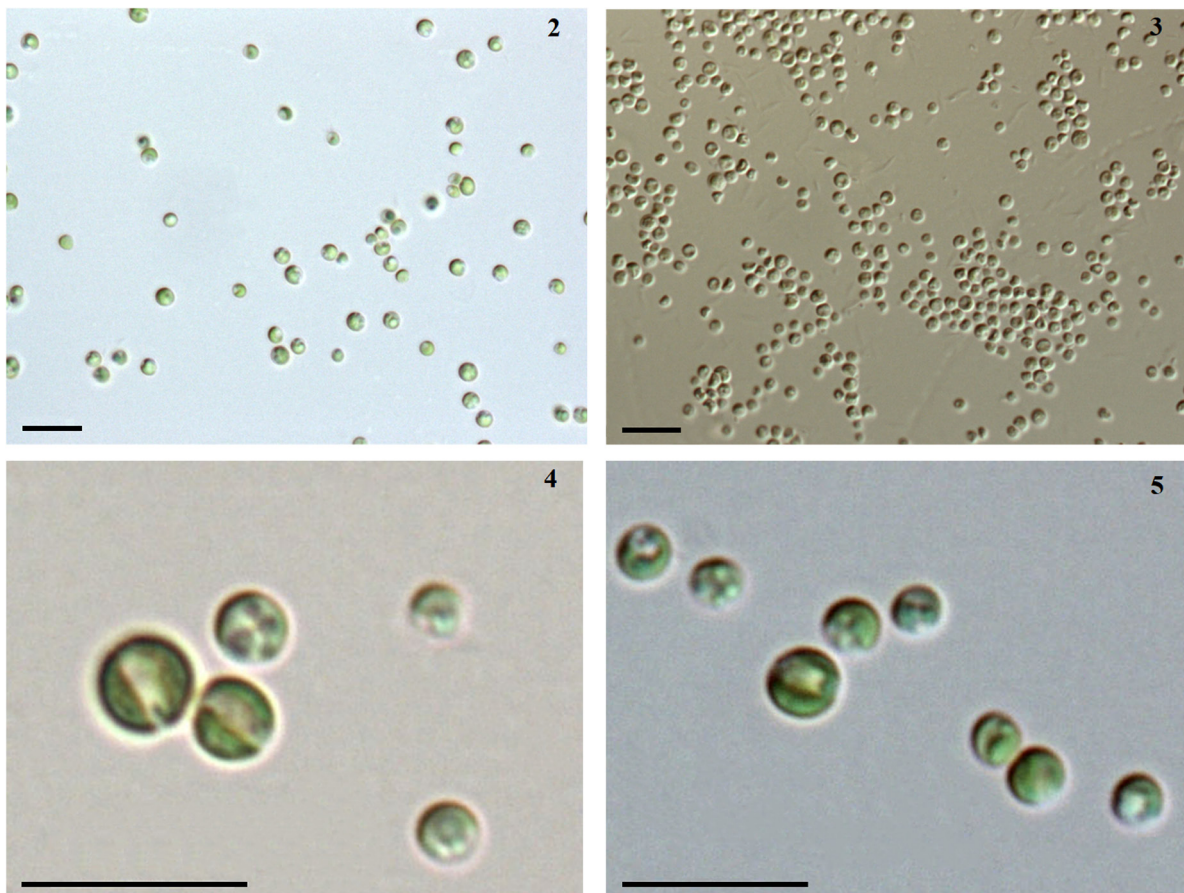
DISCUSSION

The genus *Mychonastes* includes solitary or colonial (4–64 cells or more) algae with or without mucous membrane. Mucous colonies are spherical, ovoid or irregular in shape. In the colonies, the cells are connected by gelatin filaments, which are formed by the remnants of the cell wall of the mother cell after sporulation. The cells have a spherical, slightly ovoid or ellipsoidal shape, 1.5–10 μm in diameter. The cell wall is sculptural and consists of two layers: an outer layer with a trilaminar substructure and an amorphous inner layer. A cell contains from one to four chloroplasts, disk-shaped, mantle-shaped, or,

most often, cup-shaped, without a pyrenoid; each cell has one nucleus. With aging, secondary carotenoids can form, making cultures yellow, orange, or brown. Reproduction occurs via asexual spores (usually 2–4–8 (16)), which are released by rupture of the maternal membrane. The remains of the maternal membrane are preserved in the medium (ANDREEVA 1998; KRIENITZ et al. 2011).

The genus is currently represented by 20 species in Algaebase (GUIRY & GUIRY 2020); their characteristics and habitat preferences are described in Supplementary table. Species of this genus were previously found in Scotland, Germany, Slovakia, Russia, Ukraine, Brazil, Peru, Kenya, Angola, India, USA (SIMPSON & VALKENBURG 1978; HINDÁK 1978; KOMÁREK & FOTT 1983; HINDÁK 1984; HINDÁK 1988a, b; ANDREYEVA 1998; HANAGATA et al. 1999; TSARENKO et al. 2011; KRIENITZ et al. 2011; RAMOS et al. 2015, etc).

Mychonastes frigidus clustered with *M. homospaera*, *M. pusillus*, *M. rotundus* and *M. ovahimbae* on unrooted ITS2 tree and formed a separate branch inside this cluster (Fig. 7). This result is in general accordance with the results of morphological analysis, which indicated that *M. frigidus* belongs in a group of species with solitary spherical cells. Comparison of the ITS2 secondary structures of *M. frigidus* with other species with known ITS2 rDNA sequences revealed a number of CBCs with all studied taxa, including highly conserved



Figs 2–5. Microphotographs of *Mychonastes frigidus* sp. nov. Scale bars 10 μm .

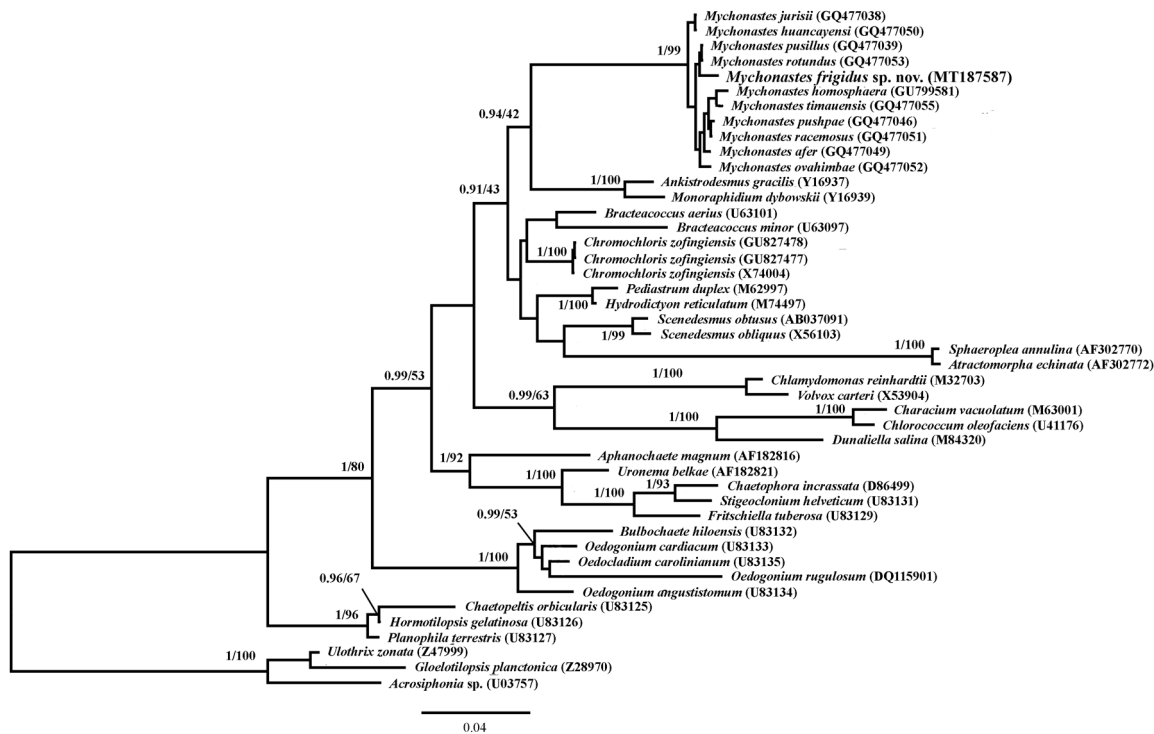


Fig. 6. Bayesian tree of the partial small subunit rDNA (SSU rDNA). The Bayesian posterior probability and maximum likelihood bootstrap value are shown left and right of the fraction line respectively. Scale bar represents: substitution per site.

areas of Helix III (Fig. 8). All *Mychonastes* taxa are characterized by variability in the length and structure of the second and third helices. *M. frigidus*, *M. pusillus* and *M. rotundus* have similar ITS2 lengths (379–382 nt). There were 2 and 3 CBCs in the Helix III comparing *M. frigidus* with *M. pusillus* and *M. rotundus*, respectively (Fig. 8). *M. homosphaera* and *M. ovahimbae* are differ in the length of ITS2 (337 and 361 nt, respectively) and have 4 and 2 CBCs in the Helix III comparing *M. frigidus*, respectively (Fig. 8). Among the discussed taxa, *M. pusillus* is known from Kenya, *M. ovahimbae* from Angola, *M. rotundus* from Kenya and Germany and *M. homosphaera* from Sweden, the Czech Republic and Germany (KRIENITZ et al. 2011).

The CBC concept was previously used for species delimitation in the *Mychonastes* clade (KRIENITZ et al. 2011). COLEMAN (2000) and MÜLLER et al. (2007) have proposed that CBCs in ITS2 correlate with speciation, and thus can be used to distinguish species. The CBC species concept states that two organisms/strains whose ITS2 sequences differ by even a single CBC in the conserved regions of Helix II and Helix III represent two different biological species (COLEMAN 2000). According to MÜLLER et al. (2007) and WOLF et al. (2013), the presence of any CBC in the whole ITS2 molecule is sufficient for distinguishing species.

Species of the genus *Mychonastes* are one of the most abundant groups of algae of picoplankton and nanoplankton of fresh and brackish waters (KRIENITZ et al. 2011). However, *Mychonastes frigidus* was isolated from epilithon on the stream bottom and differs

in ecology from other described taxa. This new species has some special preferences: it inhabits cold waters with low content of biogenic elements and shows good growth at low temperatures. It has so far been found only in the type habitat in Northern Russia. The species may therefore represent a true cryptic species, which can only be distinguished by molecular characters.

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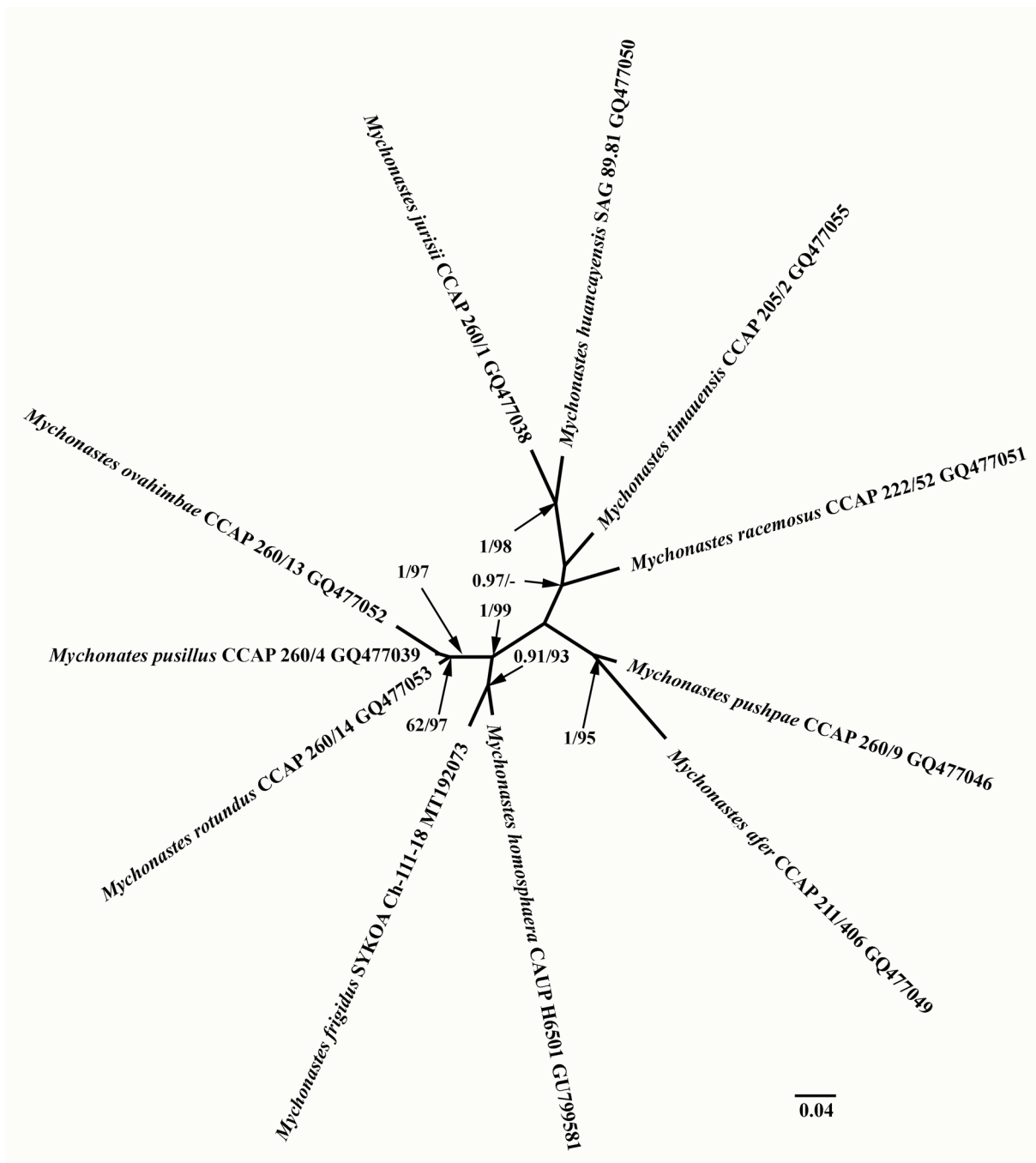


Fig 7. Unrooted Bayesian tree of the ITS2 rDNA of species from the genus *Mychonastes*. The Bayesian posterior probability and maximum likelihood bootstrap value are shown left and right of the fraction line respectively. Scale bar represents: substitution per site.

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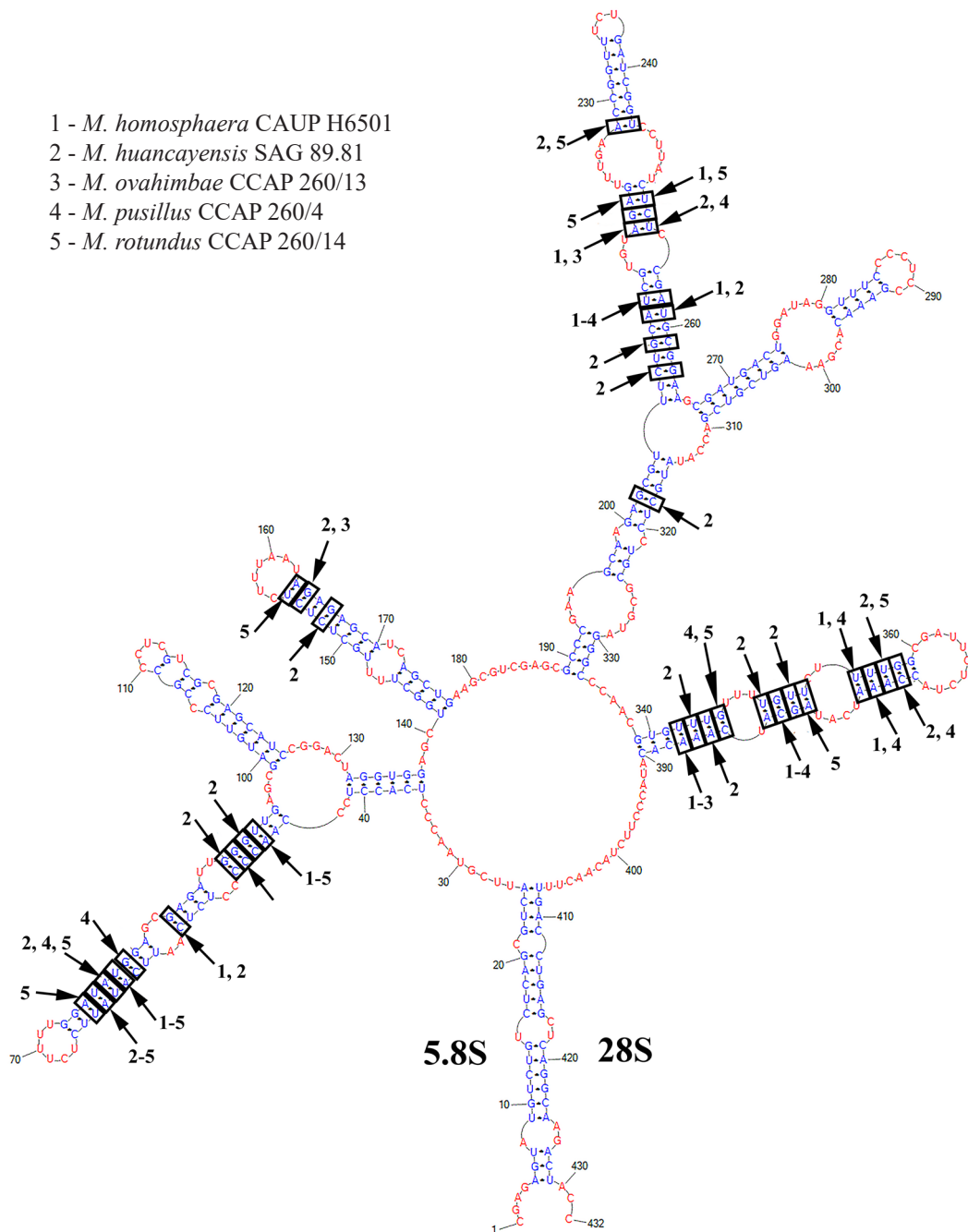


Fig 8. Predicted secondary structure of the nuclear internal transcribed spacer 2 of the strain SYKOA Ch-111–18 (*Mychonastes frigidus* sp. nov.). Black boxes – position of Compensatory Base Changes (CBC) between *M. frigidus* and other species of the genus, forming one clade on the ITS2 tree with the newly described taxon.

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Supplementary material

The following supplementary material is available for this article:

Table S1. List of known species of the genus *Mychonastes* with synonyms, morphological features and habitat preferences.

This material is available as part of the online article (<http://fottea.czechphycology.cz/contents>)