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MANTONIELLA BEAUFORTII AND *MANTONIELLA BAFFINENSIS* SP. NOV. (MAMIELLALES, MAMIELLOPHYCEAE), TWO NEW GREEN ALGAL SPECIES FROM THE HIGH ARCTIC[1]

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Members of the class Mamiellophyceae comprise species that can dominate picophytoplankton diversity in polar waters. Yet, polar species are often morphologically indistinguishable from temperate species, although clearly separated by molecular features. Here we examine four Mamiellophyceae strains from the Canadian Arctic. The 18S rRNA and Internal Transcribed Spacer 2 (ITS2) gene phylogeny place these strains within the family Mamiellaceae (Mamiellales, Mamiellophyceae) in two separate clades of the genus *Mantoniella*. ITS2 synapomorphies support their placement as two new species, *Mantoniella beaufortii* and *Mantoniella baffinensis*. Both species have round green cells with diameter between 3 and 5 µm, one long flagellum and a short flagellum (~1 µm) and are covered by spiderweb-like scales, making both species similar to other *Mantoniella* species. Morphologically, *M. beaufortii* and *M. baffinensis* are most similar to the cosmopolitan *M. squamata* with only minor differences in scale structure distinguishing them. Screening of global marine metabarcoding data sets indicates *M. beaufortii* has only been recorded in seawater and sea ice samples from the Arctic, while no environmental barcode matches *M. baffinensis*. Like other Mamiellophyceae genera that have distinct polar and temperate species, the polar distribution of these new species suggests they are cold or ice-adapted *Mantoniella* species.

Key index words: **Arctic; ITS; Mamiellophyceae; *Mantoniella*; metabarcoding; picophytoplankton; polar**

***Abbreviations*: CBC, compensatory base change; hCBC, hemi-CBC; AIC, Akaike information criterion; BIC, Bayesian information criterion; DIC, differential interference contrast; HPLC, high-performance liquid chromatography; ITS, Internal Transcribed Spacer; OSD, Ocean Sampling Day; PTFE, Polytetrafluoroethylene; SPR, Subtree Pruning and Regrafting; TEM, Transmission electron microscopy**

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Over the last few decades, the taxonomy of green algae has gone through a profound reorganization. The class Prasinophyceae, initially defined as scaly flagellates (Moestrup and Throndsen 1988), has been rearranged into several new classes including Chlorodendrophyceae, Chloropicophyceae, and Mamiellophyceae (Massjuk 2006, Marin and Melkonian 2010, Lopes dos Santos et al. 2017b), as well as clades without formal names (Guillou et al. 2004, Tragin et al. 2016), leading to the abandonment of the class name Prasinophyceae. The Mamiellophyceae are ecologically successful and particularly dominant in marine coastal waters (Lopes dos Santos et al. 2017a, Tragin and Vaulot 2018). The first scaled species of Mamiellophyceae observed were *Mantoniella squamata* (as *Micromonas squamata*, Manton and Parke 1960) and *Mamiella gilva* (as *Nephroselmis gilva*; Park and Rayns 1964). Moestrup (1984) erected the family Mamiellaceae, which included *Mantoniella* and *Mamiella,* with *Mamiella gilva* designated as the type species. Mamiellophyceae comprises three orders: Monomastigales, with one freshwater genus *Monomastix*; Dolichomastigales, with two genera *Crustomastix* and *Dolichomastix*; and Mamiellales, which currently comprises five genera *Bathycoccus*, *Mamiella*, *Mantoniella*, *Micromonas*, and *Ostreococcus*. As these genera are morphologically heterogeneous, with *Micromonas* and *Ostreococcus* lacking scales and *Bathycoccus* and *Ostreococcus* lacking flagella, the monophyly of Mamiellophyceae was established based on nuclear and plastid rRNA sequence and secondary structure analyses (Marin and Melkonian 2010).

Molecular analyses of the Mamiellophyceae have permitted the description of otherwise morphologically indistinguishable cryptic species. For example, wide genetic diversity has been shown to exist between morphologically identical *Ostreococcus* species where less than 1% difference in the 18S rRNA gene corresponds to up to 30% of variation in orthologous protein coding sequences (Palenik et al. 2007, Piganeau et al. 2011). From an early stage, 18S rRNA-defined clades of *Micromonas* and *Ostreococcus* were observed to have distinct geographic distributions, suggesting their genetic variation reflected adaptations to ecological niches (Rodríguez et al. 2005, Foulon et al. 2008) and that these clades represented distinct species. *Ostreococcus* is divided into rare species restricted to estuarine (*O. mediterraneus*) and coastal environments (*O. tauri*), as well as more abundant oceanic species (*O. lucimarinus* and clade B; Demir-Hilton et al. 2011, Treusch et al. 2012, Hu et al. 2016, Simmons et al. 2016). *Micromonas* cells were observed to be abundant in the Arctic Ocean (Throndsen and Kristiansen 1991, Sherr et al. 2003, Not et al. 2005) that subsequent 18S rRNA analyses revealed them to belong to a clade with an Arctic distribution (Lovejoy et al. 2007, Balzano et al. 2012). *Micromonas* has since been revised defining the Arctic clade as the species *M. polaris*, and species originating from lower latitudes as *M. bravo*, *M. commoda*, and *M. pusilla* (Simon et al. 2017). Similarly, in *Mantoniella*, *M. antarctica* was described from the Antarctic, whereas *M. squamata* was cosmopolitan (Marchant et al. 1989).

Three picophytoplanktonic strains (RCC2285, RCC2288, and RCC2497) were isolated in the Canadian Arctic from mesophilic surface water sampled at two sites in the Beaufort Sea in the summer of 2009 as part of the MALINA cruise (Balzano et al. 2012). A fourth strain (RCC5418) was subsequently isolated from sea ice collected in Baffin Bay in the spring as part of the Green Edge project. We performed a combination of molecular, morphological, and pigment characterization of these isolates, which we propose to constitute two novel *Mantoniella* species, *M. beaufortii* and *M. baffinensis*, restricted to polar environments.

METHODS

Culture conditions. Strains RCC2285, RCC2288, and RCC2497 were isolated from seawater collected at two sites (70°30′ N, 135°30′ W and 70°34′ N, 145°24′ W) in the Beaufort Sea in the summer of 2009 as part of the MALINA cruise as described previously (Balzano et al. 2012). Strain RCC5418 was isolated from the Green Edge project Ice Camp (http://www.greenedgeproject.info/), a sampling site on the sea ice near the village of Qikiqtarjuaq (67°28.784′ N, 63°47.372′ W). Sampling was conducted between April 20 and July 27, 2016, beginning in completely snow covered conditions followed by bare ice and ending when the ice was broken out. Sea ice from May 23, 2016 was melted overnight and 200 mL was gravity filtered (Sartorius filtration system) through 3 μm pore size polycarbonate filters (Millipore Isopore membrane, 47 mm). 500 μL of filtrate was enriched by addition to 15 mL of L1 medium (NCMA, Bigelow Laboratory for Ocean Sciences, ME, USA). The enrichment culture was purified by dilution to 10 cells per well in a 96-deep-well plate (Eppendorf) and incubated under white light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) in a 12:12 h light:dark cycle at 4°C. Cell growth was observed by the development of coloration after a few weeks. Culture purity was assessed by flow cytometry (Becton Dickinson, Accuri C6). After confirmation of the purity, the culture was transferred in a 50 mL ventilated flask (Sarstedt). Cultures are maintained in the Roscoff Culture Collection (http://roscoff-culture-collection.org/) in K/2 (Keller et al. 1987) or L1 medium at 4°C under a 12:12 h light: dark cycle at 100 μE light intensity. RCC2285 has been lost from culture since molecular analyses (described below) were performed. For pigment analysis and electron microscopy, RCC2288 was grown at 7°C under continuous light at 100 μE intensity in L1 medium prepared using autoclaved seawater from offshore Mediterranean Sea water diluted 10% with MilliQ water and filtered prior to use through 0.22 μm filters. Holotype specimens were deposited in O (Natural History Museum, University of Oslo), herbarium acronym follows Thiers (2019).

Sequences. Nuclear 18S rRNA and the Internal Transcribed Spacers (ITS) 1 and 2, as well as the 5.8S rRNA gene were retrieved from GenBank for strains RCC2288, RCC2497, and RCC2285 (Balzano et al. 2012). For RCC5418 and RCC5150 (*Mantoniella antarctica*), cells were harvested in exponential growth phase and concentrated by

centrifugation. Total nucleic acids were extracted using the Nucleospin Plant II kit (Macherey-Nagel, Düren, DE, USA) following the manufacturer's instructions. The nearly full-length nuclear 18S rRNA gene (only RCC5418) and the region containing the Internal Transcribed Spacers (ITS) 1 and 2, as well as the 5.8S rRNA gene were obtained by PCR amplification using universal primers (Table S1 in the Supporting Information). PCR products were directly sequenced at the Macrogen Company (Korea) and sequences have been deposited to Genbank under accession numbers MH516003, MH516002, and MH542162.

ITS2 secondary structure. The ITS2 secondary structure from the strains listed in Table 1 was predicted using the Mfold web interface (Zuker 2003) under the default options with the folding temperature fixed at 37°C, resulting in multiple alternative folding patterns per sequence. The preliminary structure for each sequence was chosen based on similarities found among the other structures proposed for Mamiellophyceae (Marin and Melkonian 2010, Simon et al. 2017) as well as on the presence of previously defined ITS2 hallmarks defined by Coleman (Mai and Coleman 1997, Coleman 2000, 2003, 2007). Exported secondary structures in Vienna format and the respective nucleotide sequences were aligned, visualized using 4SALE version 1.7 (Seibel et al. 2008), and manually edited through extensive comparative analysis of each position (nucleotide) in sequences from representatives of the Mamiellophyceae. The ITS2 synapomorphy analysis was confined to those positions that formed conserved base pairs in all members of the Mamiellaceae order and the resulting intramolecular folding pattern (secondary structure) of *Mantoniella* was drawn using CorelDRAW X7. A Vienna file containing the ITS2 sequences and secondary structure is available at https://doi.org/10.6084/m9.figshare.7472153.v1.

Phylogenetic analyses. Nuclear 18S rRNA sequences belonging to members of Mamiellophyceae were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). Two environmental sequences (similar to strain sequences) were included in addition to the sequences obtained from the cultures. Sequences were also obtained for the ITS2 region located between the 5S and 23S rRNA genes. However, no environmental sequences were available to be included in the 18S/ITS phylogenetic analyses.

In all, 27 nuclear 18S rRNA and 14 ITS2 sequences were aligned with MAFFT using the E-INS-i and G-INS-i algorithms, respectively (Katoh and Toh 2008). Alignments were visualized and manually edited using Geneious 10.2.5 (Kearse et al. 2012). The ITS2 alignment was further edited on the basis of conserved secondary structures (see above). The nuclear 18S rRNA and ITS2 sequences from the Mamiellaceae members were concatenated using Geneious 10.2.5 (Kearse et al. 2012). Lengths of the resulting alignments were 1,567 bp for 18S rRNA (1,242 identical sites, 295 variable, and 191 parsimony-informative sites) and 1,875 bp for concatenated 18S-ITS sequences (1,544 identical sites, 302 variable, and 179 parsimony-informative).

Phylogenetic reconstructions with two different methods, maximum likelihood (ML) and Bayesian analyses, were performed using the nuclear Mamiellophyceae 18S rRNA and Mamiellaceae concatenated 18S/ITS2 alignments.

The K2 + G + I model was selected for both sequence data sets based on the substitution model selected through the Akaike information criterion (AIC) and the Bayesian information criterion (BIC) options implemented in MEGA 6.06 (Tamura et al. 2013). ML analysis was performed using PhyML 3.0 (Guindon et al. 2010) with SPR (Subtree Pruning and Regrafting) tree topology search operations and approximate likelihood ratio test with Shimodaira-Hasegawa-like procedure. Markov chain Monte Carlo iterations were conducted for 1,000,000 generations sampling every 100 generations with burning length 100,000 using MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003) as implemented in Geneious (Kearse et al. 2012). Nodes were considered as well supported when SH-like support values and Bayesian posterior probabilities were higher than 0.8 and 0.95, respectively. The same criteria were used to represent the sequences on the phylogenetic trees. Alignments are available at https://doi.org/10.6084/m9.figshare.7472153.v1.

Screening of environmental 18S rRNA sequencing data sets. High-throughput sequencing metabarcodes (V4 and V9 hypervariable regions) were obtained from several published polar studies, as well as from the global sampling efforts Tara Oceans and Ocean Sampling Day (OSD; see Table S2 in the Supporting Information for the full details and references for each project). We screened these data as well as GenBank by BLASTn (98% identity cut-off) using RCC2288 18S rRNA gene sequence as the search query. We aligned the retrieved environmental sequences and metabarcodes with that of RCC2285, RCC2288, RCC2497, and RCC5418 using MAFFT as implemented in Geneious version 10.0.7 (Kearse et al. 2012). This allowed the determination of sequence signatures diagnostic of this species for both V4 and V9 (Figs. S1 and S2 in the Supporting Information). The oceanic distribution of stations where cultures, clones, and metabarcodes having these signatures, as well as the stations from the metabarcoding surveys where no matching metabarcodes have been found, were plotted with the R libraries ggplot2 and rworldmap. The R script is available at https://vaulot.github.io/papers/RCC2288.html.

Light microscopy. Cells were observed using an Olympus BX51 microscope (Olympus, Hamburg, Germany) with a 100× objective using differential interference contrast (DIC) and imaged with a SPOT RT-slider digital camera (Diagnostics Instruments, Sterling Heights, MI, USA).

For video microscopy, cultures from RCC2288 and RCC2497 were observed with an inverted Olympus IX70 inverted microscope using an ×40 objective and equipped with an Infinity X camera (https://www.lumenera.com/products/microscopy/infinityx-32.html). Short sequences were recorded and edited with the Video de Luxe software (http://www.magix.com/fr/video-deluxe/). Films were uploaded to Youtube (https://www.youtube.com/channel/UCsYoz-

TABLE 1. Strains used in this study.

Strain	Strain name	Oceanic region	Latitude	Longitude	Depth of isolation (m)	18S rRNA	ITS	Remark
RCC2285	MALINA E43.N1	Beaufort Sea	70°34′ N	145°24′ W	0	JF794053	JQ413368	Strain lost
RCC2288	MALINA E47.P2	Beaufort Sea	70°30′ N	135°30′ W	0	JN934679	JQ413369	
RCC2497	MALINA E47.P1	Beaufort Sea	70°30′ N	135°30′ W	0	KT860921	JQ413370	
RCC5418	GE_IP_IC_DIL_490	Baffin Bay	67°28′ N	63°46′ W	Surface ice	MH516003	MH542162	

RCC: Roscoff Culture Collection (www.roscoff-culture-collection.org). 18S rRNA and ITS show Genbank accession numbers. Strains in bold used to describe the new species.

aSJlJesyDNj6ZVolQ/videos). Video microscopy of swimming behavior of RCC2288 (https://youtu.be/CGKNxzfGUvQ), RCC2497 (https://youtu.be/rRNuk5Lx7Aw), and RCC5418 (https://youtu.be/xoxCEl1cv4Q). The recording protocol is available at dx.doi.org/10.17504/protocols.io.k24cygw.

Transmission electron microscopy. Positive-stained whole mount cells were prepared as described by Moestrup (1984), where cultures were directly deposited on formvar-coated copper grids and stained with 2% uranyl acetate. TEM thin sections was performed as previously described (Derelle et al. 2008). Briefly, fixed RCC2288 cells (1% glutaraldehyde) from an exponentially growing culture were suspended in molten (37°C) 1% low melting point agarose. The agarose cell plug was fixed, washed, dehydrated in ethanol, and embedded in Epon 812. Ultra-thin sections (80–90 nm) were placed on a 300 mesh copper grid and stained with uranyl acetate for 15 min, followed by lead citrate staining for 2 min. The cells were visualized with Hitachi H 7500 and H-9500 transmission electron microscopes.

Pigment analysis. Pigments were extracted from RCC2288 cells in late exponential phase as previously described (Ras et al. 2008). Briefly, cells were collected on 0.7 μm particle retention size filters (GF/F Whatman), pigments extracted for 2 h in 100% methanol, then subjected to ultrasonic disruption and clarified by filtration through 0.2 μm pore-size filters (PTFE). Pigments were detected using high-performance liquid chromatography (HPLC, Agilent Technologies 1200 CA, USA) over the 24 h after the extraction.

RESULTS AND DISCUSSION

Taxonomy section. Mantoniella beaufortii Yau, Lopes dos Santos and Eikrem sp. nov.

Description: Cells round measuring 3.7 ± 0.4 μm in diameter with one long (16.3 ± 2.6 μm) and one short flagellum (~1 μm). Cell body and flagella covered in imbricated spiderweb scales. Flagellar hair scales present composed of two parallel rows of subunits. Long flagellum tip has tuft of three hair scales. Scales produced in Golgi body. Golgi body located beneath and to one side of basal bodies. One green chloroplast with pyrenoid surrounded by starch and a stigma composed of a single layer of oil droplets (~0.1 μm). Ejectosomes composed of fibrils located at periphery of cell. Cell bodies with sub-quadrangular to oval scales (~0.2 μm). Body scales heptaradial, with seven major spokes radiating from center, number of spokes increasing toward the periphery. Six or more concentric ribs divide the scale into segments. Flagella with hexaradial oval scales composed of six spokes increasing in number toward the periphery. Six or more concentric ribs divide the scale into segments. Combined nucleotide sequences of the 18S rRNA (JN934679) and ITS2 rRNA (JQ413369) are species specific.

Holotype: Accession number O-A10010, plastic embedded specimen, 14 July 2009, from surface water, MALINA cruise leg 1b. Figure 4 shows the cells from the embedding. Culture deposited in The Roscoff Culture Collection as RCC2288.

Type locality: Beaufort Sea in the Arctic Ocean (70°30′ N, 135°30′ W).

Etymology: Named for its geographic provenance.

Mantoniella baffinensis Yau, Lopes dos Santos and Eikrem sp. nov.

Description: Cells measuring 4.7 ± 0.5 μm with one long flagellum of 21.8 ± 5.1 μm and one short flagellum (~1 μm). Cell body and flagella covered in imbricated spiderweb scales. Flagellar hair scales present composed of two parallel rows of subunits. Long flagellum tip has tuft of three hair scales. Cell bodies with sub-quadrangular to oval scales (~0.2 μm). Body scales octaradial with eight major radial spokes radiating from center, number of spokes increasing toward the periphery. Seven or more concentric ribs divide the scale into segments. Flagella with heptaradial, oval scales composed of seven spokes increasing in number toward the periphery. Six or more concentric ribs divide the scale into segments. Combined nucleotide sequences of the nuclear 18S rRNA (MH516003) and ITS2 rRNA (MH542162) are species specific.

Holotype: Accession number O-A10011, plastic embedded specimen, May 23, 2016, from surface sea ice, Green Edge project Ice Camp. Culture deposited in The Roscoff Culture Collection as RCC5418.

Type locality: Surface sea ice off the coast of Baffin Island in Baffin Bay (67°28′ N, 63°46′ W).

Etymology: Named for its geographic provenance.

Phylogeny and ITS signatures. The phylogenetic tree based on nearly full-length nuclear 18S rRNA sequences obtained from the novel polar strains RCC2288, RCC2285, RCC2497, and RCC5418 (Table 1), and environmental sequences retrieved from GenBank indicated that these strains belong to the family Mamiellaceae (Fig. S3 in the Supporting Information). The analysis also recovered the major genera within Mamiellales: *Bathycoccus*, *Ostreococcus*, *Micromonas*, *Mantoniella*, and *Mamiella* (Marin and Melkonian 2010). Dolichomastigales and Monomastigales were the basal orders in Mamiellophyceae with *Monomastix opisthostigma* type species used as an outgroup. Strains RCC2485, RCC2288, and RCC2497 isolated during the MALINA cruise in the Beaufort Sea and strain RCC5418 isolated from Baffin Bay during the Green Edge project Ice Camp formed a well-supported clade together with two environmental sequences (clone MALINA St320 3m Nano ES069 D8 and clone 4-E5), which also originated from Arctic Ocean samples. The two described *Mantoniella* species (*M. squamata* and *M. antarctica*) were not monophyletic in our analysis using the nuclear 18S rRNA, as reported by Marin and Melkonian (2010; fig. S3).

In contrast, the phylogenetic tree based on concatenated 18S/ITS2 alignments suggested that our strains belong in *Mantoniella* (Fig. 1). The grouping of our strains within *Mantionella* in the concatenated 18S/ITS tree was consistent with a recent nuclear multigene phylogeny based on 127 concatenated genes from related Chlorophyta species that also included RCC2288 with *Mantoniella* species (Lopes dos Santos et al. 2017b). This indicated the 18S/

ITS2 tree reflects the evolutionary history of the nuclear genome supporting the position of *Mantoniella* and our strains diverging from the same common ancestor.

The average distance between strains RCC2485, RCC2288, and RCC2497 was low (0.5% of segregating sites over the near full-length 18S rRNA gene), suggesting that these strains corresponded to a single species that we named *Mantoniella beaufortii* (see Taxonomy section). In contrast, the well-supported placement of strain RCC5418 on an earlier diverging branch within the *Mantoniella* clade, as well as the 1% average distance between RCC5418 and the other strains, suggested it represents another species, named here *Mantoniella baffinensis*.

To substantiate the description of *Mantoniella beaufortii* and *M. baffinensis* as new species, we investigated ITS2 synapomorphies of the different *Mantoniella* species. Although the use of ITS2 in taxonomy should be considered with caution (Müller et al. 2007, Caisová et al. 2011), several studies have shown the power of using ITS2 sequences in delimiting biological species, especially in microalgal studies (e.g., Coleman 2007, Caisová et al. 2011) including green algae (Subirana et al. 2013, Simon et al. 2017). For example, ITS sequencing contributed to distinguishing the Arctic diatom *Chaetoceros neogracilis* from an Antarctic *Chaetoceros* sp. that shared nearly identical 18S rRNA genes (Balzano et al. 2017). The analysis of ITS2 secondary structure in addition to molecular signatures of nuclear and plastid SSU rRNA genes supported the description of Chloropicophyceae clades as distinct species, despite the absence of clear morphological differences (Lopes dos Santos et al. 2017b). This conclusion has been further supported by recent phylogenetic analyses of chloroplast and mitochondrial genomes (Turmel et al. 2019). The

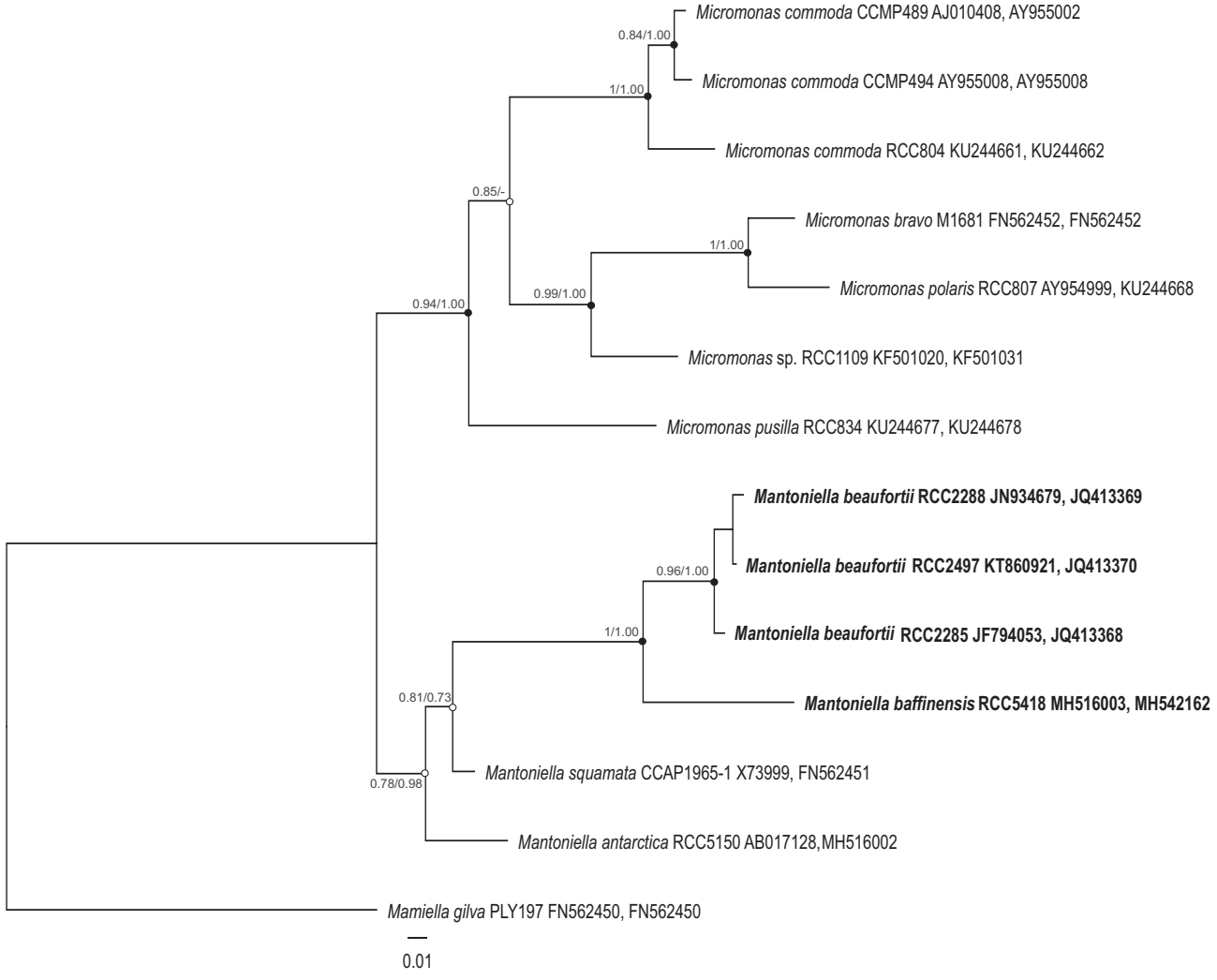

FIG. 1. Maximum-likelihood tree inferred from concatenated 18S/ITS2 sequences of Mamiellaceae. Solid dots correspond to nodes with significant support (> 0.8) for ML analysis and Bayesian analysis (> 0.95). Empty dots correspond to nodes with non-significant support for either ML or Bayesian analysis, or both.

computed ITS2 secondary structure of the new *Mantoniella* strains contained the four helix domains found in many eukaryotic taxa (Fig. S4 in the Supporting Information), in addition to Helix B9. The intramolecular folding pattern of the ITS2 transcript from *M. beaufortii* and *M. baffinensis* was very similar to the one from *M. squamata* and *M. antarctica* (Fig. S4). The universal hallmarks proposed by Mai and Coleman (1997) and Schultz et al. (2005) were present in Helices II and III of the Mamiellaceae. These were the Y-Y (pyrimidine–pyrimidine) mismatch at conserved base pair 7 in Helix II (Fig. 2) and YRRY (pyrimidine–purine–pyrimidine) motif at conserved positions 28–31 on the 5′ side of Helix III (Fig. S5a in the Supporting Information). In all four strains, the Y-Y mismatch was represented by the pair U-U and the YRRY motif by the sequence UGGU.

The structural comparison at each base pair position within the ITS2 helices identified several compensatory base changes (CBCs) and single-side changes or hemi-CBCs (hCBCs), as well as conserved base pair positions among *Mantoniella* species (Fig. S4). Note that we only considered hCBCs at positions where the nucleotide bond was preserved. No CBCs were found between the three *M. beaufortii* strains consistent with their designation as a single species. However, three hCBCs were detected in Helix II at positions 15 and 17 (Fig. 2) and Helix III at position 12 (Fig. S5A). Three CBCs were detected in Helices I (position 4), II (position 15), and IV (position 22) between *M. beaufortii* and *M. baffinensis*, supporting the separation of these strains into two distinct species (Figs. 2 and S4). When possible, the evolutionary steps of the identified CBCs and hCBCs were mapped upon branches of the Mamiellaceae phylogenetic tree that was constructed based on the concatenated 18S/ITS2 (Figs. 2 and S4) to distinguish synapomorphies from homoplasious changes (e.g., parallelisms and reversals). Few hypervariable positions showing several changes (CBCs and hCBCs) could not be unambiguously mapped upon the tree.

Morphology and ultrastructure. Under light microscopy, the cells of the new strains were green and round with one long and one short reduced flagellum (~1 μm), which were inserted almost perpendicularly to the cell (Fig. 3). They swam with their flagella directed posteriorly, pushing the cell. Occasionally, the cells ceased movement, pirouetted, and took off again in a different direction (video links in the Materials and Methods). All strains possessed a stigma, visible in light microscopy as a red eyespot located opposite the flagella. Although there are no morphological characters that are unique to the mamiellophyceans and shared by all of its members, the new strains closely resembled *Mantoniella* and *Mamiella*, which are similarly small round bi-flagellated cells (see Table S3 in the Supporting Information for morphological characters in described Mamiellophyceae). However, the flagella of *Mamiella* are of equal or near equal lengths (Moestrup 1984), so clearly the unequal flagella observed in our strains conform with described *Mantoniella* species, *M. squamata*, and *M. antarctica* (Barlow and Cattolico 1980, Marchant et al. 1989). The new strains were thus morphologically indistinguishable by light microscopy from *Mantoniella* species, supporting their placement in the genus.

The new strains were in the size range (Table 2) reported for *Mantoniella squamata* (3–6.5 μm) and *M. antarctica* (2.8–5 μm; Manton and Parke 1960, Marchant et al. 1989). Nonetheless, *M. beaufortii* strains were significantly smaller than *M. baffinensis* in cell diameter and average long flagellum length (Table 2) providing a means to distinguish the two new *Mantoniella* species from each other with light microscopy.

Transmission electron microscopy (TEM) of thin sections (Fig. 4) and whole mounts (Fig. 5) of the new strains provided details of their internal and external morphological features. The single chloroplast was cup-shaped with a pyrenoid surrounded by starch tubules running through the pyrenoid. The stigma was composed of a single layer of oil droplets (approximately 0.1 μm in diameter; Fig. 4a) and located at the periphery of the chloroplast facing the cell membrane, conforming to the description of the family Mamiellaceae (Marin and Melkonian 2010). Several large ejectosomes composed of fibrils were present at the cell periphery (Fig. 4, d and e). They are common in the Mamiellales (Moestrup 1984, Marchant et al. 1989) and are perhaps used to deter grazers.

One of the most salient features of the Mamiellophyceae is the presence of organic scales covering the cell, the most common of which comprise radiating and concentric ribs resembling spiderwebs that are present in the scale-bearing Mamiellales (*Bathycoccus*, *Mamiella*, and *Mantoniella*), as well as *Dolichomastix* (Table S3). We examined the whole mounts of the new *Mantoniella* species to establish the presence of scales and determine if they were morphologically distinguishable from related species, as *M. antarctica* (Marchant et al. 1989) and *Mamiella gilva* (Moestrup 1984) each have a unique type that differentiate them from other Mamiellales.

The flagella and cell bodies of the new strains were covered in imbricated spiderweb-like scales (Fig. 5) measuring approximately 0.2 μm. The scales were produced in the Golgi body (Fig. 4b). The body scales were sub-quadrangular to oval, whereas the flagellar scales were oval (Fig. 5). Spiderweb scales had 6–8 major spokes radiating from the center with the number of spokes increasing toward the periphery and six or more concentric ribs dividing the scale into segments. In addition, there were some small scales (approximately 0.1 μm) on the cell body composed of four spokes (increasing to eight) and separated by four, more or less concentric, ribs (Fig. 5, d and g). The flagella were also

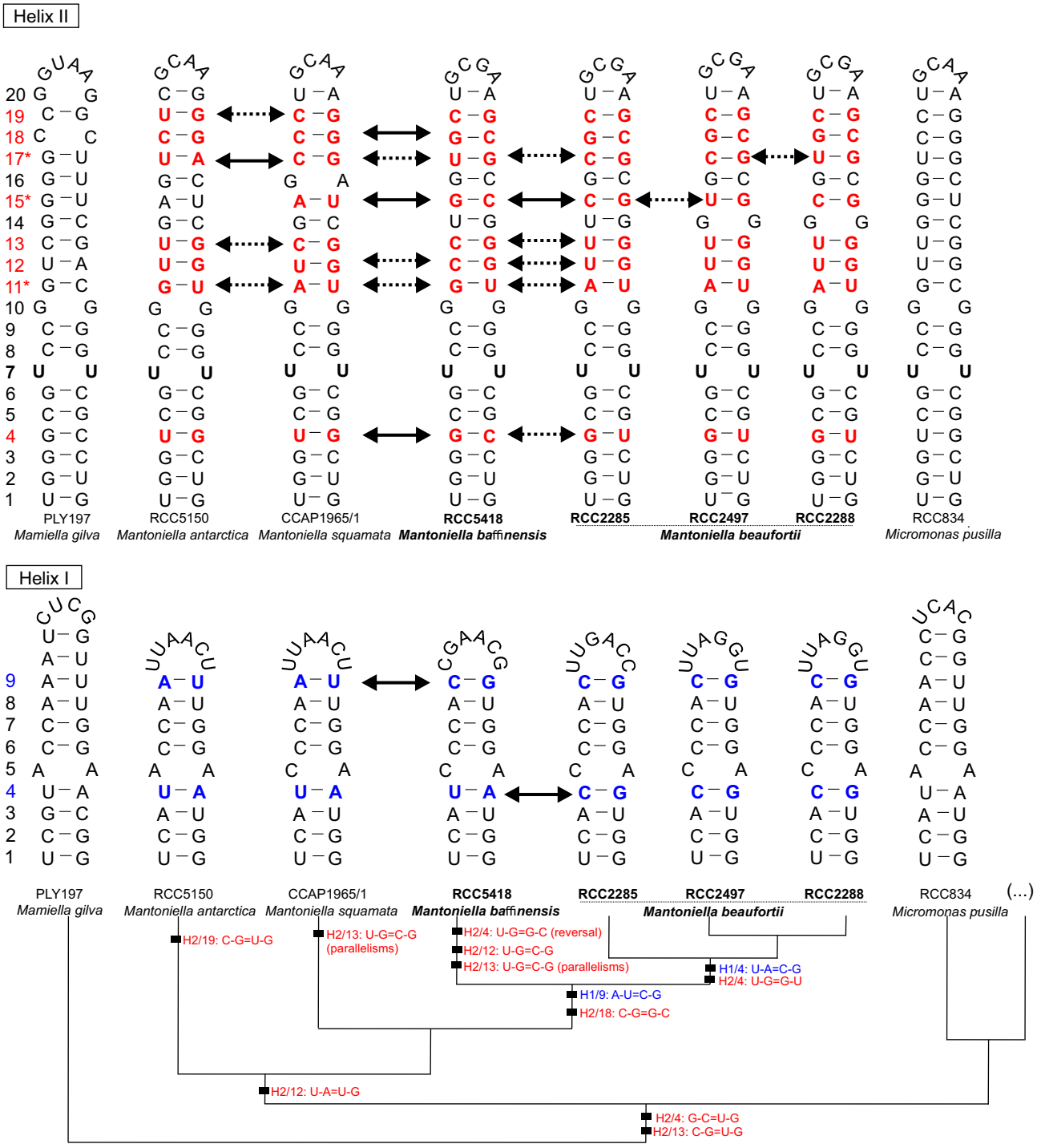

FIG. 2. Molecular signatures of *Mantoniella* species based on comparison of ITS2 secondary structures within Mamiellaceae. Signatures in Helix I and II are bolded. The conserved base pairs among the different groups are numbered. Compensatory base changes (CBCs) and hemi-CBCs (hCBSs) are highlighted by solid and dotted arrows, respectively. Hypervariable positions are marked by an asterisk (*). Ellipsis (...) represent the other clades and species of *Micromonas*. The pyrimidine–pyrimidine (Y-Y) mismatch in Helix II is shown in bold black. Single nucleotide substitutions are shown by grey nucleotides. Identified homoplasious changes are shown as parallelisms and reversals. [Color figure can be viewed at wileyonlinelibrary.com]

covered by lateral hair scales, which were composed of two parallel rows of globular subunits. At the tip of the long flagellum, there was a tuft of three hair scales, for which the subunits were more closely packed together than the lateral hair scales (Fig. 5). The hair scales of the new strains were identical to

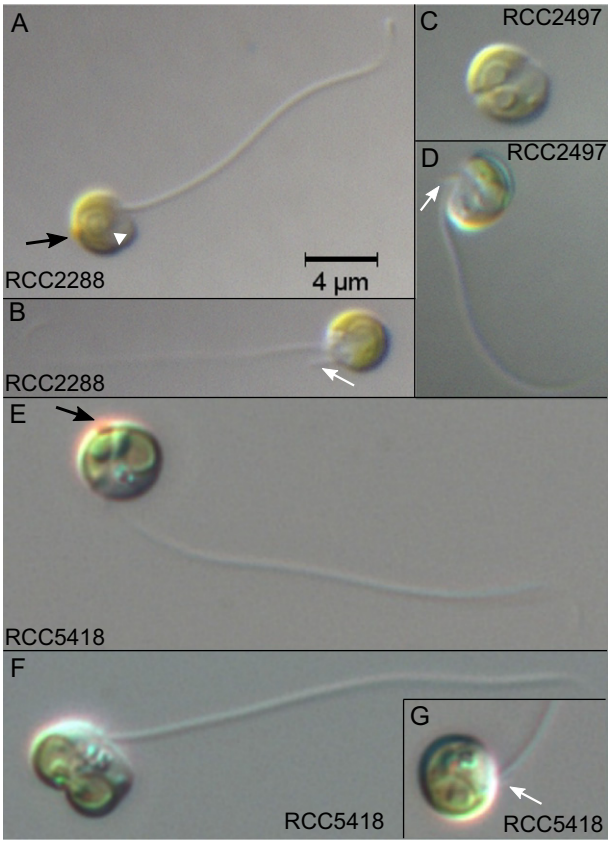

FIG. 3. Light microscopy images of the new *Mantoniella* strains. All strains have round cell morphology, visible red stigma (black arrow), a long and short flagella (white arrow), and one chloroplast with a pyrenoid (white arrowhead). Scale bar is 4 µm for all images. (A–B) *M. beaufortii* RCC2288. (C–D) *M. beaufortii* RCC2497 during cell division and single cell showing long and short flagella. (E–G) *M. baffinensis* RCC5418 single cell (E), during cell division (F) and cell showing the short flagellum (G inset). [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2. Cell diameter and long flagellum lengths measured for *Mantoniella beaufortii* (RCC2288 and RCC2497) and *M. baffinensis* (RCC5418).

Strain	Min	Max	Mean	Median	SD	*n*
Cell diameter (µm)						
RCC2288	2.89	4.98	3.77	3.70	0.41	60
RCC2497	3.15	4.74	3.87	3.77	0.39	39
RCC5418	3.54	5.69	4.66	4.66	0.51	69
Long flagellum length (µm)						
RCC2288	12.93	21.47	16.27	15.99	2.63	11
RCC2497	11.91	21.25	16.31	17.07	2.71	12
RCC5418	11.27	32.59	21.78	21.29	5.14	25

n = number of cells measured and SD = standard deviation.

the "*Tetraselmis*-type" T-hairs previously described in *Mantoniella* and *Mamiella* (Marin and Melkonian 1994). This structure is otherwise only seen in *Dolichomastix lepidota* and differs from the smooth tubular T-hairs of *Dolichomastix tenuilepis* and *Crustomastix* (Marin and Melkonian 1994, Zingone et al. 2002, Table S3).

Comparison of the spiderweb scales between *Mantoniella* species (Table 3) showed the new species differ significantly from *M. antarctica*, which possesses lace-like scales with six or seven radial ribs with very few concentric ribs (Marchant et al. 1989). Morphologically, the spiderweb scales of the new species most resembled *M. squamata*, which has large heptaradial flagellar scales, octaradial body scales, and a few additional small tetraradial body scales (Marchant et al. 1989). Indeed, the spiderweb scales of *M. baffinensis* (Fig. 5) were structurally indistinguishable from *M. squamata*. In contrast, *M. beaufortii* shared the small tetraradial body scales but possessed hexaradial flagellar scales and heptaradial body scales, potentially allowing it to be differentiated from the other *Mantoniella* based on the number of radial spokes of the spiderweb scales.

Pigment composition. Pigment to chlorophyll *a* ratios of *Mantoniella beaufortii* RCC2288 were compared to a selection of other Chlorophyta species (Fig. 6; Table S4 in the Supporting Information) from previous studies (Latasa et al. 2004, Lopes dos Santos et al. 2016), as pigments are useful phenotypic traits. Chlorophyll *a* and *b*, characteristics of Chlorophyta, were detected, as well as the basic set of carotenoids found in the prasinophytes: neoxanthin, violaxanthin, lutein, zeaxanthin, antheraxanthin, and β-carotene. The additional presence of prasinoxanthin, micromonal, and uriolide placed RCC2288 in the PRASINO-3B group of prasinophyte green algae, sensu Jeffrey et al. (2011). This pigment-based grouping showed good agreement with the molecular phylogeny of Mamiellales, where the presence of prasinoxanthin, micromonal, and the Unidentified M1 pigment are diagnostic of the order (Marin and Melkonian 2010). We did not detect Unidentified M1 in RCC2288, but as our analysis method differed from previous work (Latasa et al. 2004) and we relied on matching its chromatographic and spectral characteristics, its absence requires further confirmation. Notwithstanding, the pigment complement of RCC2288 was identical to other described Mamiellales (Fig. 6; Table S4), coherent with its classification within this order.

As noted by Latasa et al. (2004), Mamiellales pigment profiles are remarkably comparable (Fig. 6), despite strains being cultured under very different conditions. Only a few carotenoids differed substantially (at least 2-fold) in relative abundance between *Mantoniella beaufortii* and the two other *M. squamata* strains analyzed: the concentration of neoxanthin, antheraxanthin, and lutein were higher, whereas that of Mg-DVP and uriolide were relatively lower (Fig. 6; Table S4). Neoxanthin (associated with the light harvesting complex), as well as antheraxanthin and lutein (both involved in photoprotection), has previously been shown to increase significantly in *M. squamata* grown under continuous light

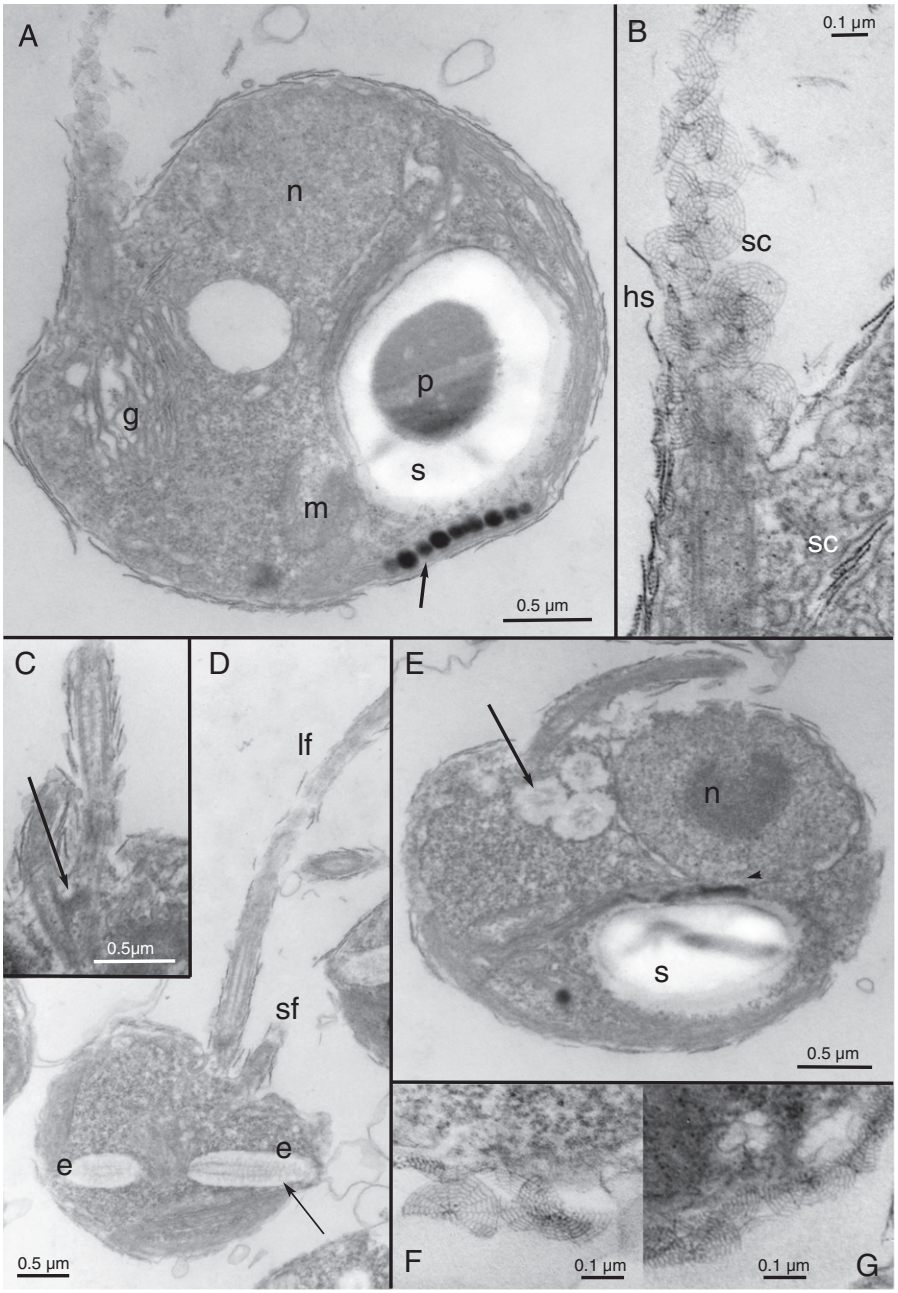

FIG. 4. TEM thin sections of *Mantoniella beaufortii* RCC2288. (A) Internal cell structure showing organelles and stigma (black arrow). (B) Detail of the hair and spiderweb scales covering the long flagellum. Scales produced in the Golgi body. (C) Detail of the flagellar base (black arrow). (D) Cell with long and short flagella and longitudinal section of the ejectosomes (black arrow). (E) Cross-section of ejectosomes (black arrow). (F) and (G) body scales made up of radiating and concentric ribs. e=ejectosome, g=Golgi, s=starch granule, m=mitochondrion, n=nucleus, p=pyrenoid, hs=hair scale, sc=scale, lf=long flagellum and sf=short flagellum.

compared to alternating light/dark cycles (Böhme et al. 2002). Therefore, the relatively high ratio of these carotenoids measured in *M. beaufortii* is consistent with growth under continuous light used with RCC2288. Uriolide and Mg-DVP have been observed to increase with light intensity in *M. squamata* (Böhme et al. 2002) and *Micromonas pusilla* (Laviale and Neveux 2011), respectively. Although more physiological data are required to interpret their relative decrease in RCC2288, these pigments are probably most responsive to light conditions (intensity and photoperiod).

Two unknown carotenoids were detected in RC2288, the first one having adsorption peaks at 412, 436, and 464 nm, and the second one at 452 nm (Table S5 in the Supporting Information). These were relatively minor components comprising 2.7% and 1.5% of total carotenoids, respectively, and may represent carotenoids unique to *Mantoniella beaufortii*.

Environmental distribution. To obtain information on the distribution of these two new species, we searched by BLAST both environmental GenBank sequences and published 18S V4 and V9 metabarcode data sets (Table S2). This allowed the retrieval

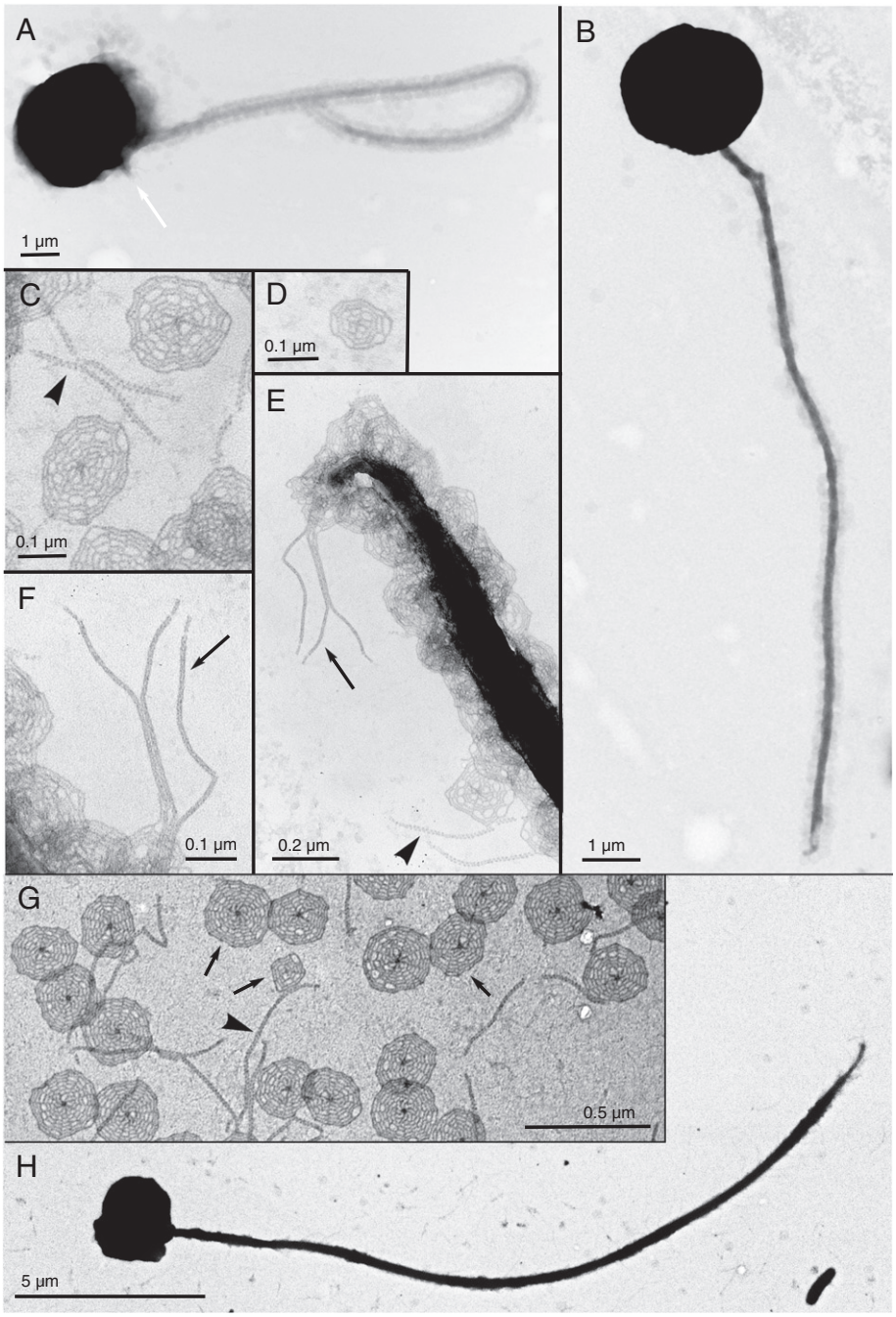

FIG. 5. Transmission electron micrographs of whole mounts of the new *Mantoniella* strains. (A–E) *M. beaufortii.* (A) Whole cells of strain RCC2288, indicating the short flagellum (white arrow) and (B) RCC2497. (C) Detached flagellar spiderweb-like scales and hair scales (black arrowhead). (D) Detail of small tetraradial body scale. (E) Imbricated scales and hair scales covering the long flagellum. A tuft of three hair scales on the tip of the long flagellum (black arrow) (F) Detail of the tuft of hair scales (black arrow). (G–H) *M. baffinensis* RCC5418. (G) Small and large body scales (black arrows) and flagellar hair scales (black arrowhead) and (H) whole cell.

TABLE 3. Comparison of *Mantoniella* spp. scale types.

Species	Flagellar scales	Body scales
Mantoniella squamata	Spiderweb-like heptaradial	Spiderweb-like large octaradial and small rare tetraradial
Mantoniella antarctica	Lace-like heptaradial	Lace-like hexaradial and smaller heptaradial
Mantoniella beaufortii	Spiderweb-like hexaradial	Spiderweb-like large heptaradial and small rare tetraradial
Mantoniella baffinensis	Spiderweb-like heptaradial	Spiderweb-like large octaradial and small rare tetraradial

of a few 18S rRNA sequences with higher than 98% similarity to the gene of RCC2288. Alignment of these sequences with other Mamiellophyceae sequences revealed diagnostic positions in both the V4 and V9 hypervariable regions permitting *Mantoniella beaufortii* and *M. baffinensis* to be

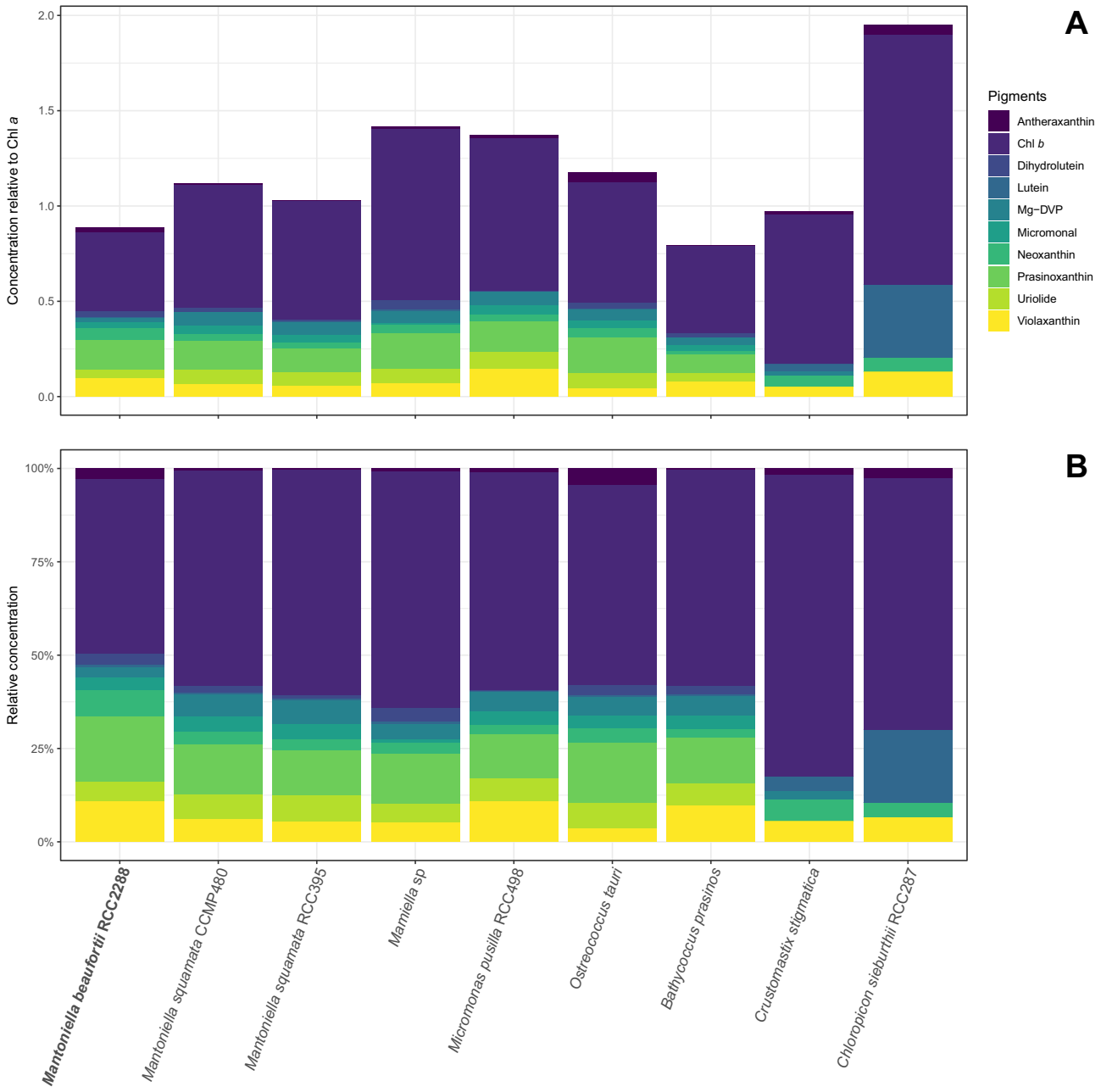

FIG. 6. Pigment to chlorophyll *a* ratios in *Mantoniella beaufortii* RCC2288 (this study) compared to other Mamiellophyceae species (data from Latasa et al. 2004). (A) Cumulative pigment to Chlorophyll *a* ratio of Chlorophyll *b* and abundant carotenoids (excluding α- and β-carotene). (B) As for A, but showing relative abundances. Mg-DVP: Mg-24-divinyl pheoporphyrin *a*5 monomethyl ester. [Color figure can be viewed at wileyonlinelibrary.com]

distinguished from other Mamiellophyceae, especially other *Mamiella* and *Mantoniella* species (Figs. S1 and S2). Signatures from the V4 region were clearer than from V9 due to the fact that for some of the strains, the sequences did not extend to the end of the V9 region (Fig. S2). In the V4 region, three signatures were observed, one common to both species (Fig. S1a), whereas the other two (Fig. S1, b and c) differed between *M. beaufortii* and *baffinensis*.

No clone library or metabarcode sequences matched exactly *Mantoniella baffinensis*. In contrast, three environmental sequences (KT814860, FN690725, and JF698785) from clone libraries had signatures similar to the *M. beaufortii* strains, two from Arctic Ocean water (Fig. 7), including one obtained during the MALINA cruise, and one from ice originating from the Gulf of Finland. V4 metabarcodes corresponding to *M. beaufortii* were found in the Ocean Sampling Day data set (Kopf

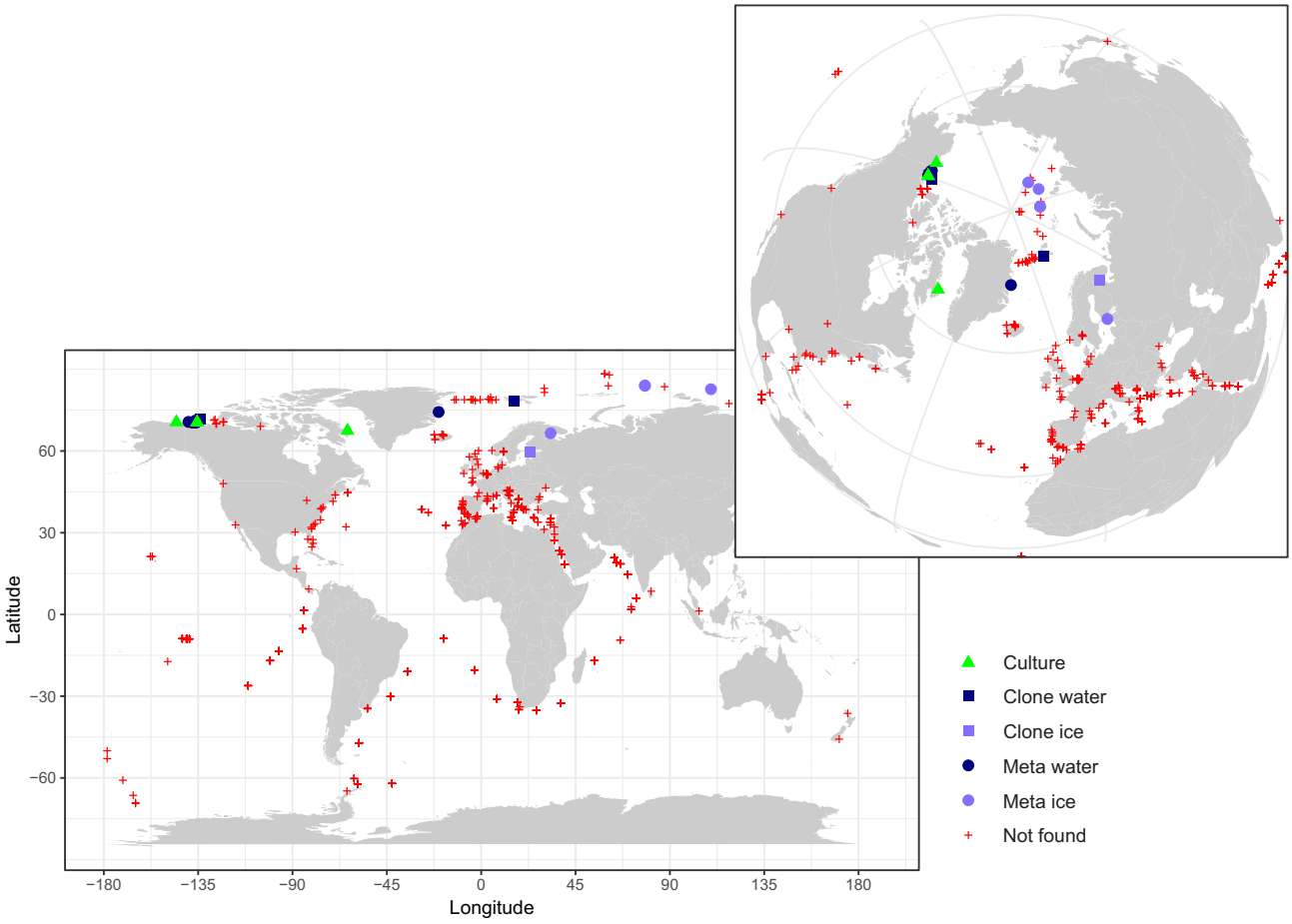

FIG. 7. Map of the distribution of *Mantoniella beaufortii* in environmental sequence data sets highlighting its prevalence in Arctic samples (inset). The isolation sites of *M. beaufortii* cultures, presence of its 18S rRNA gene sequence in clone libraries (Clone water, Clone ice), and metabarcodes from seawater and ice samples (Meta water, Meta ice) and absence in metabarcodes (Not found) are plotted. For *M. baffinensis*, only its isolation site is indicated in Baffin Bay since no similar environmental sequence was found in the data sets analyzed. Metabarcoding data sets include Ocean Sampling Day, Tara Oceans, and polar projects. See Table S2 for a full description of the metabarcoding data sets screened. [Color figure can be viewed at wileyonlinelibrary.com]

et al. 2015) that includes more than 150 coastal samples at a single station off East Greenland as well as in three metabarcoding studies in the Arctic Ocean, one in the Beaufort Sea performed during the MALINA cruise (Monier et al. 2015), one from Arctic sea ice (Stecher et al. 2016) where it was found at three stations and one from the White Sea (Belevich et al. 2017), also in the sea ice (Fig. 7). No metabarcode corresponding to these two new species were found in waters from either the Southern Ocean or off Antarctica (Fig. 7; Table S2). No metabarcodes from the V9 region corresponding to the two new species were found in the Tara Oceans data set that covered mostly temperate and subtropical oceanic regions (de Vargas et al. 2015). These data suggest that these species are restricted to polar Arctic regions (although we cannot exclude that they may be found in the future in the Antarctic which has been under-sampled until now) and are probably associated with sea ice although they can be present in the sea water, and that *M. beaufortii* is more wide spread than *M. baffinensis*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Fig. S1. Alignment of the 18S rRNA gene V4 hypervariable region from *Mantoniella beaufortii* and *M. baffinensis* strains (Red and Orange, respectively), environmental clones (Blue) and metabarcodes (Green) with a selection of sequences from closely related Mamiellophyceae. Sequence signatures diagnostic of the two new species are indicated by boxes. The A region is specific of both species while the B and C regions differ between the two species.

Fig. S2. Alignment of the 18S rRNA gene V9 hypervariable region from *Mantoniella beaufortii* and *M. baffinensis* strains (Red and Orange, respectively) and environmental clones (Blue) with a selection of closely related Mamiellophyceae sequences. Sequence signatures diagnostic of *M. beaufortii* and *M. baffinensis* are indicated by arrows.

Fig. S3. Maximum-likelihood phylogenetic tree inferred from nuclear 18S rRNA sequences of Mamiellophyceae. *Monomastix opisthostigma* was used as an outgroup. Solid dots correspond to nodes with significant support (> 0.8) for ML analysis and Bayesian analysis (>0.95). Empty dots correspond to nodes with non-significant support for either ML or Bayesian analysis, or both. GenBank accessions of the 18S rRNA sequences shown after the species name.

Fig. S4. Intramolecular folding pattern of the ITS2 molecule of *Mantoniella* (RCC2288, RCC2285, RCC2497 and RCC5418). The four major helices are labeled as Helix I – Helix IV. Blue dots represent either CBCs or hCBCs. Non-CBCs (N – N ↔ N × N) are represented in orange.

Fig. S5. Molecular signatures of *Mantoniella* species revealed by comparison of ITS2 secondary structures within Mamiellaceae. Signatures in Helix III are shown in (**A**) and Helix IV in (**B**). The conserved base pairs among the different groups are numbered. CBCs and hCBCs are

highlighted by solid and dotted arrows, respectively. Hypervariable positions are marked by an asterisk (*). Ellipsis (. . .) represent the other clades and species of *Micromonas*. The YRRY (pyrimidine-purine-pyrimidine) motif on the 5′ side arm of Helix III is shown in bold black. Single nucleotide substitutions are shown by grey nucleotides. Identified homoplasious changes are shown as parallelisms and reversals.

Table S1. Primers and PCR conditions used in this study. Abbreviations: fwd – forward, rev. – reverse, Temp. – Temperature.

Table S2. Metabarcoding datasets of the 18S rRNA gene analyzed in this study for the presence of *Mantoniella beaufortii* and *M. baffinensis* signatures.

Table S3. Morphological characters in Mamiellophyceae species.

Table S4. Pigment composition of *Mantoniella beaufortii* (RCC2288) compared to a selection of green algae. Values are shown as a ratio of pigment to Chl *a* concentration and percent contribution to total carotenoids (in italics). See Table S5 for the full names of the pigments.

Table S5. Pigments analyzed in this study. LOD, limit of detection.