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Chlamydomonas schloesseri sp. nov. (Chlamydophyceae, Chlorophyta) revealed by morphology, autolysin cross experiments, and multiple gene analyses

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Abstract

Chlamydomonas in the traditional sense is one of the largest green algal genera, comprising more than 500 described species. However, since the designation of the model organism *C. reinhardtii* as conserved type of this genus in 2007, only two species remained in *Chlamydomonas*. Investigations of three new strains isolated from soil samples, which were collected near Lake Nakuru (Kenya), demonstrated that the isolates represent a new species of *Chlamydomonas*. Phylogenetic analyses of nuclear SSU and ITS rDNA and plastid-coding *rbc*L sequences have clearly revealed that this species is closely related to *C. reinhardtii* and *C. incerta*. These results were confirmed by cross experiments of sporangium wall autolysins (VLE). All species belonged to the VLE group 1 *sensu* Schlösser. The comparison of the ITS-1 and ITS-2 secondary structures showed several compensatory base changes among the three species. In addition, the *rbc*L amino acid composition was also species-specific. The genus *Chlamydomonas* was phylogenetically closely related to the colonial families Goniaceae, Tetrabaenaceae and Volvocaceae. *Chlamydomonas debaryana* (VLE group 2) formed a separate clade among these colonial families of the Volvocales, a species of which autolysin dissolved the sporangium walls of the members of VLE group 1, suggesting its close relationship to *Chlamydomonas*. As consequence of our results, we propose *Chlamydomonas schloesseri* sp. nov. for the new Kenyan isolates. We also propose a new combination of *C. debaryana* to the newly erected genus *Edaphochlamys*.

Keywords: integrative taxonomy, *Chlamydomonas*, intraspecific variation, species concept, DNA barcoding

Introduction

Traditionally within the genus *Chlamydomonas* (including *Chloromonas*) more than 500 species have been described based on morphological and cytological characters. Many phylogenetic studies have clearly demonstrated that both genera are polyphyletic and subdivided into eight clades, some containing taxa of other flagellated and coccoid algae (i.e. Pröschold *et al.*, 2001 and references therein). Therefore, the species of *Chlamydomonas* need to be taxonomically revised, a process that was initiated by Pröschold *et al.* (2001). In that first contribution, the oogamous species of *Chlamydomonas* were transferred to the new genus *Oogamochlamys*, others to the genus *Lobochlamys*, and the genus *Chloromonas* was emended. As a result of these phylogenetic studies, the number of species remaining within the genus *Chlamydomonas* was drastically reduced; to include the model organism *C. reinhardtii* P.A. Dangeard and its close relatives. Because of the difficult nomenclatural situation of the traditional type species (*C. pulvisculus*), *C. reinhardtii* was proposed as conserved type of the genus *Chlamydomonas,* replacing *C. pulvisculus* (Pröschold & Silva, 2007), a proposal which was accepted by the International Botanical Congress in 2011 in Melbourne (Australia). The closest relatives are *C. globosa* Snow/*C. incerta* Pascher and *C. debaryana* Goroschankin. The relationship of these species was recognized by Schlösser (1976) based on the results of cross experiments of sporangium autolysins. Autolysins are enzymes, which take part in the release of reproductive stages during the cell cycle. In the life cycle of *C. reinhardtii*, two different lytic enzymes have been observed in experiments: sporangium, and gamete autolysins (see review of Matsuda, 1988). A third autolysin is probably responsible for the release of the meiospores after the meiosis from the zygote. Sporangium autolysins (vegetative lytic enzymes: VLE) are species or species-group specific, and

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can be used for classification. These enzymes are produced by zoospores for release of the sporangium cell wall and are stage-specific. Schlösser (1976, 1984) distinguished 15 VLE groups from 65 strains of different *Chlamydomonas* species examined. Phylogenetic analyses based on SSU rDNA sequences have revealed that strains belonging to the same VLE group are closely related. For example, the taxonomic placement of *C. applanata* E.G. Pringsheim (VLE group 7) was revised by Ettl & Schlösser (1992) and later confirmed by Gordon *et al.* (1995). Buchheim *et al.* (1997) demonstrated that the strains belonging to VLE group 14 (*C. noctigama* Korshikov) are almost identical in their SSU rDNA sequences. Several strains belonging to VLE groups 9 and 10 were identified as *C. segnis* Ettl and *C. culleus* Ettl, respectively. Both species are closely related and were transferred to the genus *Lobochlamys* by Pröschold *et al.* (2001) using an integrative approach (Pröschold & Leliaert, 2007). These examples clearly demonstrate that sporangium autolysins are good biochemical marker for classification of *Chlamydomonas* species.

The gamete lysin (gamete lytic enzyme: GLE) is produced during gametogenesis of *C. reinhardtii* and released after flagellar agglutination of two gametes to dissolve their cell wall before gamete fusion (Claes, 1971). This enzyme is not stage-specific in its activity. It dissolves not only the cell walls of gametes, but also can release zoospores from their sporangia, and can be used for isolation of protoplasts from vegetative cells (Schlösser *et al.,* 1976). Matsuda *et al.* (1987) demonstrated that the gamete lysin digested cell walls of several *Chlamydomonas* species (i.e., *C. reinhardtii*, *C. incerta*, *C. globosa*, *C. debaryana*) and taxa belonging to the colonial families Tetrabaenaceae and Goniaceae *sensu* Nozaki *et al.* (2014) of the Volvocales. To the Tetrabaenaceae belong the genera *Basichlamys* and *Tetrabaena*, which were originally designated as four-cell *Gonium* species, the genera *Gonium* and *Astrephomene* were assigned to the family Goniaceae. These results indicate a close relationship between the *Chlamydomonas* species and the colonial families, which was confirmed by Matsuda (1988).

Coleman (2000) showed that compensatory base changes (CBCs) in secondary structures of the internal transcribed spacer region (ITS) of the ribosomal operon are generic and species characters and can be used for taxonomic revisions as genetic signatures. She developed new generic and species concepts (Z- clade and CBC-clade concept) based on CBCs in ITS sequences (genetic signatures) compared with the mating ability of species. In contrast to the biological species concept, strains of the same Z-clade form zygotes, but they cannot germinate or produce fertile F1 generations. Both biological species or members of the same Z-clade are found to be similar in their ITS rDNA sequences and almost identical in the conserved region of ITS-2. Therefore, ITS sequences are an ideal tool for evolutionary comparisons, especially at the biological species level (Coleman, 2003, 2009).

Pröschold *et al.* (2005) studied different molecular traits of the model organism *Chlamydomonas reinhardtii* and its closest relatives. One closely related strain (CCAP 11/132) was isolated from wet soil collected on the shore of a small dam in Lake Nakuru National Park (Kenya). Two more strains were isolated from the same site two years later. The topic of this study is the characterization of these strains by comparison of morphology, cross experiments of sporangium autolysins, and SSU, ITS rDNA and *rbc*L sequences with the closest related *Chlamydomonas* species. As a results of these studies, we propose a new species of *Chlamydomonas*, *C. schloesseri* sp. nov., for the Kenyan strains.

Material and Methods

Strains used in this study, culture conditions, and light microscopy

In this study, three new strains isolated from soil samples collected at the shore of a small dam near Lake Nakuru (Kenya) were investigated. The geographic position of the sampling site is S 00°23.421', E 36°02.946' at an elevation of 1900 m. This small pond (about 0.25 acres) held by an artificial dam, was established by the park rangers for watering of the wildlife. At the sampling times (9th June 2001, and 26th March 2002) the water level was very low, only a few centimeters water, which was well trod and manured by the big animals like rhinos and waterbucks. The water was extremely turbid. Water temperature was 25–27°C, pH was 7.9–8.0 and conductivity 472–540 µS/cm. The pure culture strains were established by isolation of single cells from the samples by microcapillaries (SAG 2486) or streaking on agar plates (SAG 2484, SAG 2485). Both samples were collected by Lothar Krienitz who also isolated one strain in 2001. The second sample was handed over to Luo Wei who isolated the two strains in 2003.

These new strains were compared with strains of *Chlamydomonas incerta/globosa* and *C. reinhardtii* from public culture collections (Culture Collection of Algae, University of Göttingen, Germany - SAG, Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan - NIES, Chlamydomonas Resource Center, University of Minnesota, USA - CC). Detailed information about the habitat and origin is given in Table S1 (Supplemental Material). All of the strains were cultivated in modified Bold's Basal Medium (3N-BBM+V; medium 26a in Schlösser, 1997) under standard conditions (light : dark cycle of 14:10 hrs at 18°C and 50 µmol photons/m^2s^1 provided by daylight

fluorescent tubes, Osram L36W/954 Lumilux de lux daylight, Munich, Germany). For species identification, the identification keys of Ettl (1976, 1983) were used and the morphology of the strains was compared with the original species descriptions. For the light microscopic investigations, an Olympus BX-60 microscope was used (Olympus, Tokyo, Japan) and the micrographs were taken with a Prog Res C14 plus camera using the Prog Res Capture Pro imaging system (version 2.9.0.1), both from Jenoptik, Jena, Germany.

TABLE 1. Comparison of diacritical morphological features among the species of *Chlamydomonas*.

	C. schloesseri	***C. incerta***	***C. reinhardtii***
cell shape	spherical - broadly ellipsoid - ovoid - obovoid	spherical - broadly ellipsoid	spherical - broadly ellipsoid - ovoid - obovoid
cell size	8.7-9.3 x 9.6-11.9 µm	8.0-9.5 x 9.7-12.4 µm (11.9-15.1 µm)	8.3-9.6 x 10.9-12.9 µm (12.6-15.4 µm)
papilla	no	no	no
mucilage surrounding the vegetative cells	+	-	-
cells immobile	often embedded in mucilage	rare	rare
contractile vacuoles	2 apical	2 apical	2 apical
length of flagella	slightly longer as the cell	slightly longer as the cell	slightly longer as the cell
position of the nucleus	above the middle of the cell	above the middle of the cell	above the middle of the cell
chloroplast shape	cup-shaped, slightly lobed	cup-shaped	cup-shaped, sometimes with small incisions
pyrenoid	medium, round-slightly ellipsoid, in basal position	medium, round-slightly ellipsoid, in basal position	small, round, in basal position
eyespot	small elliptic in anterior position	elliptic in anterior position	elliptic in anterior position
cell division	longitudinal after 90° rotation of the protoplast (false traverse division)	longitudinal without 90° rotation of the protoplast	longitudinal after 90° rotation of the protoplast (false traverse division)
number of zoospores	2-4-8	2-4	2-4-8-16
size of zoospores	7.6-8.0 x 5.4-5.6 µm	10.0-10.6 x 6.6-7.4 µm	7.2-9.6 x 5.4- 6.6 µm
size of sporangia	13.3-22.7 µm	15.6-16.5 µm	12.0-17.5 µm
sexual reproduction	unknown	unknown	heterothallic isogamy

Synchronized cultures and autolysin tests

The asexual development of five strains (SAG 2485 *C. schloesseri*, SAG 7.73 *C. incerta*, SAG 11-32b and SAG 54.72 *C. reinhardtii*, SAG 26.72 *C. debaryana*) was synchronized in aerated cultures using a light thermostat (Kuhl & Lorenzen, 1964) under the following conditions: 25°C, 170 µmol photons/m^2s^1, light : dark cycle of 14:10 hrs, aeration with compressed air enriched with 2% CO_2, dilution of the cell suspension at the end of every dark period to 7.5 x 10^5/ml (SAG 2485 and SAG 26.72), 1 x 10^6/ml (SAG 7.73), 1.5 x 10^6/ml (SAG 11-32b and SAG 54.72), with nutrient medium for unicellular green algae (Kuhl medium; Kuhl & Lorenzen, 1964). After accumulation of sporangia in the dark period the cell suspension (approx. 300 ml) was harvested by centrifugation during the last two dark hours. The pellet was then resuspended into 30 ml fresh culture medium in a large petri dish and incubated at 25°C in the light.

To obtain the sporulation media, after release of the zoospores from the sporangia the suspension was made cell-free by centrifugation and then stored at 4°C. The bioassay was performed the next day as follows: 2 ml of a synchronized culture (two hours before the start of the light period) were harvested and heat-fixed (5 min at 55°C). These fixed sporangia were then incubated with 2 ml of sporulation medium at 30°C. The autolysin activity was given as the time required for quantitative release of the dead zoospores from the fixed sporangia (Schlösser, 1976).

DNA extraction, PCR and sequencing, alignment and phylogenetic analyses

The genomic DNA was extracted from most of the strains using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the instructions provided by the manufacturer. The SSU and ITS rDNA were amplified in PCR reactions using the Taq PCR MasterMix Kit (Qiagen, Hilden, Germany) with the primers EAF3 and ITS055R (Marin *et al.,* 2003). The *rbc*L gene was amplified using the primers and methods described by Nozaki *et al.* (1995). All PCR products were purified and sequenced as described by Demchenko *et al.* (2012). All sequences are available in the EMBL, GenBank and DDBJ sequence databases under the accession numbers given in Table S1.

The SSU rDNA sequences of all strains were aligned according to their secondary structures. The ITS-1 and ITS-2 sequences of all strains were folded according to the protocol described in detail in Darienko *et al.* (2015). The alignments were separated into three datasets: (i) concatenated dataset of 75 SSU and ITS rDNA sequences (2688 bp), using the strains SAG 11-10 *Vitreochlamys nekrassovii* and UTEX 167 *Paulschulzia pseudovolvox* as outgroup, and datasets of (ii) 29 ITS rDNA (915 bp) and (iii) 29 *rbc*L (1128 bp) sequences of the representatives of the Goniaceae, Tetrabaenaceae and Volvocaceae including those *Chlamydomonas* spp. that belong to the VLE groups 1 and 2. In addition, the datasets of the six genes (SSU, *rbc*L, *atp*B, *psa*A, *psa*B, and *psb*C) used by Nakada *et al.* (2016) were concatenated and re-analyzed separately. The details are summarized in the Supplemental Material.

For all data sets the best evolutionary models were calculated with the program Modeltest 3.7 (Posada, 2008) using the Akaike Information Criterion (Akaike, 1974). The settings of the best models are given in the figure legends. The following methods were used for the phylogenetic analyses: distance, maximum parsimony, maximum likelihood, and Bayesian inference using the programs PAUP version 4.0b10 (Swofford, 2002), RAxML version 7.0.3 (Stamatakis, 2006), MrBayes version 3.2.3 (Ronquist *et al.,* 2012), and PHASE package 2.0 (Jow *et al.,* 2002, Higgs *et al.,* 2003, Hudelot *et al.,* 2003, Gibson *et al.,* 2005, Telford *et al.,* 2005).

For approximately unbiased tests of user-defined trees the program CONSEL version 0.20 (Shimodaira & Hasegawa, 2001) was used. For the user-defined trees, the best trees were manipulated with the program TreeView version 1.6.6 (Page, 1996), which then were loaded into PAUP to calculate the likelihood scores for these trees.

TABLE 2. Sporangium wall autolysin activity of the sporulation media among strain of *Chlamydomonas* in bioassays. **Explanation of symbols:** Quantitative release of zoospores from fixed sporangia within 5–20 min = +++; in 20–60 min = ++, in 1–24 h = +, not quantitative in 24 h = (+).

			fixed sporangia from				
			C. schloesseri	*C. incerta*	*C. reinhardtii*		*C. debaryana*
			SAG 2485	SAG 7.73	SAG 11-32b	SAG 54.72	SAG 26.72
Sporangium wall autolysin from	*C. schloesseri*	SAG 2485	(+)	(+)	(+)	(+)	-
	C. incerta	SAG 7.73	++	+++	+++	+++	-
	C. reinhardtii	SAG 11-32b	++	+++	+++	+++	-
		SAG 54.72	++	++	+++	+++	-
	C. debaryana	SAG 26.72	(+)	+	+	+	++

ITS-2 secondary structures and ITS-2/CBC approach

The secondary structures of ITS-2 sequences were folded using computer programs mfold (Zuker, 2003), which used the thermodynamical model (minimal energy), and CONTRAfold (Do *et al.*, 2006), another program that used a stochastic approach for the RNA folding. For the folding the following three constraints were set: (1) the last 25 bases of the 5.8S rRNA and the first 25 of the LSU rRNA must bind and form the 5.8S/LSU stem, (2) the pyrimidine/pyrimidine mismatch (the first RNA processing site) in Helix II after the 5–7th base pair has to be present in the structure, and (3) the second RNA processing site, the GGU motif characteristic for green algae, has to be at the 5' site in Helix III (for details about the processing sites and constraints; see Coleman 2003, Cote *et al.* 2002).

The secondary structure models of ITS-2 derived from these foldings were then used for species delimitation within *Chlamydomonas*. For the ITS-2/CBC approach, the conserved region of ITS-2 was extracted following the procedure that was introduced for *Coccomyxa* by Darienko *et al.* (2015): it includes (1) 17 base pairs of the 5.8S/LSU stem, (2) ten base pairs of Helix I, (3) eleven base pairs of Helix II including the pyrimidine-pyrimidine mismatch, and (4) all base pairs of Helix III. The resulting data set was then manually aligned. These alignments have been translated into base pair alignment by usage of a number code for each base pair (1 = A-U; 2 = U-A; 3 = G-C; 4 = C-G; 5 = G•U; 6 = U•G; 7 = mismatch; 8 = deletion/insertion or single bases). These barcodes of each species were compared to detect CBCs, hemi-CBCs (HCBCs) and/or insertion/deletion, single or unpaired bases.

Results

Morphological observations

Chlamydomonas schloesseri Pröschold & Darienko sp. nov. (**Fig. 1**)

Diagnosis: Mature vegetative cells are spherical, broadly ellipsoidal, ovoid or sometimes obovoid 8.7 x 9.3 to 9.6 x 11.9 µm in size. Cell wall is thin, without papilla, two apical contractile vacuoles. Chloroplast is cup-shaped, with thickened basal part. The surface of chloroplast is smooth, sometimes small incisions could be observed. The single pyrenoid is medium large, spherical or broadly ellipsoidal, surrounded by several starch grains and placed in the thick part of chloroplast. Nucleus is placed in the anterior part of cell. Eyespot is small, dot-like, anterior to equator. Protoplast is often withdrawn from the cell wall. The vegetative cells (flagellated and immobile) are surrounded by layered mucilage and frequently immobile. Asexual reproduction by zoospores formation. Zoosporangia contain mostly 2–8, rarely 16 zoospores, and are 13.3–22.7 µm in diameter. The morphology of zoospores is similar to that of the vegetative cells. Sexual reproduction not observed.

Differs from *C. gelatinosa* Korshikov ex Pascher by smaller cell size and thinner mucilage layer surrounding the vegetative cells.

Type locality: Rhinopool in the Lake Nakuru National Park, Kenya.

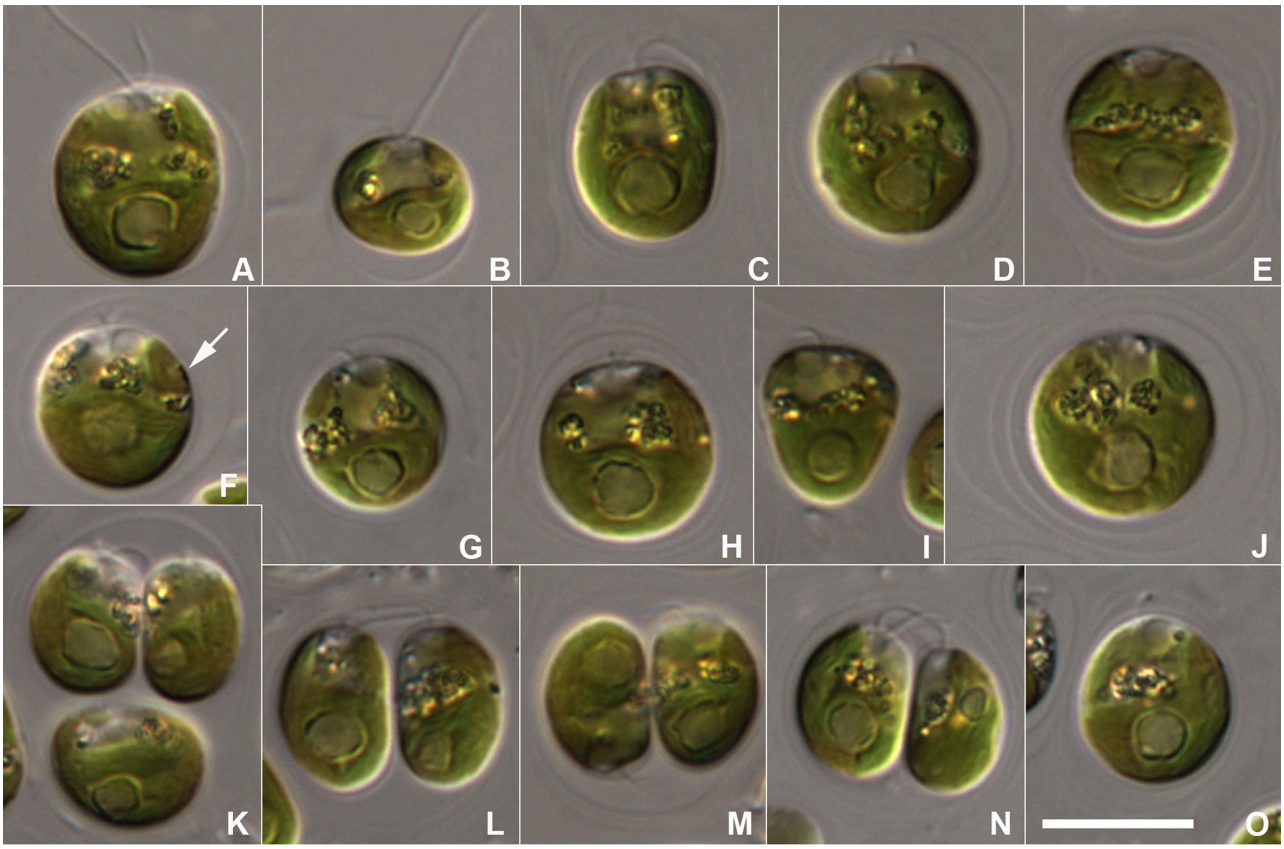

FIGURE 1. Morphology and phenotypic plasticity of *Chlamydomonas schloesseri* sp. nov. **A–J.** vegetative cells, arrow in **F.** marked the eyespot; **K–O.** different sporangia; scale bar = 10 µm.

Holotype (designated herein): The strain SAG 2485 is permanently cryopreserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the SAG, University of Göttingen, Germany.

Iconotype (designated herein in support of the holotype): Fig. 1A in this study.

ITS-2 DNA Barcode: BC1 in Fig. 7.

Etymology: The species was named in honor of Prof. Dr. Uwe Gert Schlösser for his contribution to phycology and his further development of the Culture Collection of Algae, University of Göttingen (SAG).

The three investigated strains are morphologically identical and differ mainly from other closely related species by the presence of mucilage surrounding the vegetative cells in *C. schloesseri*. The diacritical morphological features of the three *Chlamydomonas* species are summarized in Table 1. As shown in Table S1, all strains designated as *C. reinhardtii* and *C. smithii* (authentic strain SAG 54.72) were identified as *C. reinhardtii* using the identification keys of Ettl (1976, 1983). The three strains SAG 81.72, SAG 7.73 and NIES-2462 matched in morphology with original description of Pascher (1927) for *C. incerta* and the diagnoses provided by Ettl (1965, 1976). The strains of *C. schloesseri* were identified as *C. gelatinosa* using the keys of Ettl, but the comparison with original descriptions of this species by Korshikov in Pascher (1927) and Korshikov (1938) showed clear differences in morphology. The vegetative cells of *C. gelatinosa* have another cell shape (broadly ellipsoid) and size (12–20 µm x 6–10 µm) and the mucilage surrounding the cells is up to 6 µm thick.

Evidence of sporangium wall autolysins among *Chlamydomonas* species

As demonstrated above, *C. schloesseri* is similar in morphology to *C. reinhardtii* and *C. incerta*. To confirm their relationship, we tested the activity of sporangium wall autolysins in bioassays among the three species. For these bioassays, the synchronization of the asexual development was achieved in aerated cultures using the light thermostat as described in Material & Methods. Five strains were selected for these tests: SAG 2485 *C. schloesseri*, SAG 7.73 *C. incerta*, SAG 11-32b and SAG 54.72 *C. reinhardtii*, and SAG 26.72 *C. debaryana*. The activity of the sporulation media was tested on heat-fixed sporangia of each strain and microscopically observed as described above. The lytic enzymes of these strains had different activities as demonstrated in Table 2. The VLE of *C. schloesseri* was less active than those of the other strains; however, the sporangium walls were totally lysed (Fig. 2C). The mucilage that surrounded the vegetative cells was also dissolved under influence of the sporulation medium. The cross experiments clearly revealed that *C. schloesseri* belonged to the VLE group 1 *sensu* Schlösser (1976, 1984). The one-sided reaction of the sporulation medium from *C. debaryana* (VLE group 2) could be confirmed. The VLE of this species dissolved the sporangium walls of all VLE group 1 and 2 members, but no activity on sporangia of *C. debaryana* was observed using the VLE of *C. schloesseri*, *C. incerta* and *C. reinhardtii* (Table 2).

Molecular phylogeny using a multiple gene approach

To reveal the phylogenetic position of *C. schloesseri*, we sequenced the nuclear SSU and ITS rDNA and the plastid-coding *rbc*L genes of all species belonging to the VLE groups 1 and 2. For a better resolution among these strains additional volvocalean strains of the families Goniaceae, Tetrabaenaceae and Volvocaceae were sequenced. The sequences were included in three datasets: (1) SSU and ITS and (2) ITS and (3) *rbc*L. The SSU and ITS rDNA sequences were aligned according to their secondary structures. For the *rbc*L analyses, all three codon bases were included.

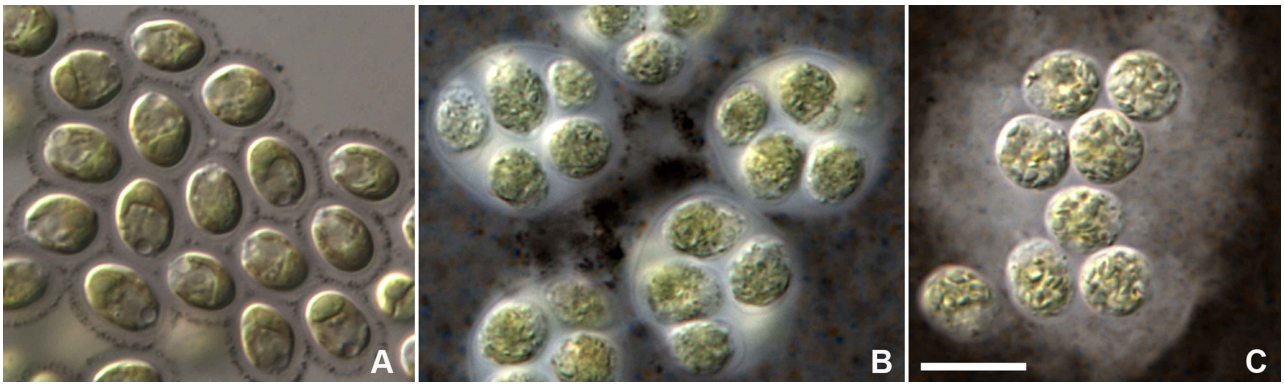

FIGURE 2. Lysis of sporangium cell wall under influence of the VLE autolysin in *Chlamydomonas schloesseri* (SAG 2485); scale bar = 10 µm.

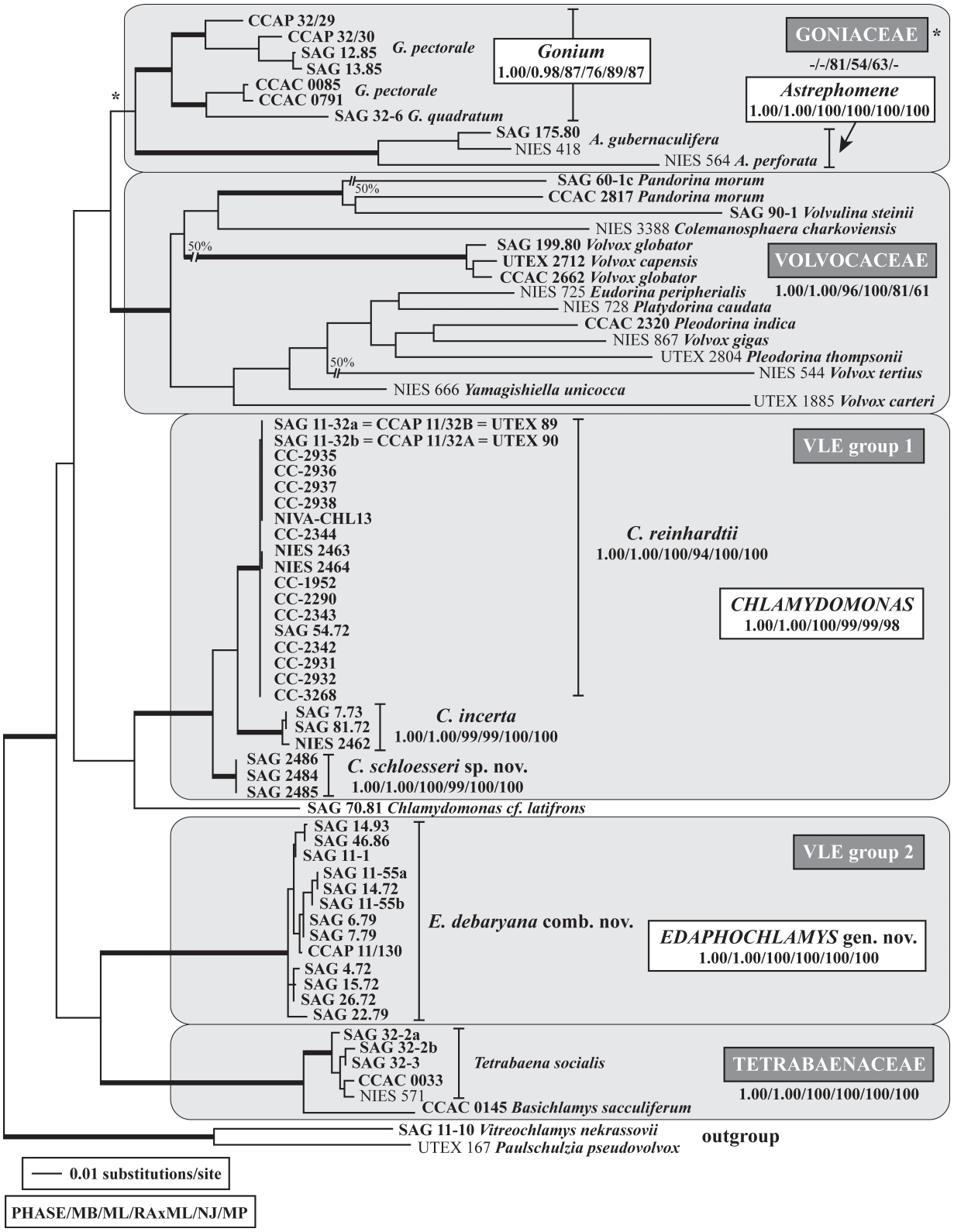

FIGURE 3. Molecular phylogeny of *Chlamydomonas* and representatives belonging to the Tetrabaenaceae, Goniaceae and Volvocaceae based on SSU and ITS rDNA sequence comparisons. The phylogenetic trees shown were inferred using the maximum likelihood method based on the data sets (2688 aligned positions of 75 taxa) using PAUP 4.0b10. For the analyses the best model was calculated by Modeltest 3.7. The setting of the best model was given as follows: GTR+I+G (base frequencies: A 0.2516, C 0.2232, G 0.2514, T 0.2738; rate matrix A-C 1.3107, A-G 2.3565, A-U 2.4706, C-G 0.4123, C-U 4.2284, G-U 1.0000) with the proportion of invariable sites (I = 0.6048) and gamma shape parameter (G = 0.3904). The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE and MrBayes; bootstrap values > 70% calculated with PAUP using maximum likelihood, neighbor-joining, maximum parsimony and RAxML using maximum likelihood). The Goniaceae are only moderated supported in bootstrap and Bayesian analyses, which is indicated by an asterisk.

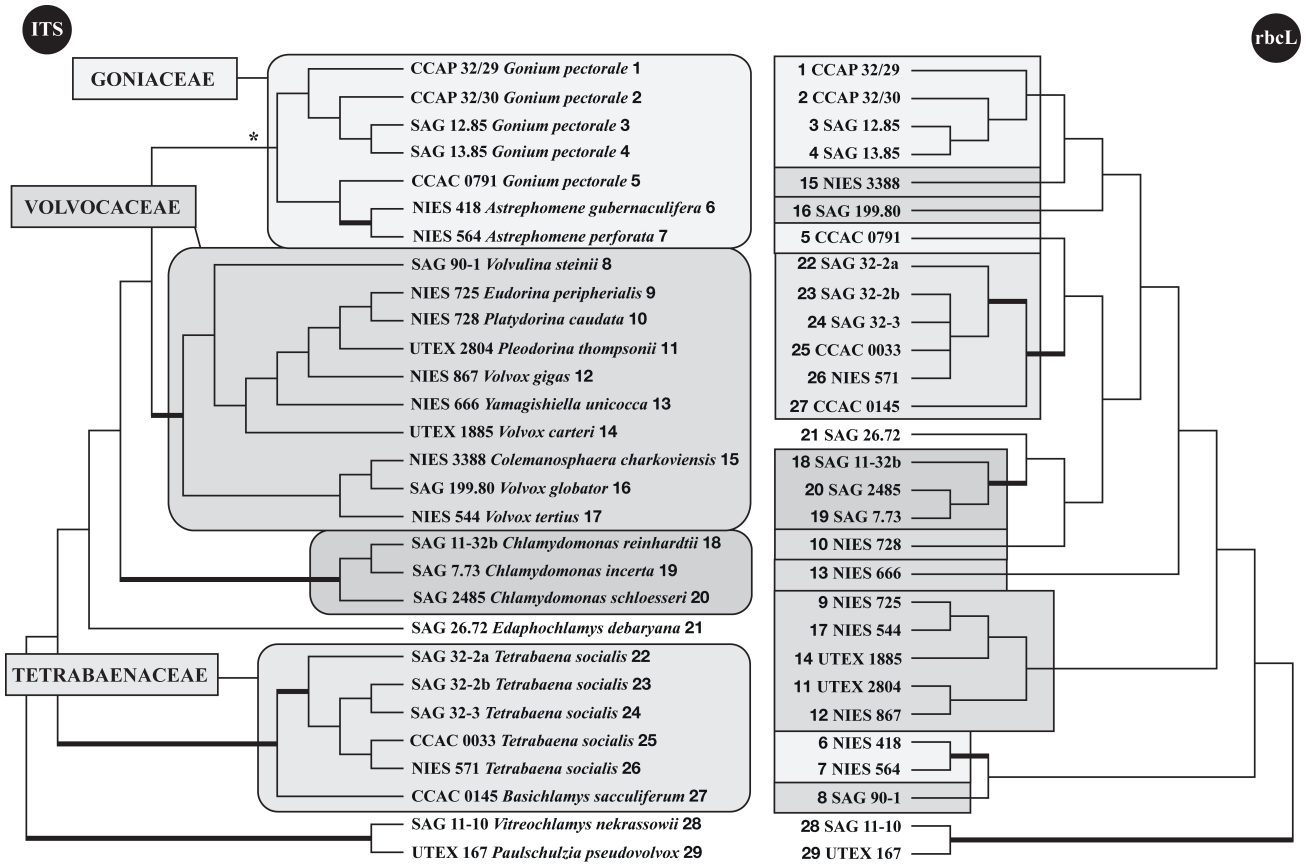

FIGURE 4. Molecular phylogeny of *Chlamydomonas* and representatives belonging to the Tetrabaenaceae and Goniaceae based on *rbc*L and ITS rDNA sequence comparisons. The phylogenetic trees shown were inferred using the maximum likelihood method based on the data sets (29 taxa: 915 aligned positions for ITS, 1128 for *rbc*L) using PAUP 4.0b10. For the analyses the best model was calculated by Modeltest 3.7. The setting of the best model was given as follows: (**ITS**) GTR+I+G (base frequencies: A 0.2634, C 0.2225, G 0.2192, T 0.2949; rate matrix A-C 1.2503, A-G 2.1014, A-U 2.0643, C-G 0.3957, C-U 3.3009, G-U 1.0000) with the proportion of invariable sites (I = 0.2682) and gamma shape parameter (G = 0.6858); (***rbc*L**) GTR+I+G (base frequencies: A 0.2777, C 0.1688, G 0.2048, T 0.3487; rate matrix A-C 0.2771, A-G 3.5238, A-U 4.0693, C-G 0.6646, C-U 7.4921, G-U 1.0000) with the proportion of invariable sites (I = 0.5523) and gamma shape parameter (G = 0.6480). The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE and MrBayes; bootstrap values > 70% calculated with PAUP using maximum likelihood, neighbor-joining, maximum parsimony and RAxML using maximum likelihood). The Goniaceae are only weakly supported in bootstrap and Bayesian analyses, which is indicated by an asterisk.

The phylogenetic analyses of all datasets clearly revealed that *C. schloesseri* represented a new species of *Chlamydomonas*, which was highly supported in all Bayesian and bootstrap analyses (Figs 3–4). The three species of *Chlamydomonas* (*C. schloesseri*, *C. incerta* and *C. reinhardtii*) are sister to the Goniaceae (containing species of the genera *Gonium* and *Astrephomene*) and the selected Volvocaceae. Strains of *C. debaryana* are almost identical in SSU and ITS rDNA sequences and formed the sister group of the Tetrabaenaceae (containing the genera *Basichlamys* and *Tetrabaena*). However, one strain labeled as *C. debaryana*, SAG 70.81, was not closely related to the other strains and was related to the three *Chlamydomonas* species, but with low bootstrap and no Bayesian support (Fig. 3). The morphology of this strain did not fit with the description of *C. debaryana* and the strain was preliminary identified as *C. cf. latifrons*; however, this needs further investigations. Despite the fact that the autolysin of *C. debaryana* dissolved the sporangium walls of the three *Chlamydomonas* species, the strains of *C. debaryana* were more closely related by DNA sequence to the Tetrabaenaceae than to *Chlamydomonas*. This was confirmed by approximately unbiased test using the program CONSEL. The tree with the artificial setting of *C. debaryana* as sister of the three *Chlamydomonas* species was significantly worse (SSU/ITS: AU 0.0348) than the best tree topology presented in Fig 3. As consequence, *C. debaryana* represents a new genus, which will be described below (*Edaphochlamys* gen. nov.).

Species delimitation using the ITS secondary structures and *rbc*L amino acid composition

To confirm that *C. schloesseri* is a new species of *Chlamydomonas*, we analyzed the secondary structures of ITS-1 and ITS-2 for detection of CBCs. Both structures showed the typical four helices (Figs 5–6). ITS-1 and ITS-2 of the three *Chlamydomonas* species are very similar. Only the helices 1 and 2 of ITS-1 and helices I, II and IV of ITS-2 are variable among these species. The other helices are identical in sequence and structure. *C. schloesseri* differed in eight CBCs and five HCBCs (marked in black and grey in Figs 6–7, respectively) from the other two species, three and two of them, respectively, are unique for this species. The conserved region of ITS-2 was extracted as demonstrated in Fig. 7 and the extracted base pairs were then translated into a number code, which is characteristic for each *Chlamydomonas* species. The resulting barcodes of the three species were nearly identical, only the base pair positions 22–28 (Helix I) were variable and represented five CBCs. These CBCs were not unique for each species, but the combination of the five CBCs separated *C. schloesseri*, *C. incerta* and *C. reinhardtii* from each other.

Analyzing the *rbc*L sequences, the alignment of the *Chlamydomonas* species varied in 34 positions. These differences were mostly located in the third codon positions that did not result in any change in amino acid. Only seven base changes in the first two codon bases were detected, resulting in six amino acid changes among the *Chlamydomonas* species. The amino acid alignment with the changes highlighted is summarized in Fig. 8.

Discussion

Many phylogenetic studies have clearly revealed that the species-rich genus *Chlamydomonas* (more than 500 described species) is polyphyletic (Pröschold *et al.,* 2001; Demchenko *et al.,* 2012 and references therein). Ettl (1976, 1981) already indicated that this genus is artificial and not a natural assemblage. Most of the original descriptions were based on microscopical investigations of field samples. The *Chlamydomonas* species are characterized by different cell sizes and shapes, cell wall papilla, different chloroplast shapes, the number and position of pyrenoids within the chloroplast, and the position of eyespot and nucleus within the cell (Ettl, 1976). To revise this genus taxonomically, comparative studies of cultured material are required; very little is known about the phenotypic plasticity of the species. For example, Ettl & Schlösser (1992) have demonstrated that the morphological features of five species of *Chlamydomonas* are very variable and overlapping. They concluded that these species belonged to one species, *C. applanata*. Besides the morphological comparison, they also showed that the investigated strains belonged to the same autolysin group (VLE group 7 *sensu* Schlösser, 1976).

Taxonomic revision among *Chlamydomonas* species using an integrative approach is still in progress (Pröschold *et al.,* 2001; Demchenko *et al.,* 2012; Nakada *et al.,* 2016). With the designation of *C. reinhardtii* as conserved type of the genus by Pröschold & Silva (2007), all species not closely related to this species need to be transferred to other or new genera. As result of this nomenclatural clarification of the type species, only two species, *C. reinhardtii* and *C. incerta*, remained in *Chlamydomonas*. *C. schloesseri* described here in this study represents the third species in the revised genus. Pröschold *et al.* (2005) already demonstrated, based on ITS rDNA sequences, that one of the Kenyan strains (SAG 2486 = CCAP 11/132) is closely related to *C. reinhardtii*, *C. incerta* and some colonial Volvocales such as *Gonium*. In our phylogenetic analyses (Figs 3–4), the comparison of the secondary structures of ITS-1 and ITS-2 (Figs 5–6) as well as the amino acid composition of the *rbc*L gene clearly support *C. schloesseri* as a new species of *Chlamydomonas*. Although the three strains of this species differed in morphology from the other species, the cross testing of sporangium wall autolysins clearly revealed that *C. schloesseri* belongs to VLE group 1. The autolysin of VLE group 2 (*C. debaryana*) also dissolved the fixed sporangia of all investigated strains belonging to VLE group 1 including *C. schloesseri*. However, the lytic enzymes of group 1 did not affect sporangia of *C. debaryana,* confirming the results of Schlösser (1976, 1984). As mentioned above the autolysins (VLE and GLE) were good biochemical markers, which indicate the natural relationship. As so far known, the 15 VLE groups discovered by Schlösser (1976, 1984) represent species or groups of species.

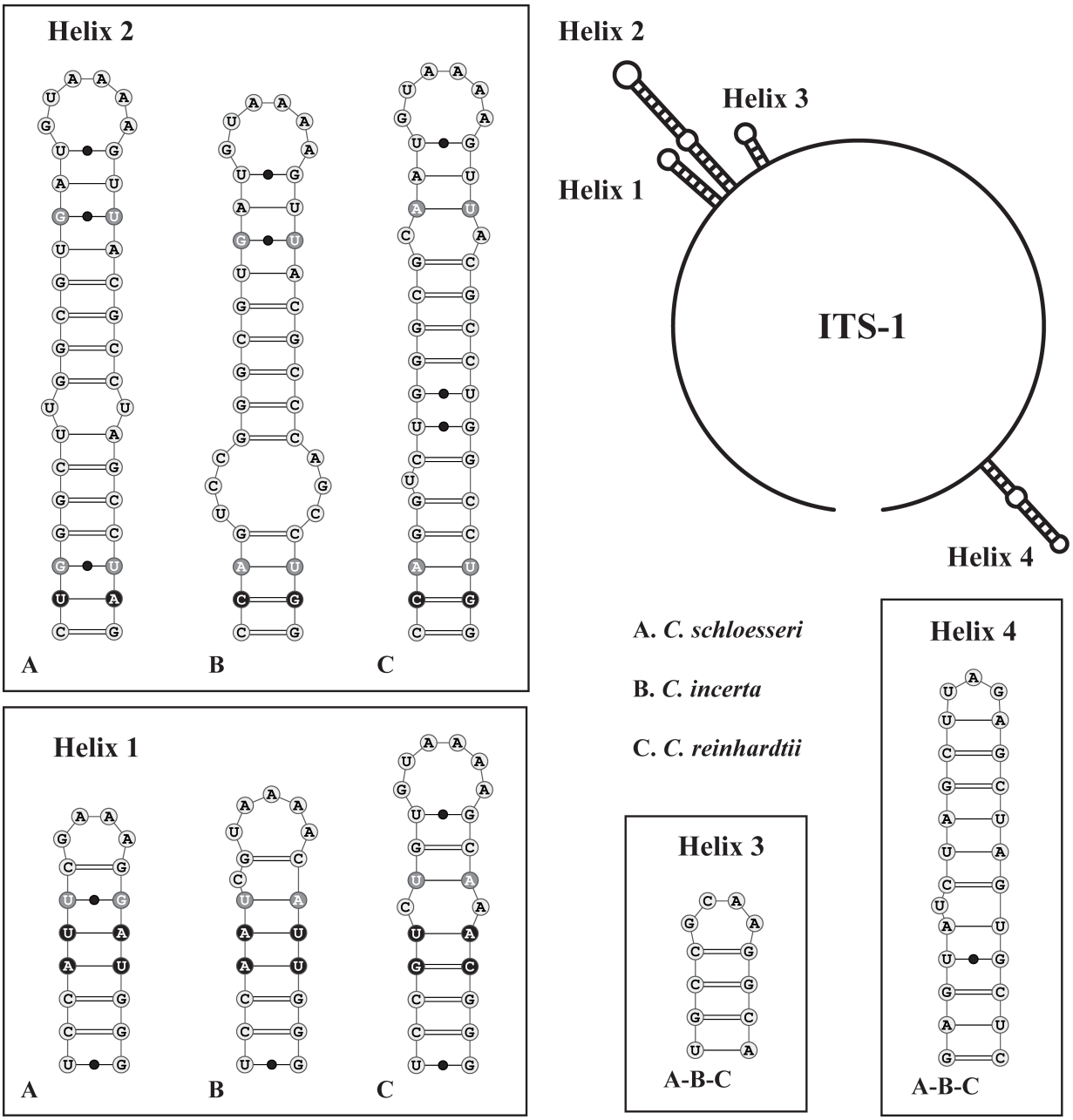

FIGURE 5. Secondary structures of ITS-1 rDNA sequences among the species of *Chlamydomonas*, **A.** *C. schloesseri*, **B.** *C. incerta*, and **C**. *C. reinhardtii.* CBCs and HCBCs are marked in black and grey, respectively.

Morphologically *C. schloesseri* is distinct from *C. reinhardtii* and *C. incerta* by the presence of mucilage surrounding the vegetative cells (Figs 1–2). From other *Chlamydomonas* species with mucilage, this species differed by cell size (*C. gelatinosa*) and chloroplast shape (both *C. segnis* and *C. culleus* are members of the genus *Lobochlamys*; see details in Pröschold *et al.,* 2001). *C. schloesseri* is somewhat similar to the species of the genus *Vitreochlamys* Batko, but these species can be recognized by the raised cell wall, where the mucilage is located between the cell wall and protoplast. Phylogenetically this genus is polyphyletic and distributed among different lineages of the colonial Volvocales, but none is closely related to *Chlamydomonas* (Nakazawa *et al.,* 2001).

Our morphological investigations of the three *Chlamydomonas* species resulted in some discrepancies about the identification of *C. incerta* versus *C. globosa*, which is described in detail in Pröschold & Darienko (2018). As a consequence of our study, we re-established *C. incerta* for the strains (SAG 7.73, SAG 81.72 and NIES-2462) and propose here the ITS-2 DNA Barcode: BC2 in Fig. 7.

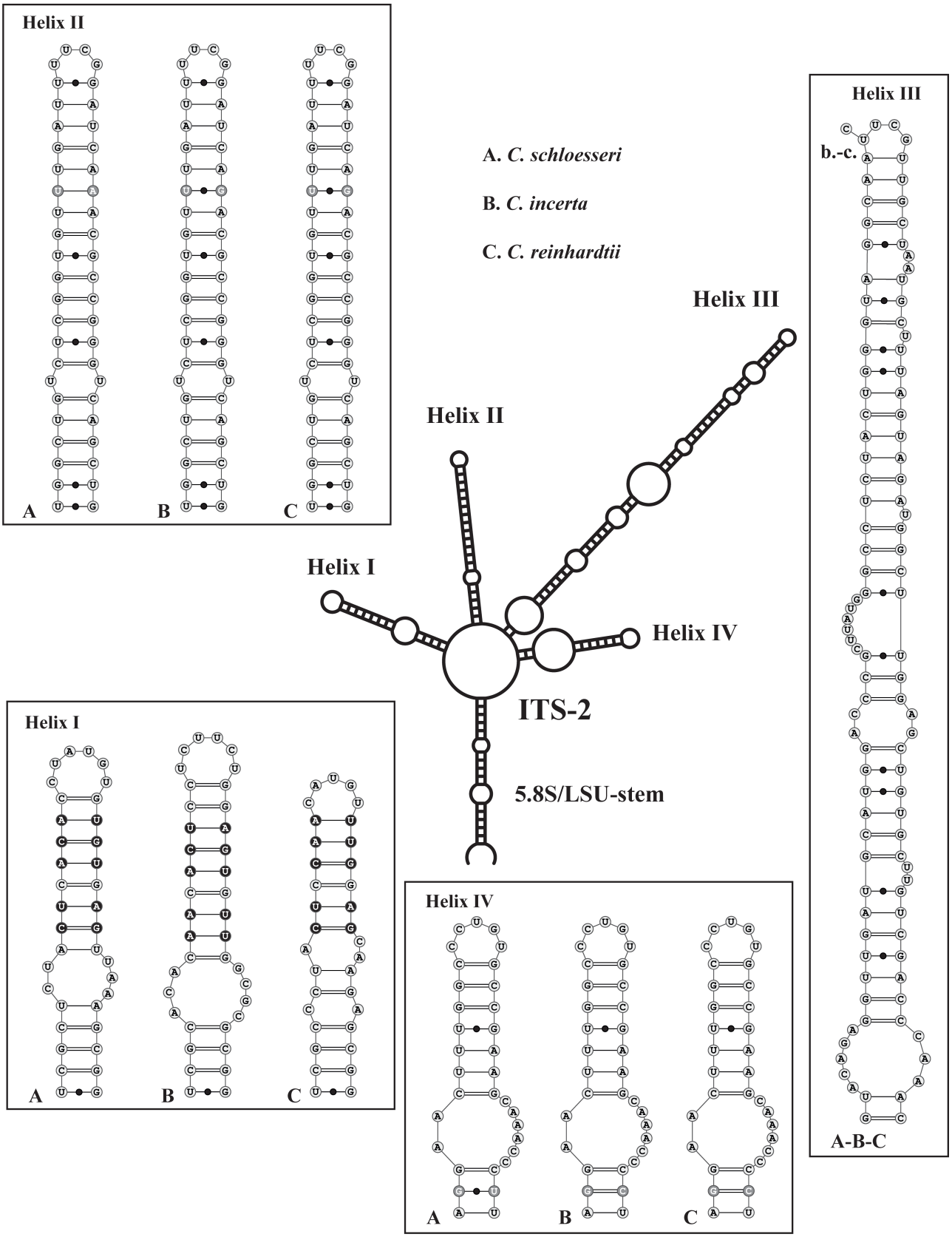

FIGURE 6. Secondary structures of ITS-2 rDNA sequences among the species of *Chlamydomonas*, **A.** *C. schloesseri*, **B.** *C. incerta*, and **C**. *C. reinhardtii.* CBCs and HCBCs are marked in black and grey, respectively.

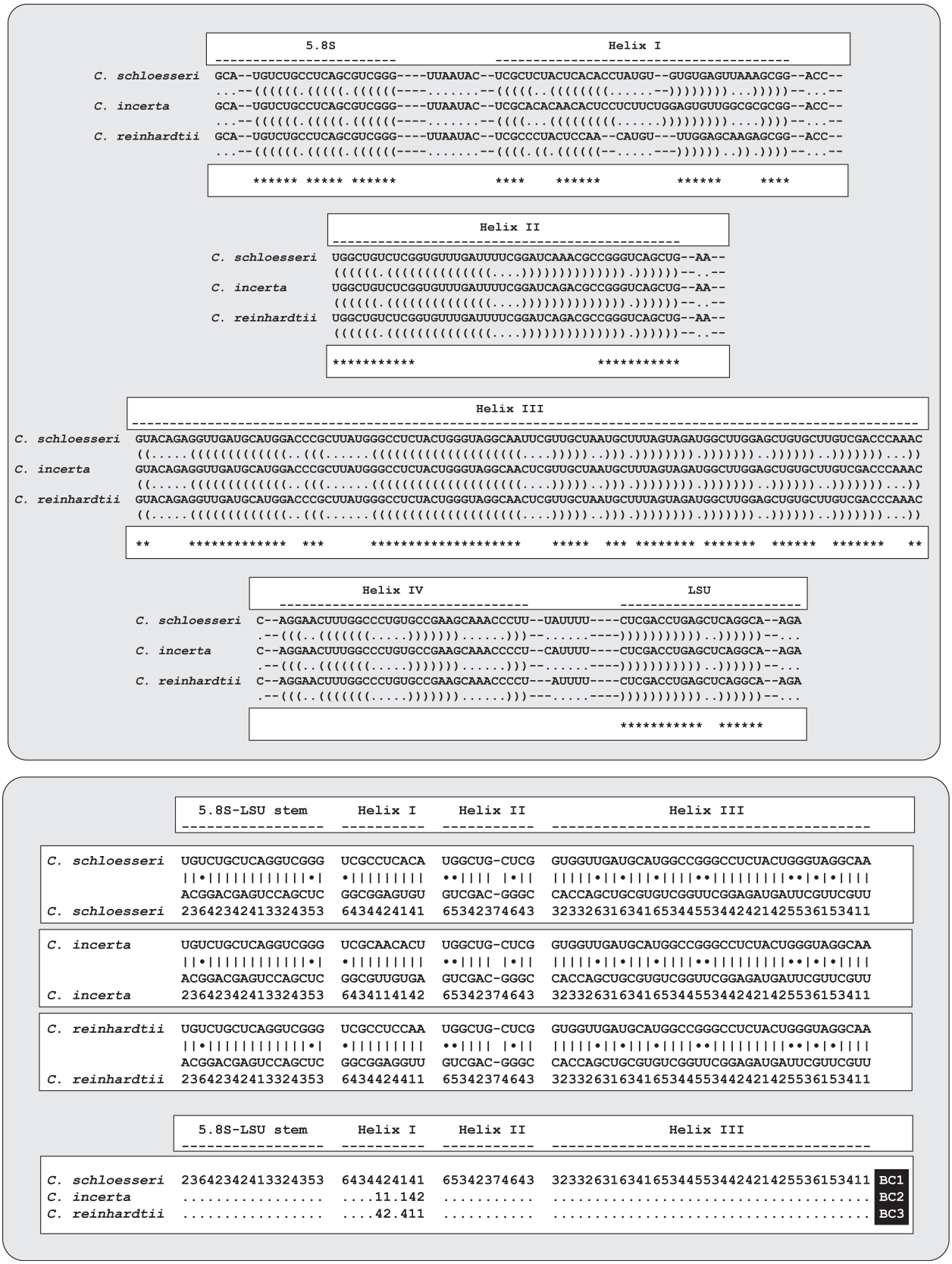

FIGURE 7. Comparison of the conserved region of ITS-2 among the species of *Chlamydomonas*. Extraction of this region and translation into a number code for its usage as barcode (extracted bases highlighted with an asterisk). Number code for each base pair: 1 = A-U; 2 = U-A; 3 = G-C; 4 = C-G; 5 = G•U; 6 = U•G; 7 = mismatch.

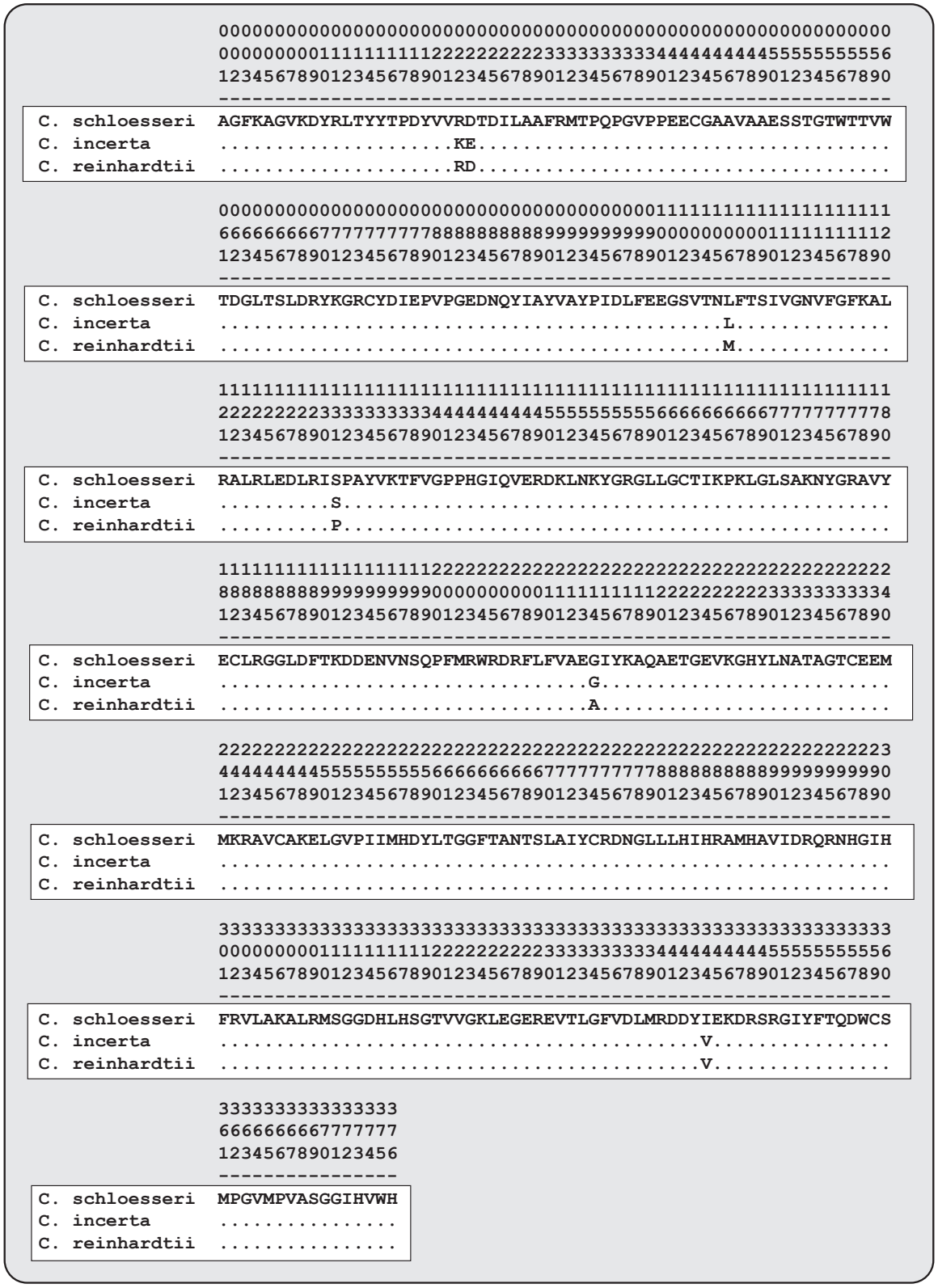

FIGURE 8. Comparison of the amino acid composition of the *rbc*L gene among the species of *Chlamydomonas.* The IUPAC symbols were used for the amino acids.

About the distribution of the three species of *Chlamydomonas* very little is known. *C. reinhardtii* was considered to be distributed only in eastern North America (Pröschold *et al.,* 2005 and references therein), but there are now new isolates found by Nakada *et al.* (2010, 2014) in Japan. *C. incerta* was found in samples collected in Cuba, Netherlands and Japan, while *C. schloesseri* is only known from Kenya so far. However, this indicates that these species of the

genus *Chlamydomonas* could have a worldwide distribution. To get a better overview about the distribution of the three *Chlamydomonas* species, we used the BLAST N search algorithm of ITS-2 sequences (100% coverage, 97% identity). No new record of the three species could be discovered. Only one new record (a specimen from Thailand, KM061449) was found, which potentially represents a new species of *Chlamydomonas* (data not shown).

Beyond *Chlamydomonas*

Our phylogenetic studies have clearly revealed that *C. debaryana* is closely related to the other species of *Chlamydomonas*, as was indicated by autolysin experiments (Schlösser, 1976, 1981, 1984, Matsuda *et al.*, 1987). The SSU/ITS phylogeny demonstrated that *C. debaryana* seems to be more closely related to the Tetrabaenaceae, which was confirmed by approximately unbiased tests of user-defined trees. The phylogenies using ITS and *rbc*L revealed that this species represents an independent lineage among the Core-*Reinhardtinia*. Therefore, *C. debaryana* is not a member of *Chlamydomonas* and represents its own genus, which we propose here as follows:

Edaphochlamys Pröschold & Darienko gen. nov.

Diagnosis: Mature vegetative cells are broadly ellipsoidal, ovoid or sometimes obovoid. Cell wall is thin, with distinct papilla, two apical contractile vacuoles. Chloroplast is cup-shaped, smooth. The single pyrenoid is medium large, spherical or broadly ellipsoidal, surrounded by several starch grains placed in the thick part of chloroplast. Nucleus is placed in the anterior part of cell. Eyespot is dot-like, anterior to equatorial position. Asexual reproduction by zoospores formation. The morphology of zoospores is similar to that of the vegetative cells. Sexual reproduction by isogamy.

Etymology: The generic name is derived from the two Greek words *edaph*, meaning "soil", and *chlamys*, meaning "mantle". This indicates preferred habitat for this genus.

Type species (designated herein): ***Edaphochlamys debaryana*** (Goroschankin) Pröschold & Darienko comb. nov.

Edaphochlamys debaryana (Goroschankin) Pröschold & Darienko comb. nov.
Basionym: *Chlamydomonas debaryana* Goroschankin (1891), Bull. Soc. Imp. Nat. Moscou, N.S. **5**: 106–108, fig. 9–12.

Comment: All strains designated as *C. debaryana* were almost identical in morphology and SSU and ITS rDNA sequences. Only the strain SAG 70.81 differed from *C. debaryana* as shown in Fig. 3. This strain could be preliminary identified as *C.* cf. *latifrons*; however, this needs further studies.

As demonstrated in our study, unicellular genera *Chlamydomonas* and *Edaphochlamys* are closely related to the colonial genera of the Goniaceae, Tetrabaenaceae, and Volvocaceae. The Goniaceae include the genera *Gonium* and *Astrephomene* (Nozaki & Kuroiwa, 1992), the Tetrabaenaceae the genera *Basichlamys* and *Tetrabaena* (Nozaki & Ito, 1994). Both families were considered as intermediate families between unicellular taxa such as *Chlamydomonas* and the Volvocaceae, which comprises the colonial genera *Pandorina*, *Volvulina*, *Yamagishiella*, *Eudorina*, *Platydorina*, *Colemanosphaera*, *Pleodorina*, and *Volvox* (Nozaki *et al.,* 2000, Nozaki, 2003, Nozaki *et al.,* 2014). However, in contrast to the phylogenies using chloroplast genes, where *Chlamydomonas* and *Vitreochlamys* species were often at the base of the Volvocales *sensu stricto*, the phylogenetic analyses of SSU and ITS rDNA sequences always demonstrated that the unicellular taxa are distributed among the colonial lineages (Nakada *et al.,* 2016; this study). Our study here revealed five lineages (*Chlamydomonas*, *Edaphochlamys*, Tetrabaenaceae, Goniaceae, and Volvocaceae) among the Core-Reinhardtinia, four of them are highly supported in bootstrap and Bayesian analyses. Only the family Goniaceae (* in Figs 3–4) was not supported in our analyses. The genera *Chlamydomonas* and *Edaphochlamys* were topologically sisters of the families Goniaceae/Volvocaceae and Tetrabaenaceae, respectively. These results were partially confirmed by the activity tests of gamete lytic enzymes (GLE) derived from *Chlamydomonas reinhardtii*. In these tests the GLE dissolved not only the cell walls of several *Chlamydomonas* species such as *C. reinhardtii*, *C. incerta*, and partly *C. debaryana* (only SAG 26.72, but no reaction by SAG 4.72 and SAG 14.72), it also degraded those of the colonial genera *Gonium*, *Astrephomene*, *Basichlamys* (= *Gonium sacculiferum*), and *Tetrabaena* (= *Gonium sociale*; Matsuda *et al.,* 1987, Matsuda, 1988). All these data indicated the close relationship of these taxa. In contrast, the gamete autolysin had no influence on the other colonial Volvocaceae.

However, these results raised the question of why the phylogenetic analyses using nuclear and plastid-coding genes showed different tree topologies. Possible explanations are: (1) The phylogenies of nuclear and plastid-coding genes were based on different data sets. Unfortunately, no congruent alignments including sequences of the same strains have

been available until now. (2) Nuclear and chloroplast genes of unicellular and colonial taxa have different evolutionary rates. Whereas the nuclear genes appear to evolve at similar rates, the plastid-coding genes evolved differently among unicellular and colonial species. To address these questions further, we created small congruent datasets (ITS and *rbc*L) of representatives of all lineages and analyzed them separately (Fig. 4). The analyses of both datasets clearly showed different tree topologies. The ITS phylogeny revealed five clades, which represented the three families (the Goniaceae is only weakly supported) and the two genera *Chlamydomonas* and *Edaphochlamys*. In contrast to highly or moderately supported lineages using ITS, the *rbc*L phylogeny did not show this topology and most lineages were not supported by bootstrap and Bayesian analyses. This clearly demonstrated that *rbc*L is too conserved to achieve a robust phylogenetic resolution. For better understanding of the plastid-coding genes, we re-analyzed the datasets used by Nakada *et al.* (2016) separately and concatenated (see Figs S1; Supplemental Material). The third codon positions were excluded from these analyses because these positions were saturated. The tree topologies using *rbc*L, *atp*B, *psa*A, *psa*B, and *psb*C were very different and showed no or only weak support for genera and families. These results were probably caused by different evolutionary rates of each gene and low genetic variations among the plastid-coding genes. The concatenated dataset of all genes also revealed high support for only some of the genera and lineages. As Wang *et al.* (2014) and references therein highlighted, if the tree topologies of single genes differ significantly, then the gene sequences should not be combined in a concatenated dataset. This resulted in incongruent species trees, which is caused by different genetic history of the selected genes. As consequence of our study, the nuclear SSU and ITS sequences combined in a concatenated dataset produce a much better resolution and therefore should be preferred for taxonomy of the volvocalean algae.

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Supplemental Material. Molecular phylogeny of the Volvocales *s.str.* based on concatenated and separate datasets of nuclear SSU rDNA and plastid-coding *rbc*L, *atp*B, *psa*A, *psa*B, *psb*C gene sequences according to Nakada *et al.* (2016). The phylogenetic trees shown were inferred using the maximum likelihood method based on these data sets using PAUP 4.0b10. For the analyses the best model was calculated by Modeltest 3.7. The settings of the best models were summarized in the table. The third codon positions of the chloroplast genes were excluded from the analyses.

Table S1. Strains used in this study.