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Molecular phylogenetic analyses, ecology and morphological characteristics of *Chloromonas reticulata* (Goroschankin) Gobi which causes red blooming of snow in the Subpolar Urals

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Abstract – The algae which cause snow blooming remain poorly studied in the northern regions of the Eastern Europe. However, the process is widely observed, especially, in the mountain regions. The growth of cryophilic algae has a strong fascilitating effect on melting of the glaciers, thus, the comprehensive study of snow algae species would help to foresee and model interrelated processes of climate change in the ecosystem.
The red snow blooming in the Subpolar Urals was investigated. The ecological characteristics of habitats where blooming occurred, as well as, chemical parameters of the meltwater were studied. The snow contained important elements for algal development, including N, P, Ca, Mg and other biogenic compounds. The density of algal cells in samples reached 0.33×10^4 cells per ml^{-1}. A *Chloromonas reticulata* strain isolated from the red snow with massive development of biflagellate algae was studied in detail. The algal strain is stored in a collection of living algae in Institute of Biology Komi Scientific Center (SYKOA Ch-054-11). Based on analysis of ITS2 sequences, morphological and ultrastructural characteristics, the studied biflagellate strain has been placed to *Chloromonas* clade, *Reticulata* group with a high bootstrap support. The set of ultrastructural characters detected in isolated strain matched with that of previously described for other *Chloromonas reticulata* strains. Minor morphological deviations that were observed in *Chloromonas reticulata* strain from SYKOA Ch-054-11 in comparison with the strains SAG 29.83, SAG 32.86 and SAG 26.90 could be attributed to a latitudinal gradient differences and ecological conditions.

Snow algae / red snow / *Chloromonas reticulata* / *Chloromonadinia* / *Reticulata* group / the Subpolar Urals

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INTRODUCTION

The blooming of snow and ice caused by algae and Cyanoprokaryota is a common natural phenomenon which can be observed in various mountain systems of the world and in the Arctic and Antarctic regions (Kawecka, 1986; Hoffmann, 1989; Matsuzaki *et al.*, 2015). Around 300 species are found in the snow, the great number of which comes from Cyanoprokaryota and green algae groups (Kawecka, 1986; Matsuzaki *et al.*, 2015). These organisms can survive under low temperatures, intensive radiation, and the lack (or excess) of the light during long polar days and nights (Hoham *et al.*, 2002, Mock & Thomas, 2008). They spend unfavorable conditions in resting stages of the cycle, also known as akinetes, aplanospores, or zygotes. Numerous studies are done on algae that cause snow blooming. The main interest is their contribution to the glacier melting in mountain and polar territories. Understanding the process (and role of algae in it) is important to estimate a related decrease of snow cover of the Earth under climatic change and evaluateits effectson the region. As some studies show, algal development has a negative effect on the glacier size: algae decrease albedo of snow and ice which accelerates the melting (Takeuchi *et al.*, 2006; Hisakawa *et al.*, 2015). The snow algae are well preserved in the ice, therefore, they have been suggested as new indicator species to infer the past climate of the Earth environments (Takeuchi *et al.*, 2006; Hisakawa *et al.*, 2015). Data on physiological characteristics of snow algae provides valuable material to use in future for a development of new methods in cryopreservation of biodiversity in vaults and banks (Andersen, 2005). The studies on ecology and physiology of cryophilous species could help to find new biotechnology objects and select organisms which are capable to transform uninhabited surfaces not only on Earth but also on other planets (Kabirov, 2010).

Due to variation in the composition of cell pigments, the algal blooming of different species gives snow wide range of colors (Kawecka, 1986; Hoffmann, 1989; Ling, 2001; Komárek & Nedbalová, 2007; Muramoto *et al.*, 2010). The most common color is red, it is the result of the massive development of species from *Chloromonas, Chlamydomonas* and *Gloeocapsa* genera. Such blooming was observed on Svalbard (Newton, 1982), in the Arctic (Gradinger, 1996), on Alaskan glaciers (Takeuchi, 2001), in the mountains of California (Painter *et al.*, 2001), Altai (Takeuchi *et al.*, 2006), the Alps (Remias *et al.*, 2010), and on Franz-Joseph land (Hisakawa *et al.*, 2015) and other regions. In Russia, the blooming of algae was noted for the first time in 1902 in the Eastern Sayan Mountains glaciers (Komarov, 1953). In many articles, the red snow blooming is attributed to the massive development of *Chloromonas nivalis* (Chodat) Hoham and Mullet. The species morphology, distribution, and ecology are well studied (Kawecka, 1986; Painter *et al.*, 2001, Komárek & Nedbalová, 2007; Mock & Thomas, 2008; Remias *et al.*, 2010; Matsuzaki *et al.*, 2015). The data on other species which cause red blooming is limited.

The area of snowfields and glaciers in the northern regions of the Urals has been decreasing in recent decades (Kotlyakov *et al.*, 2015). We believe that algal blooming on the snow surface brings a certain contribution to this process. This phenomenon is often noted in the Ural Mountains but the species diversity of snow algae has not been studied in the region.

The article summarizes the results of our study on the strain of *Chloromonas reticulata* isolated from the red snow in the Subpolar Urals (the European part of Russia). We studied its morphological characteristics and did phylogenetic analysis

based on sequences from nuclear-encoded 18S ribosomal (r) RNA and ITS1-5.8S-ITS2. The environmental factors during the mass development of this snow alga were analyzed.

MATERIALS AND METHODS

Study sites, sample collection and culturing conditions

The red snow blooming was observed in the Subpolar Urals in July 2010. The snow samples were gathered on the snowfield (65°13'49.5"N, 60°13'19.4"E) located on the northeastern slope (inclination angle 60°), in the pelvic belt, 1047 m above sea level (Fig. 1). The Subpolar Urals have a harsh continental climate with long extreme winters, cold summers and short vegetation season with relatively high precipitation. According to the Hydrometeorological Database (HMDB) (Novakovskiy & Elsakov, 2014) in 2010 the average annual temperature for the Subpolar Urals was about –2.5°C. The average temperature usually stands at –24.6°C in the coldest

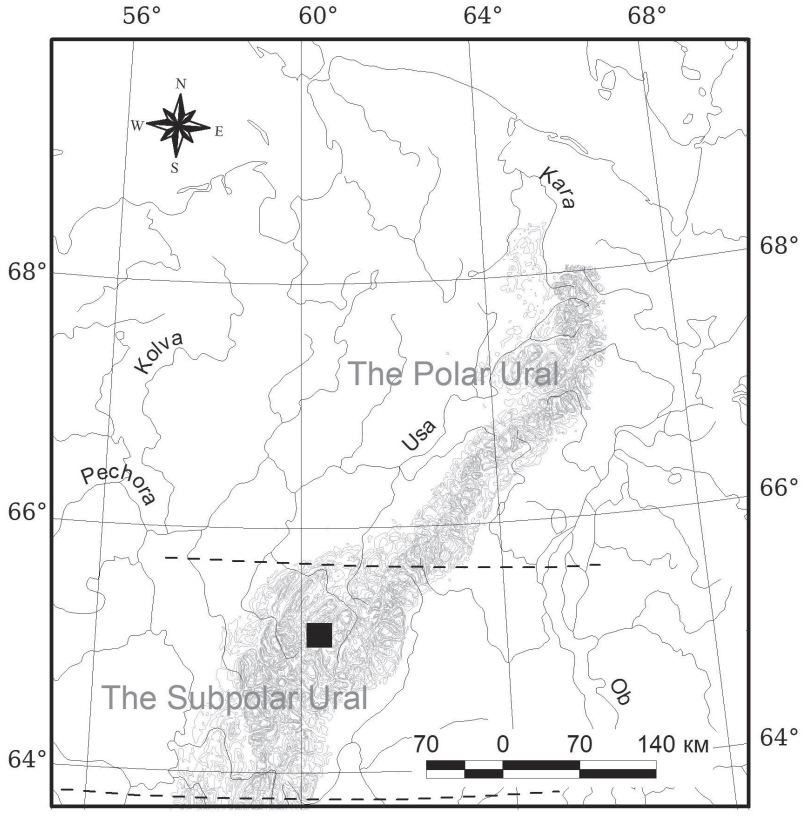


Fig. 1. The map of the studied region (Black Square – site of sampling).

month (January) and respectively 15.9°C in the warmest month (July). The vegetation period lasts for less than 80 days. The summer in the mountains is cool. The daylight lasts for 23 hours from May till mid-July, and for 18 hours during mid-July and mid-August. The most elevated regions of the western slope of the Subpolar Urals receive about 520 mm precipitations per year.

The samples of snow were picked up in the upper snow layer at the depth of 5 mm and placed in sterile plastic tubes (Greiner Bio-One, 15 ML, Austria).

The temperature of the white and red snow was estimated by Infrared Thermometer OPTRIS LS DCI (Optris GmbH, Germany).

The snow samples for chemical analysis were collected from depths of 0-5 and 5-40 cm in the area of algal blooming. The Water Test (Hanna Instruments, Germany) was used to measure the conductivity of melt water in field conditions. Analysis of other hydrochemical parameters (ions, microelements, permanganate oxidation etc.) was carried out in the ecoanalytical laboratory of the Institute of Biology, Komi Scientific Center, Ural Division, Russian Academy of Sciences (certificate No. POCC RU.0001.511257, GOST R ISO / IEC 17025 – 2009, ISO / IEC 17025 – 2005).

The samples of red snow were studied in the field by light microscope Zeiss Axiolab (Carl Zeiss, Germany). The isolation of algal cells was done by standard techniques using Pasteur pipette and unialgal culture. 3N BBM medium was applied for culturing (Andersen, 2005). The growing conditions for the isolate: 40 μmol $m^{-2}s^{-1}$ PAR (phytolamp Sylvania GRO-Lux F36W / Gro-TB, Germany), duration of light/dark period – 12/12 hours, temperature 25°C. After 7-10 days the strain was transferred to a display refrigerator (Biryusa 310ER, Russia) where it was maintained under 5 μmol $m^{-2}s^{-1}$ PAR (Camelion FSL T5 8W / 765, China), temperature + 10-14°C, duration of light/dark period – 12/12 hours.

Light and transmission electron microscopy

The algal strains were studied by a Nikon Eclipse 80i microscope (Japan) under 400 and 1000 magnification (Nikon, Japan), it was fitted with differential interference contrast (DIC) system and Axio CamICc3 (Carl Zeiss, Germany) and Nikon Digital Sight Ds-2Mv (Nikon, Japan) cameras. An image analysis system (AxioVision) was used to determine cell widths and lengths: 100 independent measurements were made for the strain every week for two months and the means and standard deviations were calculated.

For species identification, essential monographs were employed (Dedusenko-Shchegoleva *et al.*, 1959; Ettl & Gärtner, 2014). Five samples for quantitative measurements (100 ml of meltwater) of red snow were collected from the upper horizon to a depth of 5 cm. The samples were immediately fixed with 4% formalin. The count of algae cells was performed using Goryaev's chamber (Sirenko & Sakevich, 1975) under Zeiss Axiolab (Carl Zeiss, Germany) microscope, the calculation of cells was done for 1 ml of each sample in 6 replicates.

For transmission electron microscopy, cells from the five-seven-day algae cultures were taken. Algal cells, grown in strips on an agar medium, were covered with a low temperature liquid agar. After solidifying the agar strips were cut into small blocks and were fixed sequentially in 3% glutaraldehyde and 1% OsO_4, diluted in a liquid nutrient medium (Boldina, 1996). Dehydration was carried out in the acetone series of increasing concentrations. The agar blocks were enclosed in the epon-aldalite mixture. Ultrathin sections were prepared on the ultratome LKB III

(LKB, Sweden) with glass knives. The sections were placed on grids and stained with lead citrate. Cells were viewed and photographed on a transmission electron microscope Libra 120+ (Carl Zeiss, Germany) of the Core Facility Center "Cell and Molecular Technologies in Plant Science" at the Komarov Botanical Institute RAS (St.-Petersburg, Russia).

DNA sequencing

The DNA of strain cells was extracted using "FastDNASpinKit" (QBioGene, Canada) according to the instructions from the manufacturer. The extracted DNA was stored under –20°C. The amplification of fragments was performed in a total volume of 25 µl containing: 5 µl ScreenMix (Eurogen, Russia), 5 µl of each primer (0.3 µM) (Eurogen, Russia), 9.0 µl ddH_2O (Ambion, USA) and 1 µl DNA template (1÷100 ng). For the amplification of the 18S gene sequence, we used 2 sets of primers: 18sFseq (5'-TCGTAGTTGGATTTCGGGTGGGTT-3') and 18sRseq (5'-TACCGGAATCAACCTGACAAGGCA-3'), 18sL_GA2 (5'-GTCAGAGGTG AAATTCTTGGATTTA-3') and 18sR_GA2 (5'-AGGGCAGGGACGTAATCAA CG-3'). For the amplification of the ITS1-5.8S-ITS2 we used primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS-4 (5'-TCCTCCGCTTATTG ATATGC-3') (White *et al.* 1990). The amplification was carried out in Swift MiniPro (ESCO, Singapore). PCR conditions: Initial denaturation for 5 min, 95°C; followed by 35 cycles of 30 sec at 94°C, 30 sec at primer-specific temperatures (57°C for 18S and 58°C for ITS1-5.8S-ITS2), 40 sec at 72°C; and a final extension step of 2 min at 72°C. The PCR products were treated with ethidium bromide and visualized by electrophoresis on the 1.3% agarose gel on UVT-1 (Biokom, Russia). We used 100bp DNA markers as the size standard ladder (100-3000 bp) (Thermo Scientific, EU). To clean the PCR products, QIAquick Gel Extraction Kit (Qiagen, Germany) was used. The amount of the received DNA and PCR products was checked on "Fluorate-02-Panorama" (LLC Lumex, Russia). The sequencing was done using ABI Prism BigDye Terminator v.1.1 reagents on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) in the center of collective usage "Molecular biology" of Institute of biology Komi Scientific Center. The fragments of 18S rDNA and ITS1-5.8S-ITS2 strain sequences were submitted to GenBank under KF361494 and MF033356 accession numbers.

Unfortunately, GeneBank does not contain information on some regions of the 18S rDNA and ITS1-5.8S-ITS2 of *Chloromonas reticulata* strains, which caused constrains in our study. For the phylogenetic analysis we took 18S rDNA and ITS2-sequences of the *C. reticulata* strains. To build a tree we also used closely related species of *Chloromonas* genus, selected from the GenBank. Namely sequences from the Culture Collection of Algae at Goettingen University(SAG), the Culture Collection of Cryophilic Algae (CCCryo), Microbial Culture Collection (NIES) and the Culture Collection of Algae at the University of Texas at Austin (UTEX) were exploited (Table 1).

The alignments and phylogenetic trees were constructed using Mega 6 software (http://www.megasoftware.net). When comparing the sequences from 18S rDNA we could not build trees with other species from *Chloromonas* genus with highly supported branches since the gene fragment is highly conservative. In the work we present a tree which was built for internal transcribed spacer 2 (277 bp). This fragment is rapidly evolving marker and highly used in phylogenetic analysis on species and genera levels. We applied neighbor joining (NJ) and maximum

Table 1. List of taxa/strains included in the phylogenetic analyses of *Chloromonas reticulata*

Species	*Collection and strain number*	*GenBank accession number (ITS2)*
Chloromonas reticulata (Goroschankin) Gobi	SYKOA Ch-054-11	MF033356
Chloromonas reticulata	CCCryo 338-08	HQ404900
Chloromonas reticulata	CCCryo 213-05	HQ404885
Chloromonas reticulata	SAG 32.86	AB624574
Chloromonas reticulata	SAG 26.90	AB624575
Chloromonas chlorococcoides (H. Ettl & K. Schwarz) Matsuzaki, Y. Hara & Nozaki	SAG 16.82	AB624572
Chloromonas chlorococcoides	SAG 15.82	AB624570
Chloromonas chlorococcoides	SAG 12.96	AB624571
Chloromonas augustae (Skuja) T. Pröschold, B. Marin, U.W. Schlösser & M. Melkonian	SAG 5.73	AB624577
Chloromonas typhlos (Gerloff) Matsuzaki, Y. Hara & Nozaki)	CCCryo122-00	HQ404869
Chloromonas typhlos	CCCryo 214-05	HQ404886
Chloromonas fukushimae Matsuzaki & Nozaki	GsCl-11	AB906383
Chloromonas fukushimae	HkCl65	AB906384
Chloromonas tughillensis Hoham, Berman, H.S. Rogers, Felio, Ryba & P.R. Miller	UTEX SNO91	AB906387
Chloromonas hohamii H.U. Ling & Seppelt	UTEX SNO67	AB906394
Chloromonas tenuis Matsuzaki & Nozaki	UTEX SNO132	AB906395
Chloromonas rosae (H. & O. Ettl) H. Ettl	SAG 51.72	AB624576

likelihood estimation (ML) methods. Since both methods resulted in a similar outcome, here we present the trees obtained by NJ method. The default parameters for tree building were selected. Tamura-Nei model was selected for ML analysis. To align sequences we used ClustalW algorithm and the robustness of the resulting lineages was tested by bootstrap analysis with 1,000 replications. For NJ we used p-distance model.

RESULTS

The characterization of red snow

The red blooming of snow in the Subpolar Ural snowfield was caused by the massive development of biflagellate algae. The spots of the red snow were observed in the most parts of the snowfield. The depth of colored snow was more than 20 cm. The temperature of the snow surface where the red blooming was observed varied in the range of –1.1 to –3.3°C while of white snow it was lower – 2.5 – –3.8°C. The massive development of snow algae was appeared in the snow

Table 2. The chemical characteristics of red snow from the snowfield of the Subpolar Urals

Parameter	*The snow sample at the depth of 0-5 cm*	*The snow sample at the depth of 5-40 cm*	*Parameter*	*The snow sample at the depth of 0-5 cm*	*The snow sample at the depth of 5-40 cm*
pH	5.46 ± 0.20	6.20 ± 0.20	Mg, mg/dm^3	< 0.05	< 0.05
Conductivity, μS/cm	7.0 ± 0.7	8.0 ± 0.8	Na, mg/dm^3	0.21 ± 0.05	0.35 ± 0.08
Chromaticity, °	62 ± 6	40 ± 8	K, mg/dm^3	0.48 ± 0.11	0.42 ± 0.10
N-NH_4^+, g/dm^3	0.118 ± 0.026	0.15 ± 0.03	Fe, mg/dm^3	< 0.05	< 0.05
N-NO_3^-, g/dm^3	< 0.010	0.010 ± 0.006	Mn, μg /dm^3	4.0 ± 1.3	3.5 ± 1.1
$N_{total.}$, mg/dm^3	< 0.5	0.52 ± 0.09	Zn, μg/dm^3	5.5 ± 1.9	18 ± 6
HCO_3^-, mg/dm^3	< 6.1	6.2 ± 1.3	Cu, μg/dm^3	2.1 ± 0.9	1.8 ± 0.7
SO_4^{2-}, mg/dm^3	< 2.0	< 2.0	Pb, μg/dm^3	< 4.0	< 4.0
Cl^-, mg/dm^3	1.8 ± 0.4	2.0 ± 0.3	Cd, μg/dm^3	< 0.2	< 0.2
PO_4^{3-}, mg/dm^3	0.27 ± 0.04	< 0.05	Ni, μg/dm^3	< 1.0	< 1.0
$P_{total.}$, mg/dm^3	0.088 ± 0.028	< 0.020	Co, μg/dm^3	< 1.0	< 1.0
Si, mg/dm^3	0.099 ± 0.024	< 0.05	Sr, μg/dm^3	< 1.0	< 1.0
Ca, mg/dm^3	0.079 ± 0.019	0.121 ± 0.029			

Note: ± – standard deviation.

with slightly acid pH and low conductivity (Table 2). In the meltwater, the important elements and compounds for algal development were present, including N, P, Ca, Mg, etc. The accumulation of these elements in the snow occurs by dust emissions (cryoconit) from the adjacent landscapes where soil and mountain erosions happen (Takeuchi *et al.*, 2006).

The cells morphological characteristic *Chloromonas reticulata*

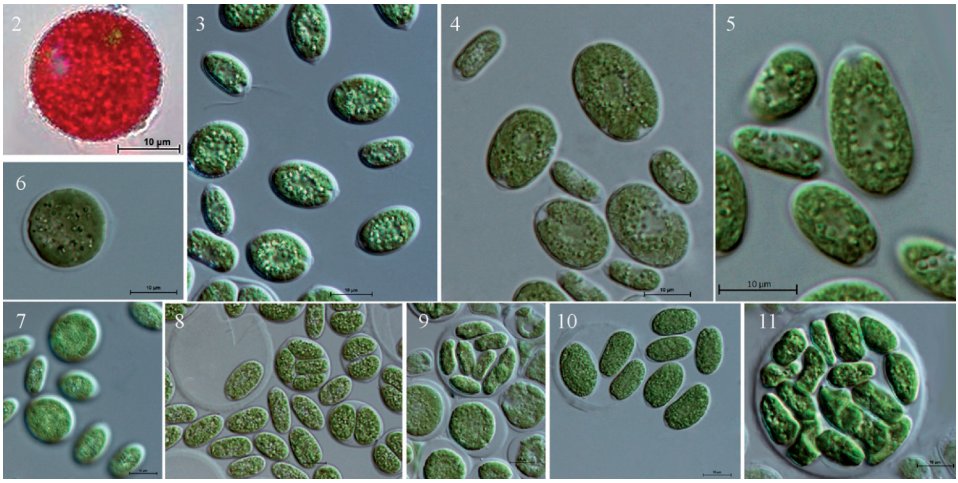
The algae cells in natural populations had round or oval shape, up to 26 μm in diameter, colored in bright red color and motionless (Fig. 2). The concentration of the cells per ml in melted water was 0.15-0.33 × 10^2-10^4, these results are close to the data obtained in the other studies – 2.2 × 10^2-10^6 cells per 1 ml^{-1} (Takeuchi *et al.*, 2006; Komárek & Nedbalová, 2007).

The strain was isolated on a standard medium 3N BBM. The strain was identified as *Chloromonas reticulata* (Fig. 3). Synonyms: *Chlamydomonas clathrata* Pascher; *Chloromonas clathrata* (Pascher) Korshikov ex H. Ettl; *Chloromonas palmelloides*. The *Chloromonas reticulata* strain is stored in the live collection of algae strains of Institute biology Komi Scientific Center RAS (Global Catalogue of Microorganisms GCM-1125) under SYKOA Ch-054-11 number (http://ib.komisc.ru/sykoa/collection/225/).

In the culture, the vegetative cells were unicellular, biflagellate, and ellipsoidal or elongate-ellipsoidal, 11-20 μm long and 5-15 μm wide; with age cells became round-shaped and reached 25 μm in diameter (Table 3).

The young cells had a slightly dorsoventral structure (Fig. 3). The cells contained one nucleus, a prominent papilla at the flagella base, a cup-shaped

chloroplast with perforations and branched incisions on the surface, the pyrenoids were not observed. The nucleus usually was located in the middle of the protoplast (Figs 4-5). The flagella were the same length as the cell (Fig. 3). The mobile algal phase was detected only for a short period. Two contractile vacuoles were located



Figs 2-11. Living cells of *Chloromonas reticulata.* **2.** Cell in the snow samples. **3-6.** The light microscopy of strain: 3-4 – Vegetative and young cells. 5 – Surface view of a cell showing eyespot (open arrowheads). 6 – The wall thickening and starch grains in the old cell (open arrowheads). **7.** Surface view of a cell showing irregular perforations and incisions. **8-10.** Zoosporangyes with zoospores: 8 – two; 9 – four; 10 – eight; 11 – aggregates of sixteen cells.

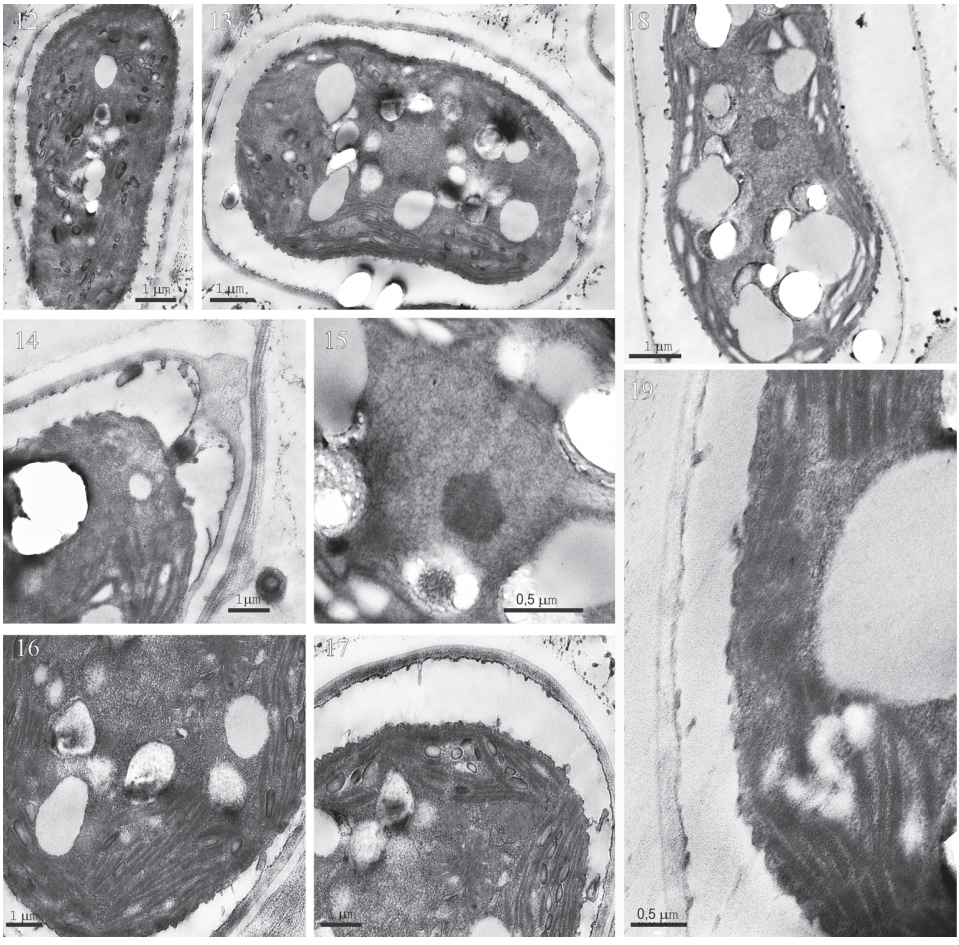
Table 3. The variation in length and width of *Chloromonas reticulata* cells in the culture during two months study period

Parameter	*Week*						
	1	*2*	*3*	*4*	*5*	*6*	*9*
Mean length (µm)	12.6 ± 2.08	13.15 ± 1.83	14.53 ± 1.81	14.53 ± 1.61	15.39 ± 2.47	16.13 ± 2.13	14.42 ± 1.71
Mean width (µm)	7.81 ± 2.06	8.0 ± 1.85	10.25 ± 2.23	10.69 ± 2.10	11.76 ± 3.61	13.37 ± 3.33	11.62 ± 2.35
The variation of cell lengths in %	17	14	12	11	16	13	12
Maximal length (µm)	18.22	20.37	21.14	19.55	23.78	24.77	20.69
Maximal width (µm)	9.82	9.10	11.09	10.90	11.12	12.36	11.16
Minimal length (µm)	17.05	14.88	20.06	17.35	23.03	24.29	20.59
Minimal width (µm)	5.33	5.27	5.93	6.42	6.09	6.50	6.5

Note: ± – standard deviation.

near the flagella base. The eyespot was narrow and oblong, on the anterior 1/3 of the cell (Fig. 5). The cell wall is thicker by 1-2 μm in the round cells (Fig. 6). The asexual reproduction occurred via zoospore formation producing 4 or 8 (sometimes 2) biflagellate zoospores within the wall of the parental cell, in old cultures were observed also aggregates of 16 cells (Figs 8-11, 20). The reproduction occurs by division in the longitudinal direction with a rotation of protoplast. The sporangia were 25 to 45 μm, 55 μm in the cultures cultivated for more than one year. The old culture remained green.

The studied strain differed from the diagnosis of the heterotypic synonym of *Chlamydomonas clathrata* (Korsch.) Pascher (Dedusenko-Shegoleva *et al.*, 1959) by the absence of mucus, the shorter flagella and narrower cells. The presence of



Figs 12-19. Cellular ultrastructure of *Chloromonas reticulata.* **12-13.** Vegetative cells with highly enlarged periplasmic space. **14.** Apical part of a cell, covered by mother cell wall, showing cell wall, prominent papilla and some details of flagellar apparatus. **15, 18.** A part of a cell with nucleus and surrounding numerous vacuoles. **16.** Cell part with numerous thylakoids organized in packs and some starch grains. **17.** Numerous starch grains localized in chloroplast lobes. See Golgi body in cytoplasm. **19.** Details of chloroplast lobe. Thylakoids packs enter in the light chloroplast stroma.

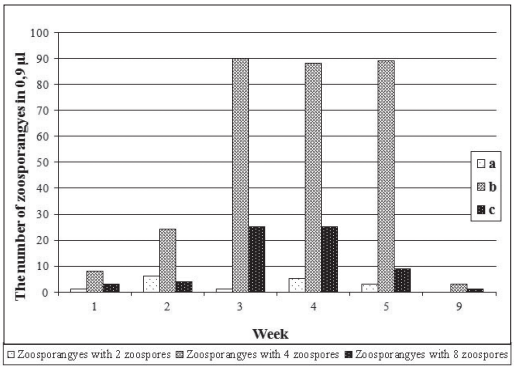


Fig. 20. The proportion of 3 groups of zoosporangyes (based on number of zoospores) in 2-month study period by weeks. Zoosporangyes with: a – 2, b – 4, c – 8 zoospores.

spherical cells and bigger cell sizes of the studied strain are not typical for the described *Chloromonas clathrata* Korshikov SAG 29.83 (Boldina, 1998) and *Chloromonas palmelloides* strains (Ettl and Gärtner, 2014). Our strain differs from the strain *Chloromonas reticulata* described in the article (Matsuzaki *et al.*, 2012) by the larger size of the old cells and variations in a number of zoospores (not only 4-8 but also 2) and by aggregates of 16 cells observed in old cultures.

The ultrastructure of the *Chloromonas reticulata* cells

The ultrastructural study of vegetative cell walls revealed that the outer capsular layer was practically invisible. Its presence was marked by thin fibrillar structures founded in the papilla area (Fig. 14) and other parts of the cell (Fig. 17). At the same time, two other layers, thin (0.03 μm, formed by two dark lamellae separated by the light one) and thick (0.3 μm, internal, fibrillar) were well developed. A characteristic subunit structure or oblique parallel striation was noticeable in the composition of the thin trilamellar layer. The thickness of the trilamellar layer was stable, although the thickness of inner layer changed and became the largest at the papilla area, reaching 1.5 μm (Fig. 14). The plasmalemma was characterized by curly outlines. It evenly surrounded the cell, did not form a protuberance, and did not participate in the formation of papilla. In some sections, the H-shaped transition zone and elements of the radial flagellate system were distinguishable. The chloroplasts contained predominantly straight and relatively short thylakoids, usually 3 (4)-6 in each pack (Figs 12-17). The larger packs were found less often. In the stroma, starch grains were small (0.1-0.3 μm) and numerous (about 40 per section). The starch grains were evenly located between the packs of thylakoids (Fig. 19). They often formed characteristic clusters in the thickenings of the chloroplast lobes. They were characterized by a geometrically irregular shape close to the fusiform. The stigma was not seen on the sections and pyrenoid was absent. The small parts of the stroma without thylakoids and increased concentration of starch grains in the thickening of chloroplast (Figs 13-17) without a change in the electron density were detected. A complex chromocentric nucleus (Fig. 15) and nucleolus with mosaic-organized fibrillar and granular components were observed. Mitochondria were noted mainly in the central part of the cell and at the base of the flagella apparatus. They had the appearance of numerous short profiles. Cristae were lamellar. The Golgi apparatus was found near the nucleus (Fig. 17): it contained 1-2 dictyosomes (per section) with up to 10 cisterns and numerous bubbles surrounding them. In addition to contractile vacuoles, vacuoles with transparent and/or fibrillar contents were seen (Figs 13, 15-17).

The comparison of the strain ultrastructure to the closely related species (which were previously placed in *Reticulata* group) is presented in the Table 4. The ultrastructure of the studied strain is almost identical to that of previously described

Table 4. Ultrastructural specificities of some *Chloromonas* spp. of *Reticulata* – group

Name	*Cell wall*	*Plasma-lemma*	*Chloroplast*			*Mito-chon-dria*
			Thylakoids	*Pyrenoid*	*Starch grains*	
Chloromonas reticulata SYKO-A	outer layer weak or invisible, median clear	curly outlines	straight, short, 3-6 in each pack	absent, but present thylakoid-lacking spaces	irregular shape close to the fusiform	central
Chloromonas reticulata SAG 29.83[2-4] SAG 32.863	outer layer weak, median clear	curly outlines	straight, short, 3-6 in each pack	absent, present thylakoid-lacking spaces	irregular shape close to the fusiform	central
Chloromonas chlorococcoides SAG 15.82[1,3]	outer layer visible, median poorly visible, sometimes clear[1]	curly outlines	straight, short, 3-5 or more in each pack[1]	present, contains pyrenoglobules[1,3] and peripheral thylakoid packs[1]	of different shape[1]	central[1]
Chloromonas rosae SAG 51.72[3]	outer layer visible, median clear, thick inner	smooth outlines, sometimes curly	straight, short	absent, but present thylakoid-lacking spaces	of different shape	central
Chloromonas typhlos SAG 26.86[3,5]	outer layer visible, median clear[3]or poorly visible[5]	curly outlines	straight and curved, long and short, 2-7 in each pack	present, contains central thylakoid packs[5]	of different irregular shape	central

Source: 1. Boldina, 2008; 2. Boldina, 1998; 3. Matsuzaki *et al.*, 2012; 4. Watanabe *et al.*, 2016; 5. Boldina, 2017.

strain SAG 29.83 of *Chloromonas reticulata* (Boldina, 1998; Matsuzaki *et al.*, 2012). Small differences revealed in the width of the inner layer in the papillae region (1.5 μm and 0.8 μm, respectively) are possibly associated with changes in intracellular pressure and the effect of plasmolysis. Differences in the amount of grain starch in the stroma (approximately 40 per cut and 80, respectively) are not basic and reflect the conditions of cultivation.

Molecular phylogenetic analyses

The analysis of ITS2 fragment has shown that the strain belongs to *Chloromonas* clade (Fig. 21) which has cryophil species of the genus: *C. reticulata* (CCCryo 338-08, CCCryo 213-08, SAG 26.90, SAG 32.86), *C. rosae* (SAG 51.72), *C. chlorococcoides* (SAG 12.96, SAG 15.82, SAG 16.82) and *C. typhlos* (CCCryo 122-00, CCCryo 214-05) (Hoham*et al.*, 2002; Matsuzaki *et al.*, 2012). This is a, monophyletic group (Matsuzaki *et al.*, 2012) which unifies similar species in molecular aspects and most of morphological characters. As phylogenetic analysis has shown, our strain was most similar to *C. reticulata* strain SAG 32.86 (99% BV in ML and NJ analyses) by a studied fragment of nuclear ITS2.

The second clade includes *Chloromonas fukushimae* (GsCl-11; HkCl65), *C. tughillensis* UTEX SNO91, *C. hohamii* UTEX SN067, *C. tenuis* UTEX SN0132 – snow-inhabiting *Chloromonas* species with elongate or ellipsoidal vegetative cells

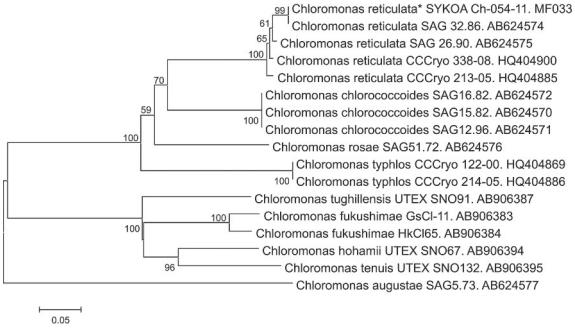


Fig. 21. Phylogenetic tree built using NJ method based on ITS2 sequence. Bootstrap values (NJ) are shown for each node with values above 50%.
*- the studied strain.

(Matsuzaki *et al.*, 2014). The strain of *C. augustae* (SAG 5.73) is separated from the main group. This species has pyrenoid and belongs to a sister group which is confirmed by other authors (Pröschold *et al.*, 2001; Matsuzaki *et al.*, 2012).

The genus *Chloromonas* Wille is isolated from the genus *Chlamydomonas* due to lack of pyrenoid, but the accepted separation is artificial, currently, the taxonomy of these genera is revised (Pröschold *et al.*, 2001; Nakada *et al.*, 2008). It is believed that the evolution of these organisms was influenced by harsh conditions of cold environments (Hoham *et al.*, 2002).

DISCUSSION

The algae causing red blooming in natural habitats have similar morphology and, thus, are hard identificate to species level. Therefore, a complex approach is exploited. It includes study of hypnospores and zygotes, collected on the snow and ice surface, as well as, the investigation of cells in culture under light and transmission electron microscopes, and by molecular and genetic methods (Muramoto *et al.*, 2008; Matsuzaki *et al.*, 2014).

The *Reticulata* group includes species with similar morphological and ultrastructural characteristics (Matsuzaki *et al.*, 2012) which makes difficulties with their identification. The detailed comparison of our strain characteristics with other species of *Chloromonas* showed that *C. reticulata* differs from the closest species by a form of the cell and papilla. In *Reticulata*-group there are some peculiarities in papilla structure: hemispherical in *C. reticulata,* obtuse and cone-shaped in *C. rosae* and hemispherical with a flattened top in *C. typhlos.* It could be separated from *C. chlorococcoides* and *C. typhlos* by the absence of pyrenoid, larger cell size, a form of stigma, and a number of zoospores (Matsuzaki *et al.*, 2012; Boldina, 2017).

The cellular ultrastructure of *Chloromonas reticulata* and many species of *Reticulata*-group is very close. Minor differences are visible only in its cell wall components, plasmalemma outlines and organization of thylakoid system in the chloroplast, as well as starch form and location in it. But *C. reticulata* can be easily distinguished from close species *C. chlorococcoides* and *C. typhlos* by the absence of normal pyrenoid with dark stroma, starch envelope and thylakoids. Besides, pyrenoids of *C. chlorococcoides* contain lipid bodies (Matsuzaki *et al.*, 2012) and peripheral thylakoid packs (Boldina, 2008). In comparison with *C. reticulata* the cells of *C. rosae* and *C. chlorococcoides* have more clearly visible outer layer but only *C. rosae* contains clear median layer and smooth outlines of plasmalemma (Table 4).

The molecular phylogenetic analyses have confirmed that the species belonged to the *Reticulata* group of *Chloromonadinia* clade with high statistical support.

Our study has shown that the red blooming of snow in the Subpolar Urals was caused by biflagellate *Chloromonas reticulata* as our morphological, ultrastructural and molecular phylogenetic analyses have shown. This microalga belongs to a highly adaptive species which has a wide distribution from the Arctic to Antarctic. The species is noted in clean and polluted waters, on the snow and in the ice, probably, it was also found in the soils (Dedusenko-Shchegoleva *et al.*, 1959; Matsuzaki *et al.*, 2012; Ettl & Gärtner, 2014). The concentration of the cells in the snow samples reached up to 0.33×10^4 cells per ml. The temperature of snow was slightly higher in places where the blooming was observed if compared to a clear white snow. The massive development of the algae is caused by the accumulation of cryoconite in upper horizons which is rich in N, P, Ca, Mg and other compounds necessary for the algal growth.

The studied strain had slight deviations from the described morphological parameters in essential monographs (Dedusenko-Shchegoleva *et al.*, 1959; Matsuzaki *et al.*, 2012, Ettl & Gärtner, 2014): it had high variation in number of zoospores in zoosporangia, the absence of mucus around the wall, the smaller flagella, and thickening of the inner layer near papilla, as well as the lower number of starch grains in stroma (around 40). Taking into account all the results, we could conclude that distinctions in morphological characteristics could be attributed to a latitudinal gradient differences and ecological conditions.

The article supplements the existing data on the species. The further investigations are needed for *Chloromonas reticulata* species to evaluate its contribution to the processes of glaciers and snowfield melting in the north regions of the Ural Mountains.

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