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A new species of *Chrysosphaerella* (Chrysophyceae: Chromulinales), *Chrysosphaerella rotundata sp. nov.*, from Finland

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Abstract

Chrysosphaerella rotundata sp. nov. is described from a small lake in Finland. Ultrastructural morphology of *C. rotundata* scales is similar, even identical, to the morphology of *C. brevispina* scales. However, *C. rotundata* possesses three types of scales: scales characterized by a circular or almost circular outline were found in addition to the larger and smaller oval scales. Significant genetic differences were recognized in ITS rDNA sequences between *C. rotundata* and *C. brevispina*. Results of the molecular analyses and the observed morphological variation of scales have been discussed, clearly showing the existence of a hidden diversity within the genus *Chrysosphaerella.*

Introduction

The genus *Chrysosphaerella* Lauterborn (1896: 16) represents free-living, autotrophic organisms covered with siliceous scales and spines and bearing two flagella of unequal length. Cells may be solitary or grouped in more or less spherical colonies. Two colonial taxa, *C. longispina* Lauterborn (1896: 16) and *C. brevispina* Korshikov (1942: 31) emend. Harris & Bradley (1958: 75) are commonly found in freshwater habitats (Kristiansen & Preisig 2001). Taxonomy of the genus *Chrysosphaerella* is based on ultrastrucutre of siliceous scales and spines, and the images of *C. longispina* and C. *brevispina* include both transmission and scanning electron micrographs (e.g. Nicholls 1980, Cronberg & Kristiansen 1980, Siver 1993). The phylogenetic position of the genus *Chrysosphaerella* was reported by Andersen (2007) based on uncultured and unidentified isolates. Subsequently, SSU rDNA and *rbc*L sequences of cultured *C. brevispina* and *C. longispina* corroborated the phylogenetic position of *Chrysosphaerella*, which is firmly placed into the clade comprising naked chrysophyte genera *Chrysamoeba* Klebs (1893: 407), *Chromulina* Cienkowski (1870: 435), and *Oikomonas* Kent (1880: 230, 250) (Škaloud *et al.* 2013).

The detailed ecological study of *C. brevispina* and *C. longispina* has been published by Siver (1993), who investigated their distribution along seasonal, temperature, pH, specific conductance, and total phosphorus gradients. *Chrysosphaerella brevispina* and *C. longispina* have been proposed to be distributed primarily in the winter and the summer/autumn months, respectively. Both taxa were found to be primarily distributed at pH 5 to 7, and in waters having low specific conductance. However, *C. brevispina* has been proposed to tolerate higher trophic and conductance conditions, which could explain its more common occurrence (Siver 1993).

Colonial *Chrysosphaerella* species are widely distributed worldwide (Siver 1993). Both species have been reported from Finland as well (e.g. Hällfors & Hällfors 1988, Ikävalko 1994). During our investigation of Finnish chrysophyte flora in the spring of 2012, we reported *Chrysosphaerella* taxa in several investigated localities. Besides *C. brevispina* colonies bearing morphologically typical silica scales, some isolated colonies were partially covered by distinct, rounded scales. Therefore, we aimed to further investigate the morphology and phylogenetic position of this organism.

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Material and methods

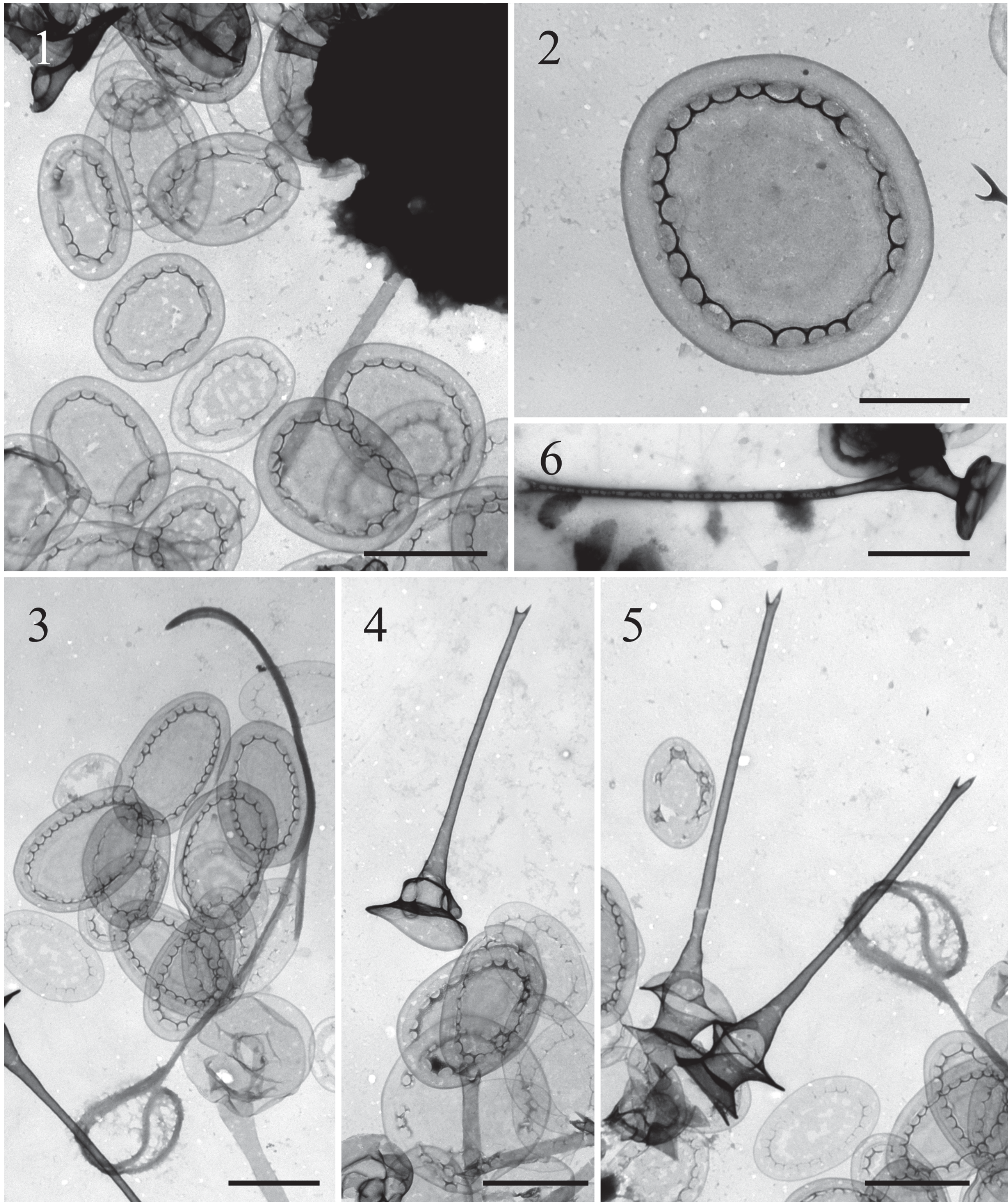
The material was collected on 1 May 2012 from a small, unnamed lake near the Alalampi lake in Central Finland (62° 15' 1.07" N, 26° 34' 48.00" E, water temperature: 5.6 °C, pH: 7, conductivity: 40 µS cm^{-1}), using a plankton net with a mesh size of 20 µm. The *Chrysosphaerella* colonies were isolated by pipetting, and were subsequently cultured at 15 °C in Erlenmeyer flasks filled with DY IV medium (Andersen *et al.* 1997). In addition, we investigated the culture of *C. brevispina* S 74.D5 isolated from Dráchovské pools in the Czech Republic (Škaloud *et al.* 2013). For transmission electron microscopy (TEM) investigations, the samples were dried onto Formvar-coated copper grids and gently rinsed with distilled water. The TEM grids were examined with a JEOL 1011 transmission electron microscope.

For DNA isolation, ca. 2000 cells were centrifuged in PCR tubes (6000 rpm for 3 minutes), and 50 mL of InstaGene matrix (Bio-Rad Laboratories) was added to the pellet. The solution was vortexed for 10 s, incubated at 56 °C for 30 min, and heated at 99 °C for 8 min. After vortexing a second time, the tubes were centrifuged at 12000 rpm for 2 min, and the supernatant was directly used as a PCR template. Two molecular markers were amplified by PCR: nuclear SSU rDNA and chloroplast *rbc*L. The amplification of SSU rDNA was performed as described by Škaloud *et al.* (2013), using the primers 18S-F and 18S-R (Katana *et al.* 2001). The amplification of the *rbc*L marker was performed according to Jo *et al.* (2011), using the newly designed primers *rbc*L-Chrys-F1 (5'-TTG GAC AGA TTT ATT AAC-3'), *rbc*L-Chrys-F2 (5'-TTA TTA ACW GCT TGT GAT-3') and *rbc*L-Chrys-R (5'-TCC ATR TCR AAG AAA ATW CC-3'). The amplification of ITS rDNA was performed as described by Kynčlová *et al.* (2010). The PCR products were purified and sequenced at Macrogen in Seoul, Korea. The newly obtained sequences of the ITS rDNA, SSU rDNA and *rbc*L regions of *C. rotundata* and *C. brevispina* were deposited in GenBank with the accession numbers HG315742 - HG315746.

A multiple alignment of the newly determined SSU rRNA and *rbc*L gene sequences and other sequences selected from the GenBank/EMBL/DDBJ databases was built using MAFFT, version 6, applying the Q-INS-i strategy (Katoh *et al.* 2002). The sequences were selected to encompass all known Chrysophyte lineages, with the exception of Paraphysomonadales, for which the *rbc*L sequences are unavailable. The final matrix contained 52 taxa (51 SSU rDNA and 48 rbcL sequences, respectively). *Nannochloropsis limnetica* (Eustigmatophyceae) and *Synchroma grande* (Picophagea/Synchromophyceae) were selected as ougroup. Poorly aligned regions of the SSU rDNA partition, as well as the saturated positions of the *rbc*L partition, were removed as described in Škaloud *et al.* (2013). The final concatenated alignment consisted of 2591 bp comprising 1687 bp of SSU rDNA and 904 bp of *rbc*L. Suitable substitution models for the entire SSU rDNA dataset and individual *rbc*L codon positions were selected using MEGA 5 (Tamura *et al.* 2011). The GTR+G+I model was estimated as the most appropriate for all partitions.

The phylogenetic trees were inferred with Bayesian Inference (BI) by using MrBayes v. 3.2.1 (Ronquist & Huelsenbeck 2003), maximum likelihood (ML) analysis using GARLI v. 2.0 (Zwickl 2006), and weighted maximum parsimony (wMP) analysis using PAUP v. 4.0b10 (Swofford 2002), respectively. BI analysis was carried out on a partitioned dataset to differentiate among SSU rDNA gene and individual *rbc*L codon positions. All parameters were unlinked among partitions. Two parallel Markov Chain Monte Carlo (MCMC) runs were carried out for 5 million generations each with one cold and three heated chains. Trees and parameters were sampled for every 100 generations. Parameter stability and run convergence were inspected using Tracer v1.4.1 (Rambaut & Drummond 2003). The first 10% of samples were discarded as burnin, using the "sumt" command. ML analysis was carried out on a partitioned dataset, with the GTR+G+I model applied to each partition, using default settings, five search replicates, and the automatic termination set at 100000 generations. The wMP analysis was performed using heuristic searches with 1000 random sequence addition replicates, TBR swapping, and random addition of sequences (the number was limited to 10000 for each replicate). The weight to the characters was assigned using the rescaled consistency index on a scale of 0–1000. New weights were based on the mean of the fit values for each character over all of the trees in memory. ML and MP bootstrap support values were obtained from 100 and 1000 bootstrap replicates, respectively. Only one search replicate was applied for the ML bootstrapping.

The ITS2 secondary structures of investigated *Chrysosphaerella* strains were constructed using the mfold computer program v. 2.3 (Zuker 2003), with folding temperature set to 18 °C. Consensus secondary structure was drawn using VARNA (Darty *et al.* 2009), and the compensatory base changes (CBC) were manually derived.



FIGURES 1–6. *Chrysosphaerella rotundata, sp. nov.* Fig. 1. Large oval and less or more circular scales. Fig. 2. Large circular scale. Fig. 3. Large and small oval scales. Fig. 4. Spine with a thick shaft, and both oval and circular scales. Fig. 5. Two spines with a thick shaft. Fig. 6. Spine showing a variation in a thickness of a shaft. Scale bars: Figs. 1, 3–6: 2 µm; Fig. 2: 1 µm.

Taxonomy

Chrysosphaerella rotundata Škaloudová & Škaloud, *sp. nov.* (Figs. 1–6)

Colonies spherical, consisting of cells bearing two flagella. Cells covered by numerous scales and spines. Three size classes of scales occur: large circular scales (3.0–3.5 × 2.2–3.1 µm), large oval scales (2.0–3.1 × 1.3–2.4 µm), and smaller oval scales (1.5–1.7 × 1.2 µm). Scales patterned with a series of short ridges forming a scalloped shaped pattern. Spines with a shaft joining the two plates of the bobbin-like structure. The length of the spines varies from 4 µm to 10 µm. Cysts unknown.

Type:—FINLAND. Keski-Suomi, 62° 15' 1.07" N, 26° 34' 48.00" E, a small, oligotrophic unnamed lake, water temperature 5.6 °C, pH 7, conductivity 40 µS cm^{-1}, coll. *Škaloudová & Škaloud*, 1 May 2012 (holotype: Strain S89.C4, frozen material deposited at the Culture Collection of Algae of the Charles University in Prague, Department of Botany, Benátská 2, 12801 Prague 2, Czech Republic). Fig. 2 is an illustration of the holotype.

Cells were grouped into spherical colonies. Individual cells were spherical to pyriform, about 12–13 µm long and 11 µm wide, bearing two flagella. Cells were covered by numerous scales and spines. Three size classes of scales might be discerned; however, a continuous transition in size of scales existed. The majority of the scales were larger and oval in outline (Figs. 1, 3). The second type of the scales, larger and circular in outline, were produced less frequently (Fig. 2). However, presence of the large, circular-shaped scales was a main distinguishing character of *Chrysosphaerella rotundata.* Both large circular and oval scales were patterned with a series of short ridges forming a scalloped shaped pattern. The pattern of smaller oval scales (Fig. 3) was the same as that of larger scales, but often less distinct. The spines had a thick shaft joining the two plates of the bobbin-like structure (Figs. 4–5); however, a variation in the thickness of the shaft was observed and spines with a thinner shaft were found as well (Fig. 6).

Etymology:—The specific epithet 'rotundata' refers to the rounded shape of scales.

Phylogenetic analyses, ITS2 secondary structures:—Bayesian inference, Maximum Likelihood, and Maximum Parsimony analyses inferred from the concatenated SSU rDNA and *rbc*L sequences resulted in highly similar phylogenetic trees, recognizing the six main lineages within Chrysophyceae (Fig. 7). According to their members, the lineages could be recognized as traditionally defined orders: Chromulinales, Chrysosaccales, Hibberdiales, Hydrurales, Ochromonadales, and Synurales. With the exception of the Ochromonadales, all lineages were also significantly supported by ML and wMP analyses. Both analyses resolved the Ochromonadales as monophyletic, but without any statistical support. The genus *Chrysosphaerella* formed a firmly supported monophyletic lineage within the Chromulinales (Fig. 7). It was divided into two supported subclades. *Chrysosphaerella rotundata* was inferred as closely related to morphologically similar *C. brevispina*, with which it formed the first inferred subclade. The species differed by eight and eleven nucleotide substitution changes in the SSU rDNA and *rbc*L sequences, respectively. The second subclade consisted of *C. longispina* and two environmental *Chrysosphaerella* isolates. Since the isolates were molecularly characterized by only the SSU rDNA (summer isolate) or *rbc*L (winter isolate) sequence, we could not exclude that they in fact belong to the same genotype. The environmental isolates differed by two nucleotide substitution changes in the SSU rDNA and *rbc*L sequences from the *C. longispina* sequences.

To further evaluate the degree of genetic differentiation of the closely related *C. rotundata* and *C. brevispina*, we additionally sequenced the ITS1-5.8S rDNA-ITS2 region. The ITS1 region was hardly alignable, and the sequences differed significantly in their length (342 bp in *C. rotundata* vs. 294 bp in *C. brevispina*). Within the aligned regions, the divergence between the ITS1 rDNA sequences was approximately 15.5%. The length of the ITS2 region was similar in both species (293 bp in *C. rotundata* vs. 287 bp in *C. brevispina*), and the overall divergence between the ITS2 rDNA sequences was approximately 12.5%. In addition, we mapped the differences in the ITS2 sequence on a predicted ITS2 secondary structure (Fig. 8). Most of the differences were located in terminal and internal hairpin loops. Four compensatory base changes (CBCs) were found in the stem regions of helices I and II.

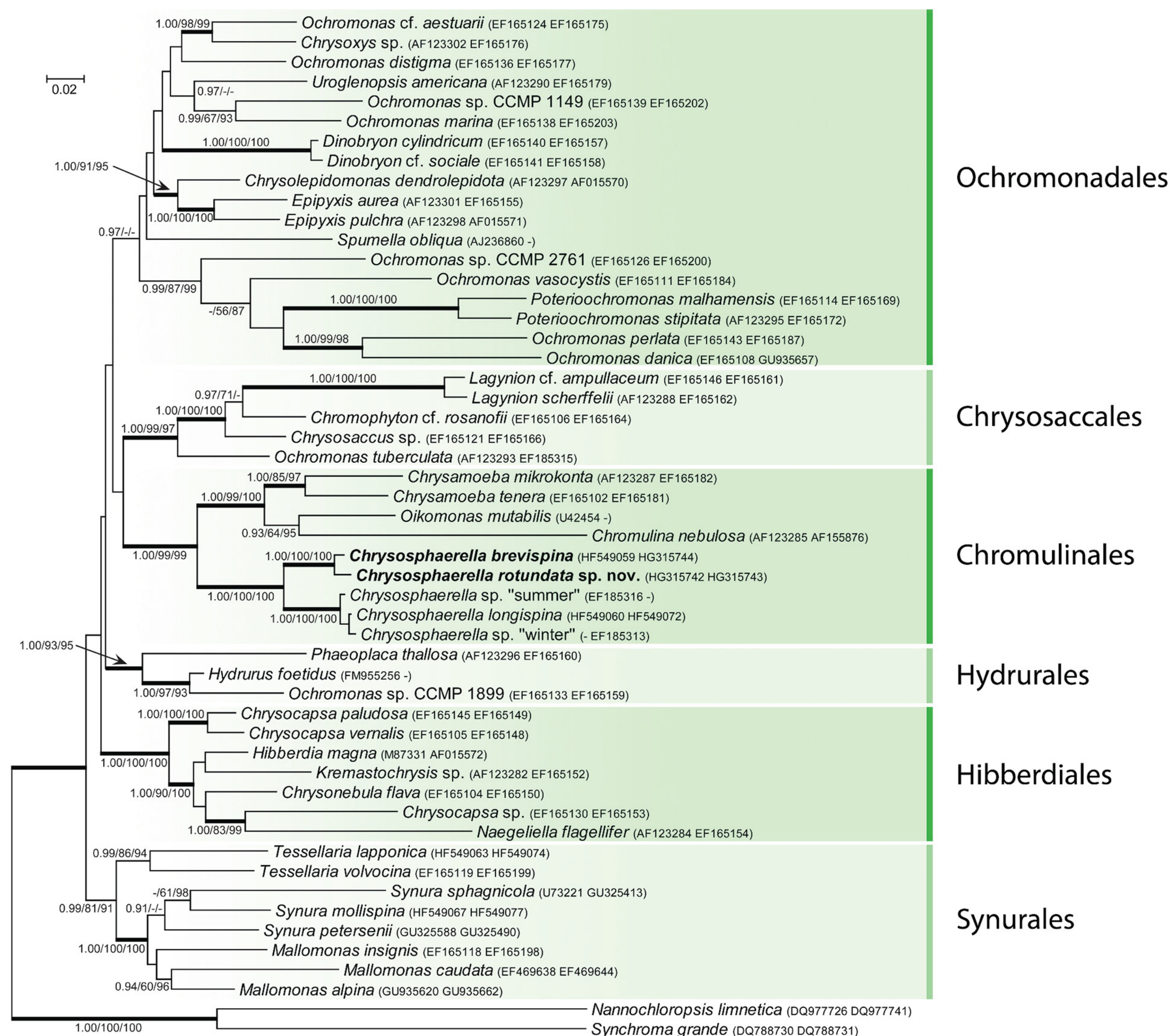


FIGURE 7. Bayesian analysis of Chrysophyceae, based on the combined and partitioned SSU rDNA + *rbc*L dataset using a GTR+G+I model for all partitions. Values at the nodes indicate statistical support estimated by three methods – MrBayes posterior-node probability (left), maximum-likelihood bootstrap (middle), and maximum parsimony bootstrap (right). Thick branches represent nodes receiving the highest PP support (1.00). Newly obtained sequences are given in bold. Accession numbers for the concatenated sequences (SSU rDNA and *rbc*L, respectively) accompany each species name. Scale bar shows the estimated number of substitutions per site.

Discussion

Chrysosphaerella brevispina is characterized by two types of scales, differing by their size. Siver (1993) has measured both types of scales found in several North American localities: the dimension of the larger oval scales was 2.9–4.0 × 1.4–2.5 µm, and the mean size of the smaller oval scales was 1.7 × 0.8 µm. This observed range of scale sizes of both the large and the small types is in agreement with the *C. brevispina* scales found in Europe (own unpublished data). In *Chrysosphaerella rotundata*, both large and small scale types are also produced. Morphologically, they are identical to *C. brevispina* by their oval shape, but they tend to be slightly shorter and wider. Besides the larger and smaller oval scales, *C. rotundata* possesses the third, newly recognized type of scales, characterized by a circular or almost circular outline. Interestingly, large, more or less circular *Chrysosphaerella* scales have been recorded by Nicholls (1984), who considered them as belonging to *C. brevispina*. However, only oval scales were mentioned and illustrated in Korshikov's original description of *C. brevispina* (Korshikov 1942), based on light microscopic investigations. Subsequently, Harris & Bradley (1958) have emendated Korshikov's description by characterizing the detailed ultrastructural morphology of the *C. brevispina* scales and spines observed by electron microscope. Congruent with the original description, they have recorded the oval shaped scales only.

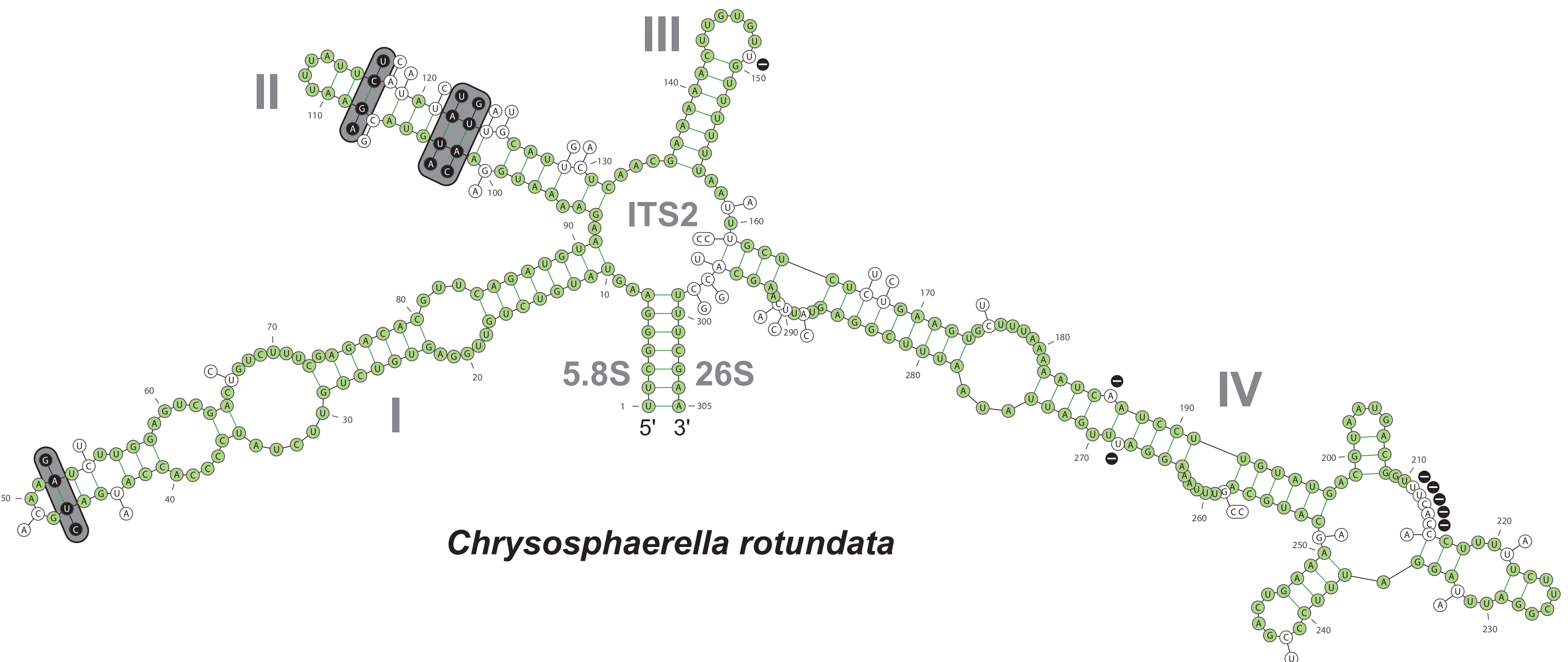


FIGURE 8. Comparison of the ITS2 sequences and predicted secondary structures of *Chrysosphaerella rotundata* and *C. brevispina*. Base numbering is indicated every 10 bases, and the four helices are numbered with Roman numerals. The structure shown corresponds to *C. rotundata*; positions conserved in *C. brevispina* are portrayed in green, bases substituted in *C. brevispina* are shown by the structure and connected to the respective position by a short line, deletions are indicated with minus symbols. Four base pairs marked in grey boxes are compensatory base changes (CBCs).

The above-mentioned morphological differences warrant the recognition of *C. brevispina* and *C. rotundata* as two distinct species. The existence of these two species is further supported by significant genetic differences found in their ITS rDNA sequences. Within the past two decades, the ITS rDNA region has become the single most frequently utilized DNA marker to assess species diversity in various groups of microalgae, including chrysophyte algae (Wee *et al.* 2001, Kynčlová *et al.* 2010, Boo *et al.* 2010, Škaloud *et al.* 2012). The observed sequence divergence in both the ITS1 rDNA (15.5%) and the ITS2 rDNA (12.5%) region is much higher or comparable to the differences observed among the recently described cryptic species within the *S. petersenii* complex (Škaloud *et al.* 2012), where it varies from 2 to 13% in the ITS1 region and 5 to 12% in the ITS2 region, respectively). In addition, a comparison of ITS2 secondary structures of *C. brevispina* and *C. rotundata* revealed the presence of four compensatory base changes (CBCs) in the stem regions of helices I and II. According to the CBC species concept, the presence of even a single CBC in helices II and III should correspond to incompatibility to sexually cross, and thus determines the limit between biological species (Coleman 2000). However, since the application of the CBC species concept in species delimitation is still speculative (Caisová *et al.* 2011), we rather consider the presence of three CBCs as an attribute of elapsed evolutionary time, indicating that sufficient time has passed to produce a speciation event (Müller *et al.* 2007).

Finally, our results provide the first clear evidence of the existence of hidden diversity within the genus *Chrysosphaerella*. Observations of morphologically exceptional *Chrysosphaerella* scales were occasionally reported in the past. Apart from the large, circular scales (Nicholls 1984), morphologically distinct triangular shaped scales were reported by Siver (1993) in Connecticut, U.S.A. and by Wujek *et al.* (1981) in Minnesota, U.S.A. It is highly probable that all these scales in fact belong to a yet undescribed, hidden species. Recently published studies provide clear data that hidden species could significantly differ in their ecological niche (e.g., Amato *et al.* 2007, Pfandl *et al.* 2009). Therefore, characterization of these species is extremely important as it may provide the key to correctly interpret the distribution, abundance, and biology of planktonic organisms.

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