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Research Article

RESEARCH ARTICLE

Diversity of carotenogenic microalgae in the White Sea polar region

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One sentence summary: Strains of carotenogenic microalgae were isolated in the White Sea polar region.

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ABSTRACT

Carotenogenic microalgae are unicellular photosynthetic organisms with the ability to accumulate carotenoids. Carotenoid accumulation is a protective reaction against environmental stress factors, such as bright light and extreme temperatures. It makes the survival of these microorganisms under harsh environmental conditions possible. The diversity of carotenogenic microalgae has been described in detail for Central Europe and North America, as well as for tropical and subtropical latitudes with relatively favorable environments. However, data about these microorganisms in polar and subpolar latitudes are scarce and restricted to few reports. We isolated several strains of carotenogenic microalgae from the coastal zone of the White Sea, where they were abundant. The obtained microalgae related to four species of Chlorophytes: *Haematococcus lacustris*, *H. rubicundus*, *Coelastrella aeroterrestrica* and *Bracteacoccus aggregatus*. The last three species have been reported for polar latitudes for the first time. Most likely, carotenogenic algae in the White Sea coast are abundant due to their high physiological and metabolic plasticity, which is essential for surviving under adverse conditions of the northern regions. Pigment composition of the strains is provided. Their predominant carotenoids were astaxanthin and β-carotene. Further, the obtained strains may be considered as potential producers of natural pigments for biotechnology.

Keywords: carotenogenic algae; biodiversity; *Bracteacoccus*; *Coelastrella*; *Haematococcus lacustris*; *Haematococcus rubicundus*

Abbreviations

BI:	Bayesian inference-based approach of phylogenetic analysis
CBC:	compensatory base changes
ITS:	internal transcribed spacers of nuclear ribosomal gene cluster
ITS1-5.8S rRNA-ITS2:	fragment containing ITS 1 and 2 and 5.8*S*rRNA gene of the nuclear ribosomal gene cluster
ML:	maximum likelihood algorithm
MP:	maximum parsimony algorithm
NJ:	neighborhood joining algorithm
PCR:	polymerase chain reaction
R_f:	retardation factor
TLC:	thin layer chromatography

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INTRODUCTION

As a rule, in a photoautotrophic cell, carotenoids are coupled with photosynthetic apparatus (Lichtenthaler 1987; Cunningham and Gantt 1998). Pigment–protein complexes involved in light-driven electron transport are characterized by exact stoichiometry between different photosynthetic pigment groups. Consequently, the amount of carotenoids is under strict control of the cell (Lichtenthaler 1987; Giuliano, Bartley and Scolnik 1993; Cunningham and Gantt 1998; Takaichi 2011). However, some organisms are able to synthetize secondary carotenoids, which are not functionally and structurally coupled with a photosynthetic apparatus. This phenomenon is called secondary carotenogenesis. In this case, it is possible for a cell to accumulate a high amount of carotenoids (Boussiba 2000; Jin, Lee and Polle 2006; Lemoine and Schoefs 2010; Han, Li and Hu 2013; Solovchenko 2013).

Secondary carotenogenesis has been reported for some species of unicellular algae (so-called carotenogenic microalgae) (Boussiba 2000; Orosa *et al.* 2000; Jin, Lee and Polle 2006; Lemoine and Schoefs 2010; Takaichi 2011; Han, Li and Hu 2013; Solovchenko 2013). Carotenogenic microalgae are widely distributed among Chlorophyceae (Orosa *et al.* 2000; Kostikov *et al.* 2001). Each order of Chlorophyceae includes at least one representative accumulating high amount of carotenoids (Kostikov *et al.* 2001). They accumulate β-carotene or xanthophylls (ketocarotenoid astaxanthin and/or intermediates of its synthesis) (Orosa *et al.* 2000; Takaichi 2011; Minyuk *et al.* 2017). Due to the high practical value of carotenoids, microalgae are a potential subject of biotechnological cultivation (Olaizola and Huntley 2003; Han, Li and Hu 2013; Solovchenko and Chekanov 2014). However, at present, only two species of carotenogenic microalgae are cultivated on commercial scale: *Haematococcus lacustris* (formerly *H. pluvialis*) (Olaizola and Huntley 2003) and *Dunaliella salina* (Borowitzka and Borowitzka 1990)—for obtaining astaxanthin and β-carotene, respectively. These microorganisms are characterized by some limitation for industrial use, such as low growth rate or low resistance to contamination (Solovchenko and Chekanov 2014). Thus, screening of new natural isolates and assessment of their biotechnological potential are essential. Particularly, the ability to undergo secondary carotenogenesis and pigment accumulation under stress has been described for microalgae from the *Chromochloris* (Chromochloridaceae) (Ip and Chen 2005), *Coelastrella* (former *Scotiellopsis*) (Scenedesmaceae) (Abe, Hattori and Hirano 2007; Minyuk *et al.* 2016, 2017) and *Bracteacoccus* (Bracteacoccaceae) (Minyuk, Chelebieva and Chubchikova 2014) genera.

Carotenogenic algae are cosmopolitan. A number of such microorganisms are found in tropical and temperate latitudes with a relatively mild climate (Droop 1953; Proctor 1957; Kostikov *et al.* 2001; Chelebieva *et al.* 2018). Due to a high degree of phenotypic plasticity and high resistance to a wide range of adverse conditions, such as high light intensity, osmotic stress and mineral nutrient deficiency, carotenogenic microalgae are observed in different extreme environments (Takaichi 2011; Solovchenko 2013). They develop there due to the absence of natural competitors. Therefore, massive growth of carotenogenic algae colonies is often observed under adverse conditions, e.g. snow and ice algae (in Arctic and snow highlands) (Tschaikner, Gärtner and Kofler 2008; Uzunov *et al.* 2008; Klochkova *et al.* 2013; Chelebieva *et al.* 2018). The presence of the cells of *Chloromonas nivalis* (Volvocales) on the snow and ice causes a well-known phenomenon, 'blood snow' (Remias, Lütz-Meindl and Lütz 2005). *Dunaliella salina* is a typical microorganism in water ponds with a very high salinity (Borowitzka and Borowitzka 1990).

The White Sea is located on the border of the northern polar region and includes subpolar zones. Biodiversity of the microalgae from the White Sea region is scarcely described. Especially, there are few studies with data on the molecular identification and systematics of microalgae from this region. Previously, green microalgae (Gorelova *et al.* 2012, 2015) and cyanobacteria (Gorelova *et al.* 2009) associated with White Sea invertebrates and lichens (Chekanov, Feoktistov and Lobakova 2017), as well as free-living green microalgae (Chekanov *et al.* 2014; Solovchenko *et al.* 2016; Ismagulova *et al.* 2018) were characterized. Cryptomonads from the *Rhodomonas* genus were identified in the meromictic lakes (Krasnova *et al.* 2014, 2015). Based on metagenomic data, picoalgae of the White Sea were evaluated (Belevich *et al.* 2015). Kublanovskaya *et al.* (2019) described cyanobacterial diversity in the rock baths on the coast of Kandalaksha Bay. In the present study, we focused on the isolation and characterization of the strains of carotenogenic microalgae gained from White Sea region located in the vicinity of N.A. Pertsov White Sea Biological Station of Lomonosov Moscow State University (66°34′N; 33°08′E). The study gives new data on the distribution of carotenogenic microalgae and presents new strains, which can be used for carotenoid production.

MATERIALS AND METHODS

Samples collection

Samples containing microalgae were collected in the summers (July, August) of 2011–2016 in the vicinity of N.A. Pertsov White Sea Biological Station (WSBS) of Lomonosov Moscow State University (66°34′N; 33°08′E) located in Kandalaksha Bay of the White Sea (Louhi Region, Republic of Karelia, Russia). There were the following sample stations: WSBS territory, Bays on the Kindo peninsula near WSBS (Biofilters' Harbor and Probkina Gubka Bay), Upper Yershovskoye Lake (a duct to the lower one), a stone quarry near the village of Nil'moguba, Pokormezhny and Kast'yan Islands (Fig. 1, Table 1). The sampling was conducted in the presence of orange or red coloration. The samples collected were water from ponds or rock baths in the coastal zone of the sea or dry crusts on the rock surface. Some of them were colonies of carotenogenic algae (natural monoalgal cultures) and some were associations of different phototrophs. Liquid samples were collected in the 15-ml sterile plastic tubes. Dry crusts were scraped by a steel scalpel or a razor and transferred into plastic bags.

Algal strains isolation

Two methods of algal strains isolation were used in the work (for dry and liquid samples) (Fig. 2). In the case of the liquid samples (Fig. 2A and B) 100–500 μl of each of them were inoculated onto solid mineral BG-11 medium (Stanier *et al.* 1971) containing 1.5% (wt/v) agar immediately after collection. Dry samples (Fig. 2C and D) were transferred into 2–5 ml of sterile BG-11 medium and incubated for one to two months under daylight (~40 μmol/m^2/s) at room temperature. Then, 100–500 μl of algae-containing suspension were inoculated onto solid BG-11 medium. In both cases, cells were incubated in Petri dishes with solid BG-11 medium from one week to three months. After single algal colonies on the surface of the medium appeared, they were transferred into 2 ml of liquid BG-11 medium by a micromanipulator and incubated for one month at 25°C under daylight.

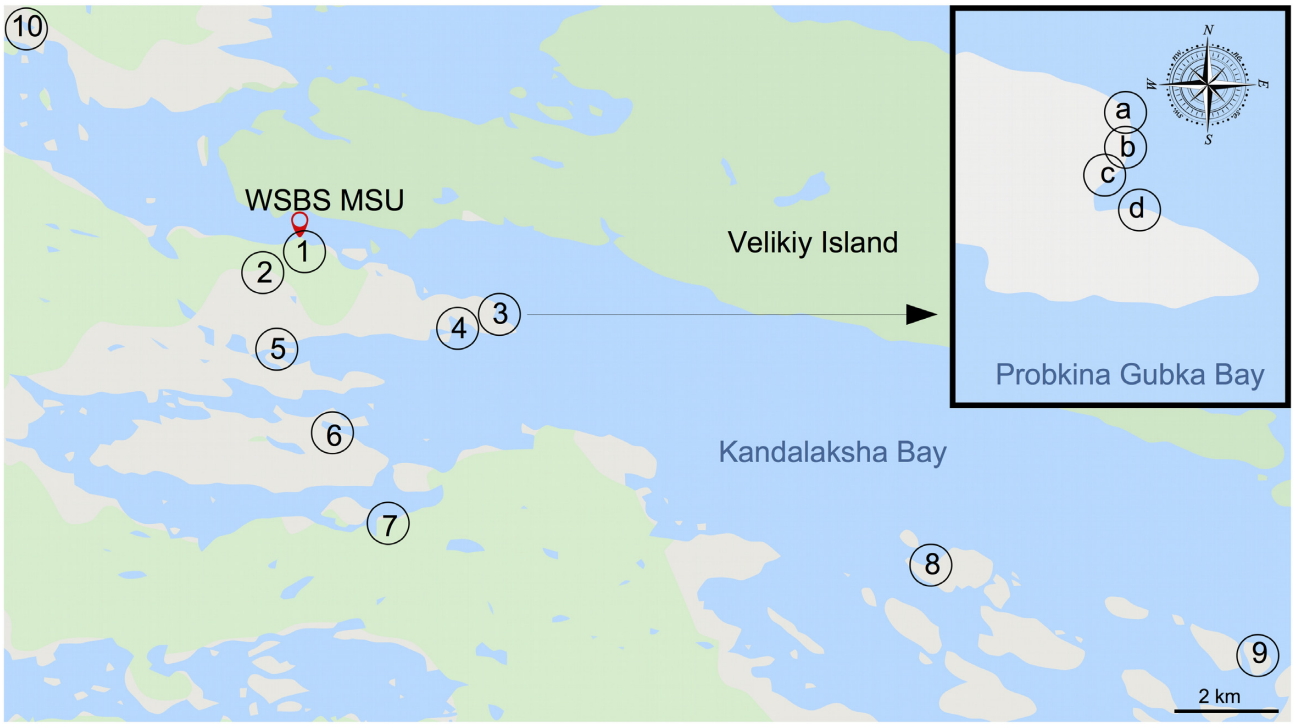

Figure 1. Sampling locations of the study: map of the Rugozerskaya Gulf of the Kandalaksha Bay, White Sea. 1 and 2: WSBS MSU, 3: Probkina Gubka Bay, Maria's Stones (a: BMM1, b: BMM2, c: BMM3, d: BMK), 4: Biofilters' Harbor, 5: Upper Yershovskoye Lake, 6: Olenevsky Island, 7: Nil'moguba, 8: Kast'yan Island, 9: Pokormezhny Island, 10: Trehtsvetnoye Lake (three-colored lake).

Table 1. Summary of the sampling locations of carotenogenic microalgae in the region of Kandalaksha Bay of the White Sea.

Sample	Location	Coordinates	Sample description	Date
BM1	Kast'yan Island	66°29′47.3′N 33°22′57.4′E	Drying rock pond filled with salty water with red crust	2011
BM7/13	Nil'moguba (Career)	66°30′10.2′N 33°06′11.8′E	Dry crust on the rocks	2013
BM4	Olenevskiy Island	66°30′51.5′N 33°04′53.4′E	Red crust on the rocks	
BM6/13	WSBS	66°33′12.9′N 33°06′08.3′E	Biofilm in reddish water (0 ‰)[a]	
BMM1/14	Maria's Stones	66°32′28.1′N 33°11′07.3′E	Water in the birdbath (Maria's stone) (1 ‰)	2014
HELL-1	Harbor of Biofilters	66°32′27.8′N 33°10′01.5′E	Reddish crust on the piece of styrofoam	
HELL-2	Harbor of Biofilters	66°32′27.8′N 33°10′01.5′E	Reddish crust on the piece of styrofoam	
BMM1/15	Maria's Stones	66°32′28.1′N 33°11′07.3′E	Water from a birdbath (Maria's stone) (2 ‰)	2015
Lake	Lake Trehtsvetnoye (three-colored lake)	66°35′31.8′N 32°58′46.1′E	Water from the upper layer of meromictic lake (0 ‰)	
BMK/15	Probkina Gubka Bay	66°32′25.5′N 33°11′01.8′E	Reddish water with a skeleton of the finfish (0 ‰)	
BM5/15	Upper Yershovskoye Lake, the flow through	66°32′07.6′N 33°03′58.6′E	Water from the lake (6 ‰)	
BMM2/15	Maria's Stones	66°32′28.1′N 33°11′07.3′E	Water from a birdbath (Maria's stone) (2 ‰)	
BM7/15	WSBS	66°33′12.9′N 33°06′08.3′E	Fresh water on the rocks (0 ‰)	
BMP/16	Pokormezhny Island	66°28′43.0′N 33°31′24.5′E	Water from a birdbath (130 ‰)	2016
BMK/16	Probkina Gubka Bay	66°32′25.5′N 33°11′01.8′E	Water from a birdbath (0 ‰)	
BMM1/16	Maria's Stones	66°32′28.1′N 33°11′07.3′E	Water from a birdbath (Maria's stone) (3 ‰)	
BMM2/16	Maria's Stones	66°32′28.1′N 33°11′07.3′E	Biofilm from a birdbath (Maria's stone) (3 ‰)	
BMM3/16	Maria's Stones	66°32′28.1′N 33°11′07.3′E	Water from a birdbath (Maria's stone) (5 ‰)	

[a]For liquid samples, salinity is also provided.

Microscopy and pigment analysis

The cells from the cultures and environmental samples were studied by bright field microscopy on an Eclipse 90i (Nikon, Tokyo, Japan) motorized photomicroscope. Algal colonies on the solid medium were investigated using a Nikon SMZ745T stereomicroscope (Nikon, Tokyo, Japan) with a white LED illuminator Nikon C-FiD and a MMC31C12-M camera (Nikon, Tokyo, Japan) (magnification up to 3.5).

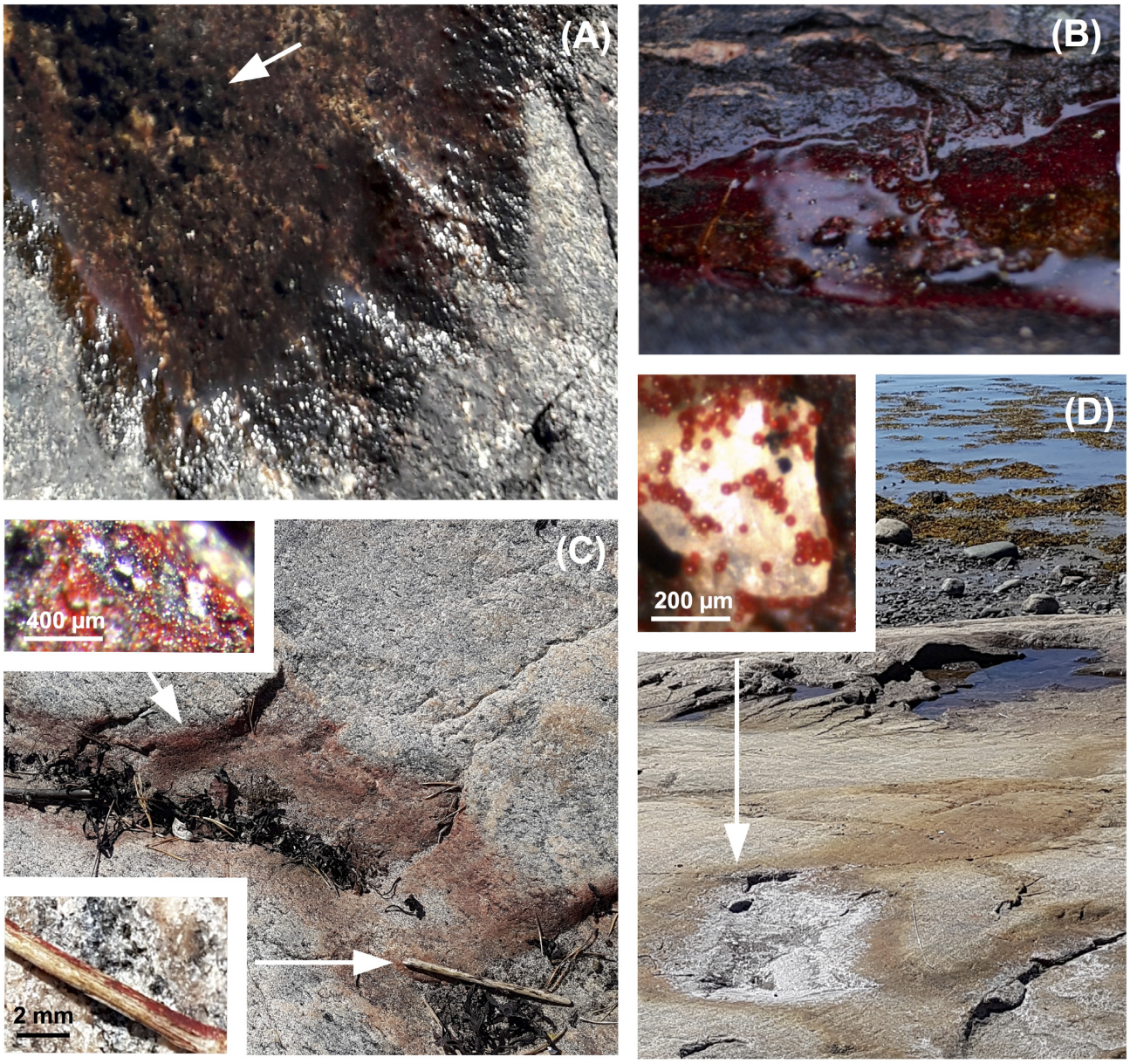

Figure 2. Locations of the carotenogenic microalgae containing samples collection: rock baths with fragments of a biofilm (an arrow) and soil **(A)** and reddish water **(B)** on the Maria's Stones, dry crusts formed by carotenogenic microalgae colonies on the Olenevsky Island on the surfaces of coastal rock and a wooden stick **(C)**, a dried saline rock bath on the Pokormezhny Island with carotenogenic algae attached to the sea salt crystal surface **(D)**.

For carotenoid synthesis induction 40 ml of algal suspensions in the BG-11 medium in sterile polypropylene cell culture flacks T25 (Eppendorf, Hamburg, Germany) were transferred to bright cold-white light (500 μmol quanta/m^2/s) as measured with a LiCor 850 quantum sensor (LiCor, Lincoln, NE, USA) in front of the flack and incubated for 5 days. Pigments were extracted with a chloroform–methanol mixture according to Folch, Lees and Sloane Stanley (1957). Pigment separation was performed by thin layer chromatography (TLC) on silica gel plates Silufol (Kavalier, Prague, Czech Republic) using the separation system of organic solvents from Chekanov *et al.* (2014): hexane:chloroform:benzene = 10:20:1 (by volume). Separated pigments were eluted from silica gel by chloroform. Pigments were determined by the shape of their absorbance spectra in chloroform (Britton 2008) on an Agilent Cary 300 (Agilent, Santa Clara, CA, USA) spectrophotometer in standard 1-cm quartz cuvettes and by their retardation factor (R_f). Their concentration in chloroform solutions was determined spectrophotometrically using the equations from Wellburn (1994).

Phylogenetic analysis

Molecular identification of the strains was performed based on fragments containing internal transcribed spacers (ITS) 1 and 2 and the 5.8*SrRNA* gene of the nuclear ribosomal gene cluster (ITS1-5.8S rRNA-ITS2 fragment). Genomic DNA was extracted using a MagJET Plant Genomic DNA Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The fragment ITS1-5.8S rRNA-ITS2 was amplified by polymerase chain reaction (PCR) and sequenced as described previously

(Ismagulova et al. 2018). In addition, some strains were pre-identified based on 18S rRNA (Chekanov et al. 2014) and/or rbcL (Gorelova et al. 2015) gene fragments.

Homologous sequences were searched in the NCBI GenBank database using the heuristic local alignment algorithm BLAST (http://blast.ncbi.nlm.nih.gov/) (Altschul et al. 1997). ITS1-5.8Sr RNA-ITS2 sequence data was analyzed in MEGA X (Kumar et al. 2018). Multiple alignment was obtained by the MUSCLE (Edgar 2004) global alignment algorithm with default settings. There were three datasets of aligned sequences for subsequent phylogenetic analysis: for families Scenedesmaceae (13 sequences, 631 positions), Haematococcaceae (31 sequences, 650 positions) and Chlorococcaceae (21 sequences, 904 positions). All positions with <5% site coverage were not included in subsequent analyses. Phylogenetic trees were constructed by the neighborhood joining (NJ) algorithm (Saitou and Nei 1987), maximum parsimony (MP) algorithm (Swofford 2003) with initial tree obtained by random addition of sequences, and maximum likelihood-based (ML) algorithm (Aldrich 1997) with an initial tree obtained by NJ. Heuristic search of optimal trees was performed by using subtree-pruning-regrafting method (Nei and Kumar 2000) approach. The best DNA evolution models for ML phylogenetic analysis were selected under Bayesian criterion (Nei and Kumar 2000). They were as follows: Tamura three-parameter model (Tamura 1992) for Haematococcaceae and Bracteacoccaceae datasets and Kimura two-parameter model (Kimura 1980) for the Scenedesmaceae dataset. In all the models, a discrete Gamma distribution was used for model evolutionary rate differences among sites (five categories). Robustness of topology of the trees obtained by NJ, MP and ML was estimated by the bootstrap method (Felsenstein 1985) with 1000 replicates. In addition, phylogenetic relationships were confirmed by the stochastic Bayesian inference (BI) using the Metropolis coupled Markov chain Monte Carlo method (Altekar et al. 2004) in MrBayes 3.2 (Ronquist et al. 2012) software. Two independent runs (three heated and one cold chain) were started. Total of 10 000 000 generations were included in each run. Chain sampling was performed every 100 generations; the first 25% samples of the cold chain run were excluded from consideration. At the end of the analysis, the average standard deviation of split frequency was <0.005. Optimal model of DNA evolution in BI analysis was selected as explained by Ismagulova et al. (2018).

ITS secondary structure prediction

The sequences of ITS1-5.8S rRNA-ITS2 were annotated based on multiple alignments with the sequences from the NCBI GenBank with provided annotations. In addition, for annotation of ITS2 fragment the ITS2 rRNA database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) was used (Ankenbrand et al. 2015).

Secondary structures of ITS fragments were predicted by the Zuker energy minimization on an mfold 2.3 (http://unafold.rna.albany.edu/) server (Zuker 2003) at a folding temperature of 300 K and ionic strength provided by the addition of 1 M NaCl. Folding Gibbs free energies were calculated by energy rules from SantaLucia (1998). Secondary structures were aligned using LocARNA (Will et al. 2012) on the http://rna.informatik.uni-freiburg.de/ server (Smith et al. 2010) under the conditions set as default. Compensatory base changes (CBC) (Coleman 2003) were calculated in CBCAnalyzer (Wolf et al. 2005). The structures were visualized using the ViennaRNA 2.0 package (Lorenz et al. 2011).

RESULTS AND DISCUSSION

Distribution of carotenogenic microalgae in the vicinity of N.A. pertsov biological station

Colonies of carotenogenic microalgae were abundant in the coastal zone of the Kandalaksha Bay region of the White Sea. There were a total of 18 samples containing unicellular green carotenogenic microalgae (Table 1). As a rule, they were represented by dry crusts or reddish water in the coastal ponds (bird-baths). In one sample station (Maria's Stones), dry or wet crusts were observed for several years in a row. In some cases, reddish color characterized biofilms in the water ponds. In the most of the samples, there were natural monoalgal cultures, however sometimes other phototrophs were also present (cyanobacteria, diatoms, conjugates and other chlorophytes). Based on the cell morphology and the ability to accumulate red pigment, most of the microalgae were described as *Haematococcus* spp. (Chlorophyceae, Volvocales). In the coastal ponds they were represented by motile biflagellated zoospores, green coccoid vegetative cells and autosporangia. In the dry samples, microalgae were represented only by red-colored aplanospores (so-called haematocysts). Cells of carotenogenic microalgae were attached to various substrates. In some cases, they were associated with sea salt crystals and formed colonies on the surface of rocks, a wooden stick, soil and a piece of styrofoam (Fig. 2, Table 1). The samples strongly differed by their properties: the presence of water and its salinity, and the substrate from which they were isolated (Table 1). Thus, carotenogenic microalgae were adapted to survive under a wide range of environmental conditions.

Isolation of the strains and morphological characteristics

In most of the samples, the carotenogenic microalgae were almost the only phototrophic microorganisms. In some cases the samples contained cyanobacteria typical for this habitat (Kublanovskaya et al. 2019) and heterotrophic microorganisms, such as amoebas and ciliates, as well as micromycetes and lichen spores (Chekanov, Kublanovskaya and Lobakova 2019). During the cultivation on solid BG-11 medium red-colored colonies of carotenogenic microalgae were formed (Fig. 3A and C). In the case of cyanobacteria-containing samples, their colonies also presented (Fig. 3A). It was necessary to perform additional separation of cyanobacteria and carotenogenic algae. It was easy to obtain monoalgal cultures of carotenogenic microalgae due to the red coloration of the cells. After reseeding of individual microalgal cells on new Petri dishes, vegetative green cells appeared first and became red during the development of aplanospores in two weeks (Fig. 3B and C).

Based on morphological analysis, most of the strains (except for HELL-2 and BM5/15) corresponded to the description of the genus *Haematococcus* (Chlorophyceae, Volvocales) of carotenogenic microalgae (Fig. 3D–G and J) (Buchheim et al. 2013; Allewaert et al. 2015; Guiry and Guiry 2019). They were represented by red spherical aplanospores (also termed haematocysts) (Fig. 3J). Their diameter varied from 10 to 90 μm. The large size of these cells was due to the low division frequency and vigorous accumulation of triacyl glycerols (Chekanov et al. 2014). Most of the liquid samples also contained green coccoid vegetative cells (Fig. 3G), autosporangia with dividing cells (Fig. 3D) and motile cells (zoospores) (Fig. 3E and F). Vegetative cells were characterized by a smaller variability of their diameter (10–40 μm). Sometimes they formed palmelloid clusters. Two

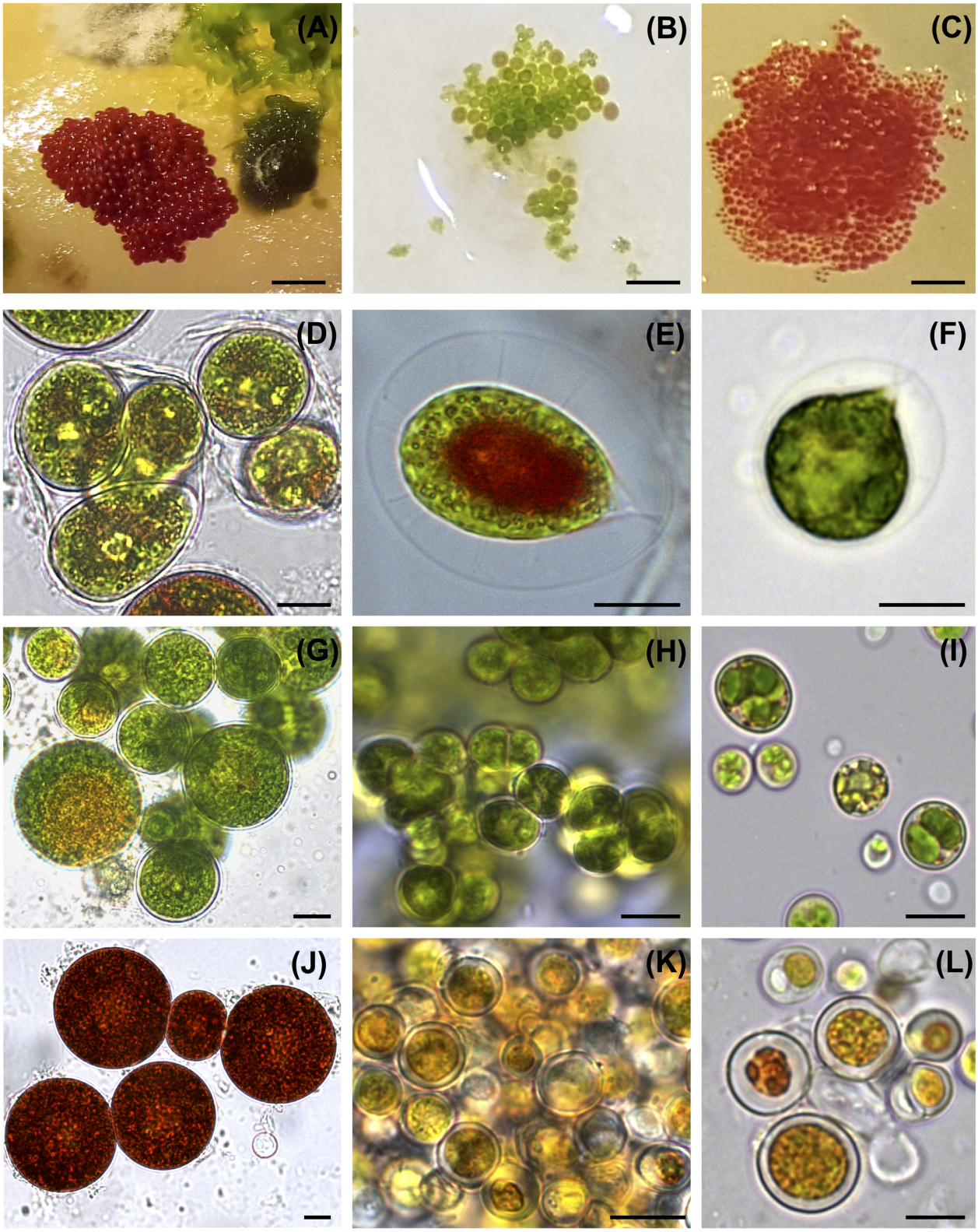

Figure 3. Representative micrographs of the cells of carotenogenic microalgae at the agar surface in the Petri dishes with solid BG-11 medium **(A–C)** and in the obtained cultures of algal strains **(D–L)** produced by the methods of light microscopy. Colonies of a carotenogenic microalga (the red one) and cyanobacteria (the green one) after the inoculation of a natural liquid cyanobacteria-containing sample to Petri dishes with solid BG-11 medium (A), colonies of a carotenogenic microalga formed from individual red cells after being transferred to a new Petri dish with solid BG-11 medium in 2 weeks (B) and after 1 month (C). The cells of carotenogenic microalgae of the White Sea coast: an autosporangium of *H. lacustris* BMM3/16 (D), zoospores of *H. lacustris* BMM3/16 (E) and *H. rubicundus* BM7/13 (F), vegetative cells of *H. lacustris* BM4 (G), *Coelastrella aeroterrestrica* HELL-2 (H), and *B. aggregatus* (BM5/15) (I), the cells of carotenogenic microalgae after induction of carotenoid accumulation: *H. rubicundus* BM7/13 (J), *C. aeroterrestrica* HELL-2 (K) and *B. aggregatus* (BM5/15) (L). Scale bar: A and C: 500 μm, B: 160 μm, D–L: 10 μm.

Table 2. GenBank IDs of the DNA sequences of carotenogenic algae strains isolated from the White Sea polar region.

Strain	GenBank accession number		
	ITS1-5.8SrRNA-ITS2	18S rRNA	rbcL
Haematococcus lacustris BM1	MH191370.1	JQ867352.1	KR296734.1
Haematococcus rubicundus BM7/13	MH188835.1	None	None
Haematococcus lacustris BM4	MH188839.1	KF644445.1	KU707909.1
Haematococcus lacustris BM6/13	MH191371.1	KY049901.1	None
Haematococcus lacustris BMM1/14	MH188840.1	KY049899.1	None
Haematococcus lacustris HELL-1	MH188836.1	None	None
Coelastrella aeroterrestrica HELL-2	MH205945.1	None	None
Haematococcus lacustris BMM1/15	MH188834.1	None	None
Haematococcus lacustris Lake	MH191372.1	None	None
Haematococcus lacustris BMK/15	MH229858.1	KY049900.1	None
Bracteacoccus aggregatus BM5/15	MH205944.1	None	None
Haematococcus lacustris BMM2/15	MH188838.1	None	None
Haematococcus lacustris BM7/15	MH188844.1	None	None
Haematococcus lacustris BMP/16	MH188841.1	None	None
Haematococcus lacustris BMK/16	MH191369.1	None	None
Haematococcus lacustris BMM1/16	MH188837.1	None	None
Haematococcus lacustris BMM2/16	MH188842.1	None	None
Haematococcus lacustris BMM3/16	MH188843.1	None	None

isokont flagella presented on the apex of zoospores (Fig. 3E and F). Zoospores were spherical (10–15 μm in diameter) (Fig. 3E) or pear-shaped (Fig. 3F) with more or less extended apical part. These cells were covered by a layer of gelatinous matrix. In some cases it was connected with protoplast by cytoplasmic strands (Fig. 3E), which are typical for this genus (Buchheim *et al.* 2013; Allewaert *et al.* 2015).

The strain HELL-2 was represented by small unicellular coccoid algae (Fig. 3H and K). Its cells were spherical and ∼1–2 μm in diameter. During cultivation in liquid BG-11 medium at relatively high division frequency, they were green (Fig. 3H). These cells became orange-reddish when growth rate decreased or transfer to solid BG-11 (Fig. 3K). In this case, the cells formed clusters and their diameter increased up to 4 μm (Fig. 3K).

The strain BM5/15 was represented by green coccoid cells (approx. up to 9 μm in diameter) in liquid BG-11 medium (Fig. 3I). As a rule, they formed multicellular clusters. Growth rate decline was accompanied by the appearance of orange coloration of the cells and the increase cell diameter up to 20 μm (Fig. 3L). For HELL-2 and BM5/15 a motile stage was not observed.

Identification of the strains

Due to low morphological variability, light microscopy observations cannot solve the task of identification of unicellular coccoid green algae (Krienitz and Bock 2012), and genetic data is necessary.

For all the strains, putative sequences of the ITS1-5.8S rRNA-ITS2 ∼850 bp long were obtained by PCR (see the section 'Materials and Methods'). Based on homologs search, highest similarity for most of the strains was observed with the family Haematococcaceae of green microalgae, while one strain was related to Scenedesmaceae, and one to Bracteacoccaceae. The sequences were submitted to GenBank NCBI under accession numbers presented in Table 2.

Results of analysis of sequence data were in accord with morphological observations of Haematococcus, Bracteacoccus and Coelastrella (Fuíková, Flechtner and Lewis 2013; Kaufnerová and Eliáš 2013; Minyuk, Chelebieva and Chubchikova 2014; Minyuk *et al.* 2016, 2017; Mamaeva *et al.* 2018).

Phylogenetic trees constructed by different algorithms were similar in their topology. Therefore, ML phylograms corresponding to the highest values of maximum likelihood function are presented (Fig. 4). Due to the low number of ITS1 sequences in the GenBank database, only the ITS2 region was included in the analysis.

On the Haematococcaceae phylogenetic tree (Fig. 4A), four strains (BM-1, HELL-1, BM6/13 and Lake) formed a common clade with the Swedish strain SAG 34–1a and Belgian strain BE02_09. Other strains (BM4, BM7/15 and BMK/15) nested with Belgian BE03_05 and German SAG 192.80. The strains from the same location (Maria's Stones) formed their own clade. It also included the strains BMK/16 and BMP/16. The strain BM7/13 was significantly far from the others in terms of phylogenetic distances. It was close to Belgian strains BE10_03 and BE05_06 and Dutch strains NL01_05 and NL02_08. The strain BM7/13 was far from other Haematococcaceae White Sea coastal strains in terms of phylogenetic distances (Fig. 4A). It clustered with Dutch and Belgian strains. Notably, the strains isolated in the same place (Maria's Stones) in different years were close related to each other. Thus, colonies of carotenogenic algae are relatively stable, and the same population occupies its own habitat for a long period.

The system of the *Haematococcus* genus was significantly reconsidered over past few years. Two species of the genus *Haematococcus*, *H. lacustris* (Girod-Chantrans) Rostafinski and *H. pluvialis* Flotow, were proposed by Droop (1959). However later, in physiological and phylogenetic studies, it was commonly accepted that they were synonymous, and *H. pluvialis* was more useful. Based on multilocus phylogenetic analysis, *H. pluvialis* was recognized as a separate species in this genus (Buchheim *et al.* 2013). Nakada and Ota (2016) noticed that *H. lacustris* was the first proposed name; therefore, it is more correct. Currently, the name *H. lacustris* is used in the databases and publications. Recently accepted correct name 'Haematococcus lacustris' is

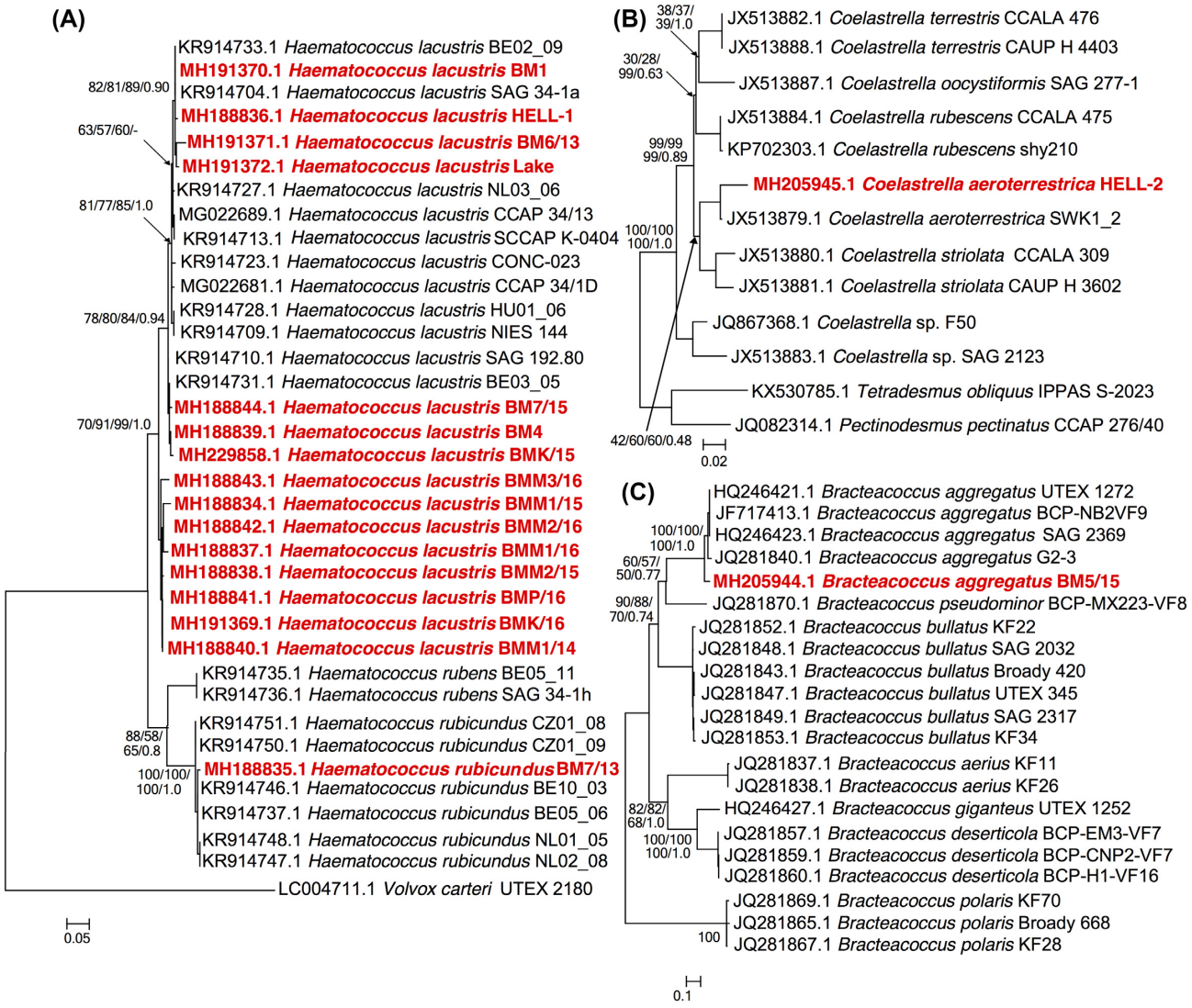

Figure 4. Phylogenetic relationship of carotenogenic algae isolated from the White Sea coastal zone: phylogenetic trees of Haematococcaceae **(A)**, Scenedesmaceae **(B)** and Bracteacoccaceae **(C)** obtained by ML algorithms. Significance of the elements of trees topology is indicated as ML/NJ/MP/BI, where ML, NJ and MP are percentage of bootstrap support in ML-, NJ- and MP-trees, respectively, BI: probability in Bayesian inference analysis. Scale: number of substitutions per site.

used instead of its obsolete synonym '*Haematococcus pluvialis*'. There is no difference between the data obtained for '*H. pluvialis*' in previously published papers and '*H. lacustris*'. In 2015, based on the strains isolated in Europe, two new species, *H. rubicundus* and *H. rubens*, were described (Allewaert *et al.* 2015). In addition, a strain of *H. alpinus* from New Zealand was described in 2018 (Mazumdar *et al.* 2018). Based on the obtained ITS2-based phylogenetic tree, most of the strains are related to H. lacustris (Fig. 4A). Single strain BM7/13 isolated from freshwater near the village Nil'moguba was identified as *H. rubicundus* Allewaert & Vanormelingen (Fig. 4A). Most likely, *H. rubicundus* is a rarer species of the *Haematococcus* genus in the White Sea coastal region.

Optimal secondary structures of ITS1 and 2 of Haematococcus strains, including helices I, II, III and IV, were typical for this genus (Fig. 5; Buchheim *et al.* 2013; Allewaert *et al.* 2015). The values of standard Gibbs free energy of these structures' formation were in the range of −127 to −92 kcal/mol. European species of the Haematococcus genus may differ in several CBCs in ITS secondary structures: one CBC in the helix III of ITS2, two CBCs in the helix I and one CBC in the helix III of ITS1 (Allewaert *et al.* 2015). The ITS1 secondary structure of the White Sea strains differed by two CBCs in the helix I (Fig. 5A). White Sea species differed by one CBC in the apical part of the helix, which was absent in European strains: C-G in *H. rubicundus* and U-A in *H. lacustris* (Fig. 5A). Some strains of *H. lacustris* (BM7/13, HELL1, Lake, BM6/13, BM1) have a complementary pair G-C (Fig. 5A) in helix I typical for this species (Allewaert *et al.* 2015). The strains from the White Sea region differed by one CBC in the helix III (Fig. 5B) of ITS2 (U-A in *H. rubicundus* and C-G in all *H. lacustris* from Maria's Stones (BMM1/14, BMM1/15, BMM2/15, BMM1/16, BMM2/16, BMM3/16, BMP/16) and Pokormezhny Island—strain BMP/16), which was absent in the European strains. In addition, one complementary base pair G-C (Fig. 5B) typical for *H. lacustris* presented in the same helix of all strains of this species.

The sequence of ITS1-5.8SrRNA-ITS2 of the BM5/15 strain was closely related to microalgae of the Bracteacoccaceae family including a single genus Bracteacoccus. On the phylogenetic tree, it formed a common cluster with representatives of *B. aggregatus* Tereg (type species of the genus) and *B. cohaerens*

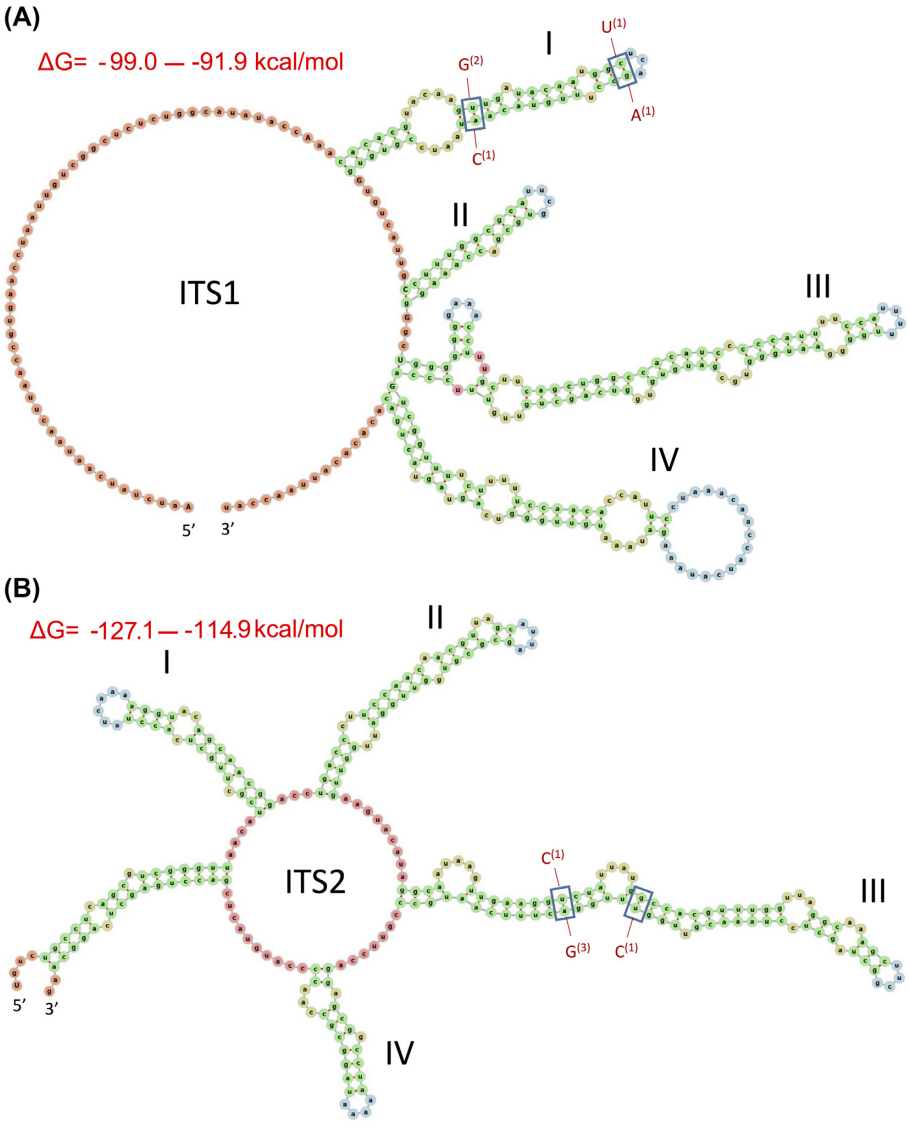

Figure 5. Models of secondary structures of ITS1 **(A)** and ITS2 **(B)** of *H. rubicundus* BM7/13 corresponding to the lowest values of Gibbs free energy of their formation. Positions of CBC, which may be observed between different *Haematococcus* species, are outlined in the frames. Helixes are commonly numbered as I–IV. The ranges of Gibbs free energy of the ITS structures formation are provided.

H.W.Bischoff & Bold (Fig. 4C). After a revision of Fuíková, Flechtner and Lewis (2013), *B. cohaerens* was recognized as synonym of *B. aggregatus*. Thus, *B. aggregatus* BM5/15 is a more correct name for the strain.

Based on BLAST search, the sequence of ITS1-5.8S rRNA-ITS2 of the strain HELL-2 was closely related to the sequences of algae of the genera Coelastrella, Scotilellopsis and Scenedesmus of Scenesesmaceae family (95–99% similarity). As reviewed by Punčochářová and Kalina (1981), Scotiellopsis differs from Coelastrella by ultrastructural features. Hanagata (1998) based on a high similarity of 18S rRNA sequences proposed to transfer these two genera into the genus Scenedesmus. However, based on compensatory base change analysis in ITS2, it was shown, that the genus Scotiellopsis is paraphyletic and some strains should be transferred into the genus Coelastrella (Kaufnerová and Eliáš 2013). The strain HELL-2 was most closely related to Coelastrella microalgae. It was nested with microalgae *C. aeroterrestrica* that Tschaikner, Gärtner and Kofler previously described in alpine soil from Tyrol (Austria) (Tschaikner, Gärtner and Kofler 2008) and Pirin Mountains (Bulgaria) (Uzunov *et al.* 2008) (Fig. 4B).

ITS2 secondary structures of strains BM5/15 (Fig. 6A) and HELL-2 (Fig. 6B) were characterized by branched helix I typical for Sphaeropleales (Fawley, Fawley and Hegewald 2011; Fuíková, Flechtner and Lewis 2013; Kaufnerová and Eliáš 2013). There were no CBCs observed comparing structures of BM5/15 and HELL-2 with *B. aggregatus* (Fuíková, Flechtner and Lewis 2013) and *C. aeroterrestrica* (Kaufnerová and Eliáš 2013), respectively. Thus, they were identified as representatives of these species.

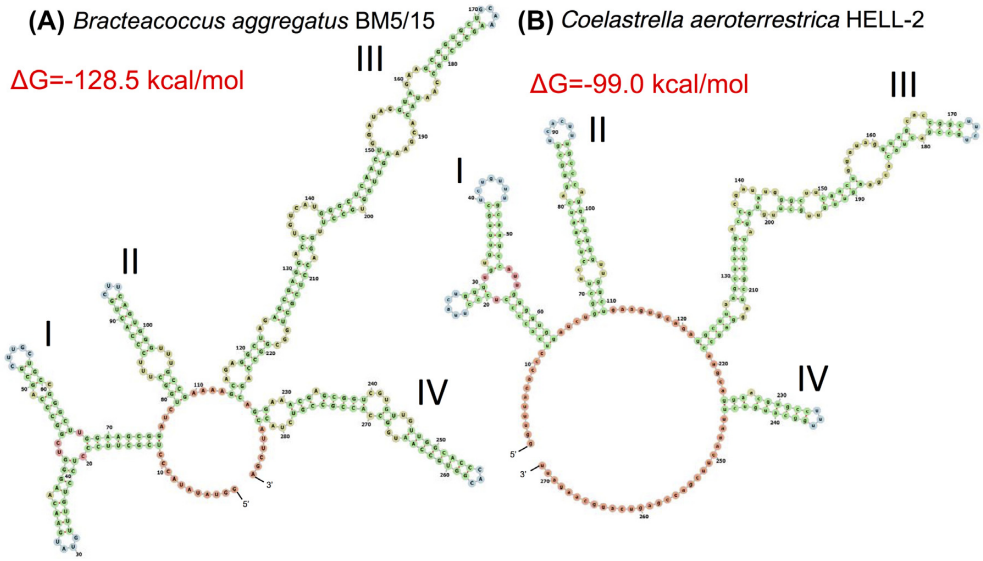

Figure 6. Models of secondary structures of ITS2 region of *B. aggregatus* BM5/15 **(A)** and *C. aeroterrestrica* HELL-2 **(B)** strains. The values of Gibbs free energy of ITS2 secondary structures formation are provided.

Pigment composition of the White Sea carotenogenic microalgae strains

The following pigment fractions were identified in the algal extracts based on the absorbance spectra and R_f values (Table 3). All microalgal strains contained ketocarotenoid astaxanthin with two hydroxyl groups and two ketogroups. It is a terminal product of ketocarotenoid biosynthesis in chlorophytes (Cunningham and Gantt 2011; Takaichi 2011; Han, Li and Hu 2013). All strains, except *B. aggregatus* BM5/15, contained canthaxanthin with two ketogroups. Some strains contained β-carotene. In addition, extracts also contained minor fractions of photosynthetic pigments, typical for Chlorophyta (zeaxanthin, violaxanthin, anteraxanthin, lutein, neoxanthin as well as chlorophyll *a* and *b*) and adonirubin (an intermediate of astaxanthin synthesis from canthaxanthin) (Lichtenthaler 1987; Cunningham and Gantt 1998; Takaichi 2011).

All Haematococcus strains were characterized by similar pigment profiles. There were no differences between *H. lacustris* and *H. rubicundus* pigment profiles. Their main carotenoid was astaxanthin (Table 3). It was predominantly in the form of mono- and diesters of fatty acids (R_f of 0.32–0.34 and of 0.54, respectively). Most of astaxanthin fractions (66–86%) were formed by monoesters with fatty acids. The fraction of free astaxanthin in the cells was significantly lower than esterified pigment (Table 3). These results were in accordance with the previously published data about pigment profile of Haematococcus (Boussiba and Vonshak 1991; Chekanov *et al.* 2014, Chelebieva *et al.* 2018). Astaxanthin was a predominant pigment in *C. aeroterrestrica* HELL-2 also. However, the ratio of its mono- to diesters was lower than in *H. lacustris* and *H. rubicundus* (Table 3). Another feature of this strain was a high fraction of other carotenoids including intermediates of astaxanthin synthesis. Similar data was obtained previously for *Coelastrella* (Hu *et al.* 2013; Minyuk *et al.* 2016, 2017). High content of β-carotene was a pronounced feature of *B. aggregatus* BM5/15 (Table 3). Its fraction was comparable with astaxanthin. Two industrial species of carotenogenic microalgae, *D. salina* and *H. lacustris*, produce only one predominant carotenoid, β-carotene or astaxanthin, respectively. Therefore, two different pigment profiles were obtained in the same separation system for these two microalgae previously (Chekanov *et al.* 2014; Solovchenko *et al.* 2015). At the same time, *B. aggregatus* BM5/15 produced both carotenoids. Therefore, this microalga combines the features of *D. salina* and *H. lacustris*. Thus, this strain may be used for combined production of this two pigments. High diversity of secondary carotenoids in Bracteacoccus microalgae was previously described for other strains (Minyuk, Chelebieva and Chubchikova 2014; Minyuk and Solovchenko 2018).

Collectively, the data for pigment profiles of White Sea carotenogenic microalgae indicate that it was similar to that of other strains of the same genera of chlorophytes. Thus, it may play a role of an additional taxonomic marker, and it is possible to predict a pigment profile based on genetic identification.

Distribution of carotenogenic microalgae

Haematococcus lacustris is widely spread all over the world. It was found throughout Europe, where it is distributed from Scandinavia to Venice, in America from Vermont to Texas and from Massachusetts to Nebraska and, possibly, even further (Hazen 1899). There are strains from equatorial, subequatorial and tropical latitudes with hot climates (Pocock 1960; Buchheim *et al.* 2013; Chelebieva *et al.* 2018), temperate latitudes (Droop 1956; Allewaert *et al.* 2015; Chelebieva *et al.* 2018), as well as northern, cold-resistant representatives, able to grow at low temperatures (Klochkova *et al.* 2013). Two polar strains of *H. lacustris* (from Blomstrandhalvøya (Svalbard, Norway) and from the White Sea coastal rocks) have been isolated (Klochkova *et al.* 2013; Chekanov *et al.* 2014). Currently, it is known that *H. lacustris* is widespread on all the continents, except Antarctica (Droop 1956; Guiry and Guiry 2019). However, the most common natural habitats of this microalga, as a rule, are small temporary ponds (Proctor 1957) filled with fresh or moderately saline water (up to 3 ‰). It is commonly accepted that *H. lacustris* is a freshwater species (Guiry and Guiry 2019). However, we also detected this microalga in pools with salinity as high as 130 ‰. New *Haematococcus* species were recently found in Europe (Allewaert *et al.* 2015) and New Zealand (Mazumdar *et al.* 2018). The present

Table 3. Fractions of carotenoid pigments in the extracts of the microalgal cells after induction of secondary carotenogenesis obtained by TLC separation. n/d—not detected.

Strain	Pigment (% of total carotenoid)					
	Astaxanthin (diester)	Astaxanthin (monoester)	Astaxanthin (free)	Cantaxanthin	β-carotene	Others
Haematococcus lacustris BM1	22.4	66.4	5.2	3.5	1.1	1.4
Haematococcus rubicundus BM7/13	19.5	77.1	1.5	<1	n/d	1.9
Haematococcus lacustris BM4	10.0	85.1	2.3	1.5	n/d	1.2
Haematococcus lacustris BM6/13	19.4	70.1	2.2	3.0	n/d	2.2
Haematococcus lacustris BMM1/14	15.0	74.8	4.7	3.1	n/d	2.4
Haematococcus lacustris HELL-1	12.3	86.3	<1	<1	n/d	<1
Coelastrella aeroterrestrica HELL-2	19.2	22.4	8.0	5.6	9.7	35.1
Haematococcus lacustris BMM1/15	18.1	72.5	4.4	2.1	n/d	1.1
Haematococcus lacustris Lake	11.1	83.3	1.9	<1	n/d	2.8
Haematococcus lacustris BMK/15	17.6	80.5	<1	<1	n/d	<1
Bracteacoccus aggregatus BM5/15	15.7	44.1	n/d	n/d	40.2	<1
Haematococcus lacustris BMM2/15	9.8	81.0	3.4	0.8	n/d	5.1
Haematococcus lacustris BM7/15	17.1	80.7	<1	<1	n/d	1.1
Haematococcus lacustris BMP/16	17.9	80.0	<1	<1	n/d	<1
Haematococcus lacustris BMK/16	16.1	78.8	1.0	1.5	n/d	2.0
Haematococcus lacustris BMM1/16	12.5	84.2	<1	1.7	n/d	<1
Haematococcus lacustris BMM2/16	18.8	79.8	<1	<1	n/d	<1
Haematococcus lacustris BMM3/16	19.9	70.2	4.6	3.1	n/d	2.3

study provides new data on the distribution of polar and subpolar *H. lacustris* strains and the first mention of *H. rubicundus* in the White Sea region.

Coelastrella and *Bracteacoccus* are known as freshwater and soil microalgae (Tsarenko and John 2011; Guiry and Guiry 2019). *Bracteacoccus* was also mentioned as a member of Arctic cryptoendolithic habitats (Omelon, Pollard and Ferris 2007) and phototrophic biofilms (Roldán *et al.* 2004). However, in the last two cases, the data were not confirmed by molecular identification; therefore, these microorganisms also could be related to other similar groups, such as *Pseudomuriella* or *Chromochloris*. *Bracteacoccus* was observed in the soils of Pakistan (Hussain *et al.* 2011) and Ukraine (Kostikov *et al.* 2001). In general, different genera of Scenedesmaceae are ubiquitous. They are cosmopolitan and spread over different regions of the world (Hegewald, Bock and Krienitz 2013). Non-carotenogenic Scenedesmaceae algae have been previously described for the White Sea region (Gorelova *et al.* 2015; Ismagulova *et al.* 2018). *Coelastrella* strains previously have been found exclusively in Europe (Guiry and Guiry 2019). Especially, *C. aeroterrestrica* is known as alpine species isolated in Bulgaria and Austria (Tschaikner, Gärtner and Kofler 2008, Uzunov *et al.* 2008). Recently, based on ITS1-5.8SrRNA-ITS2, *Coelastrella* was also identified at the Yucatan Peninsula (North America) (Ancona-Canché *et al.* 2017). Based on *18SrRNA* sequencing data, it was also found in Tigris River sediment in Iraq (Al-Rawi *et al.* 2018).

CONCLUSION

Diversity of unicellular carotenogenic microalgae was described in the White Sea coastal region. They were represented by different species of chlorophytes: *H. lacustris*, *H. rubicundus*, *C. aeroterrestrica* and *B. aggregatus*. Colonies of carotenogenic algae are relatively stable, and the same population occupies its own habitat for a long period. Samples containing carotenogenic algae were represented by natural monoalgal cultures and freshwater associations. They were dry samples, water from temporary ponds and lakes. They differed by their salinity and microalgal substrate (soil, rock surface, salt crystals, synthetic materials). Most likely, the ability for carotenogenesis is an important feature that makes the survival of these microalgae in various biotopes in polar regions possible.

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Conflicts of interest. None declared.

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