J. Phycol. **44,** 1586–1603 (2008)

DOI: 10.1111/j.1529-8817.2008.00606.x

NEW STREPTOPHYTE GREEN ALGAE FROM TERRESTRIAL HABITATS AND AN ASSESSMENT OF THE GENUS *INTERFILUM* (KLEBSORMIDIOPHYCEAE, STREPTOPHYTA)[1]

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Sarcinoid aeroterrestrial green algae were isolated from three arid locations in Ukraine and the Czech Republic. Although gross morphology suggested an affinity with *Desmococcus* (for taxonomic authorities, see Table S1 in the supplementary material), the cellular morphological characteristics were reminiscent of those of *Geminella terricola*. However, the presence of a complex of ultrastructural features indicated that these isolates were members of the streptophyte lineage in the green plants. 18S rDNA sequence phylogenies provided evidence of a close relationship with *Klebsormidium* in the Streptophyta, while the position of *Desmococcus* was within the Trebouxiophyceae. In the internal transcribed spacer (ITS) rDNA phylogeny, the sarcinoid isolates were closely related with strains of *G. terricola* and *Interfilum paradoxum*. Strains of that clade were morphologically united by a specific type of cell division that involves the association of persistent, cap-shaped remains of the mother cell wall with daughter cells. Consequently, these strains were assigned to a redefined genus *Interfilum*, and a new species, *I. massjukiae,* was described to accommodate one of the sarcinoid isolates. As the position of the genus *Geminella* was in the Trebouxiophyceae, the streptophyte *G. terricola* was transferred to *Interfilum*, as *I. terricola* comb. nov., but the ITS rDNA analyses proved inconclusive to resolve its affinities with other species of *Interfilum* due to intragenomic polymorphisms. The species of *Interfilum* had a closer relationship with *K. flaccidum* than with other species of *Klebsormidium*. The latter genus may not be monophyletic in its present circumscription.

***Key index words:* *Desmococcus*; *Geminella*; *Interfilum*; intron; *Klebsormidium*; morphology; rDNA; Streptophyta; Trebouxiophyceae; ultrastructure**

***Abbreviations:* ACKU, Algal Culture Collection at Kyiv University, Ukraine; AIC, Akaike information criterion; DGGE, denaturing gradient gel electrophoresis; ITS, internal transcribed spacer; ME, minimum-evolution distance; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining; RI, rescaled consistency index; SAG, Culture Collection of Algae at Göttingen University, Germany; TBR, tree-bisection-reconnection branch swapping; UTEX, The Culture Collection of Algae at the University of Texas**

[1]Received 11 December 2007. Accepted 19 June 2008.
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The identification of filamentous and sarcinoid green algae generally presents a challenge for most algal systematists and ecologists due to the scarcity of visual characters that are available for diagnostic purposes and the less than perfect taxonomies that are available for these groups. Recent molecular studies have led to the recognition that there is rampant morphological homoplasy in these groups and that only a tiny proportion of the genetic diversity that is revealed by DNA sequencing can be captured by microscopic observation of cellular and reproductive features. The general lack of understanding of the taxonomic diversity in these groups not only creates difficulties when attempting to accurately assess the species richness of habitats that they dominate, such as soil and other terrestrial habitats, but also leads to a significant underestimation of the number of species of coccoid and sarcinoid algae that exist. Relatively new technologies, such as separation of PCR amplified rDNA from heterogenous environmental samples with denaturing gradient gel electrophoresis (DGGE), have shown considerable promise to help reveal more of the genetic diversity of microbial and microalgal communities, and the results of these analyses can be useful pointers for microscopists to identify morphological diagnostic markers that were previously overlooked (Head et al. 1998, Diez et al. 2001, Sigler et al. 2003). However, despite the increasing popularity of and dependency on molecular genetic methods to catalogue the taxonomic diversity of microalgae, discoveries of new taxa by careful observations of experienced microscopists still occur.

An example of new green microalgae that were discovered recently by careful microscopical screening of natural samples are aerophytic organisms that were isolated from rock outcrops of the steppe zone and Mountain Crimea in Ukraine, and from soil in the Czech Republic. While showing morphological similarity in cellular structure to the filamentous green alga *G. terricola*, their sarcinoid packets were reminiscent of those typically produced by *Desmococcus*. To assess their taxonomic affinities and phylogenetic relationships within the green algae more accurately, we undertook a detailed study involving LM and TEM as well as rDNA sequence analyses. The results of these studies represent the main subject of this paper. They led to the conclusion that the three new sarcinoid isolates as well as an isolate of *G. terricola* are representatives of the genus *Interfilum*. The two Ukrainian isolates had previously been assigned to a new genus *Massjukia* (as *M. desmococcoidea*, Mikhailyuk et al. 2005). Ultrastructural and rDNA sequence evidence indicated that *Interfilum* belongs to the streptophycean lineage in the green algae, with a close relationship with *Klebsormidium, Hormidiella,* and *Entransia*. These conclusions applied also to strains from the SAG culture collection that represented *I. paradoxum* and to an unidentified strain of the genus. To accommodate these new observations in a new taxonomic framework, the diagnosis of the genus *Interfilum* and that of the type species *I. paradoxum* were emended, and one of the new isolates was assigned to a new species, *I. massjukiae* sp. nov. Finally, the diagnosis of *G. terricola* was emended, and this species was transferred to *Interfilum* as *I. terricola* comb. nov.

MATERIALS AND METHODS

Culture strains. Two isolates with sarcinoid growth habit isolated from subaerial outcrops in Ukraine, a third sarcinoid isolate from soil in the Czech Republic, and a new isolate representing *G. terricola* (see Table S1 for details about the localities) were the focus of this study. The four strains have been accessioned by the SAG culture collection as strains SAG 2101, SAG 2102, SAG 2147, and SAG 2100. The strains representing *G. terricola* and the two sarcinoid strains from Ukraine are also available from the ACKU culture collection at Kyiv University (Table S1). For comparisons at the morphological and molecular levels, additional strains representing the genera *Interfilum*, *Klebsormidium*, and *Geminella* were used as listed in Tables S1 and S2 (in the supplementary material).

Culture conditions, microscopy, ultrastructure. The cultures were maintained in agar slants with Bold Basal Medium with vitamins and triple nitrate (Starr and Zeikus 1993) at 18°C under a light:dark (L:D) regime of 14:10 and a photon fluence rate of ~25 μmol photons·$m^{-2} \cdot s^{-1}$ from white fluorescent bulbs. Microscopic observation was accomplished using an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics and with a LOMO Mikmed-2 bright field microscope (St. Petersburg, Russia) on cultures not older than 5 weeks. Micrographs were taken with the Olympus BX60 microscope with an attached ColorView III camera (Soft Imaging System GmbH, Münster, Germany) and processed with the Cell^D image programme (Soft Imaging System GmbH). Mucilage of algal cells was stained with an aqueous solution of methylene blue.

For TEM, colonies were removed with a spatula from the surface of 1- to 2-month-old agar cultures, fixed for 1 h in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.1 at room temperature, and processed further according to Massalski et al. (1995).

DNA extraction, PCR amplification, and sequencing. The strains for which rDNA sequences were determined in this study are listed in Tables S1 and S2. DNA was extracted from the strains listed in Tables S1 and S2 using the Invisorb Spin Plant Kit (Invitek, Berlin, Germany) as recommended by the manufacturer after cells were broken in a 2 mL microcentrifuge tube three-fourths filled with 425–600 μm glass beads using a MiniBeadBeater (BioSpec Products, Bartlesville, OK, USA) at 38,000–50,000*g* for 30–50 s. Nuclear-encoded 18S rDNAs were amplified using primers NS1 and 18L (Hamby et al. 1988), and for amplification of the ITS-1, 5.8S, and ITS-2 regions, primers NS7m and LR1850 (Friedl 1996) were used. For some strains, an rDNA region containing the 18S, ITS-1, 5.8S, and ITS-2 regions was amplified using the primers NS1 and LR1850 (Friedl 1996). In case of a culture contaminated with fungi, the PCR products were cloned (see below), and the clone sequences checked for their algal origin using the BLASTn search tool (http://www.ncbi.nlm.nih.gov/BLAST/). As an alternative, a PCR primer that preferentially binds to a region at the 3′-end of algal 18S rDNAs, LR 1650 (5′-TCACCAGCACACCCAAT-3′), combined with primer NS1 was used to amplify the algal 18S rDNA, or primer combination AL1500af (Helms et al. 2001) and LR1850 to amplify the algal ITS-1–5.8S–ITS-2 regions. The PCRs took place in a 50 μL volume containing 2 U of Bioline-DNA-polymerase in 1 × PCR buffer (Bioline, Luckenwalde,

Germany), 2 μL 2 mM $MgCl_2$, 100 μM of dNTP mix, the two primers (each 2 μL 0.5 μM), and ~30 ng of the extracted DNA. PCR conditions were as follows: initial denaturation for 5 min at 95°C followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 120 s. A final extension at 72°C for 10 min completed the PCR. The size and yield of the PCR products were analyzed by electrophoresis in a 1% agarose gel stained with Gelstar® (Lonza, Basel, Switzerland). The positive PCR products were pooled, purified using the NucleoSpin-Extract-Kit (Macherey-Nagel, Düren, Germany), and eluted in 30 μL elution buffer provided by the kit. For cloning, the purified PCR products were ligated into the PCR® 2.1-TOPO®-vector-system (Invitrogen, Karlsruhe, Germany) and cloned in *E. coli* TOP 10. Plasmid DNA was extracted from positive clones with the NucleoSpin-Plasmid-Kit and was used as template for sequencing reactions. Cycle sequencing was performed using the Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) with the following PCR conditions: initial denaturation for 1 min at 96°C followed by 35 cycles of denaturing at 96°C for 30 s, annealing at 50°C for 45 s, and extension at 60°C for 180 s. For the 18S rDNA sequencing, a set of nested standard primers (Elwood et al. 1985) was used, for the ITS-1–5.8S–ITS-2 regions the primers 1800F (Friedl 1996), 5.8SbF (5′-CGATGAAGAACGCAGCG -3′), 5.8SbR (5′- CGCTGCGTTCTTCATCG-3′), and ITS4 (White et al. 1990). When no clear sequences of the ITS-1–5.8S–ITS-2 regions were obtained from sequencing the PCR products, they were cloned, and ~15 positive clones were sequenced. Sequencing reactions were separated on an ABI Prism 3100 (Applied Biosystems) automated sequencer. The sequences were assembled using the programme SeqAssem (Hepperle 2004). All sequences determined in this study were deposited in GenBank (accession numbers EU434016-EU434040, Tables S1, S2).

Sequence alignment. To assess the phylogenetic position of three new sarcinoid isolates, *G. terricola* SAG 2100 and the two strains of *I. paradoxum* available from the SAG culture collection their 18S rDNA sequences were compared to a broad selection of corresponding sequences from members of the Streptophyta and Chlorophyta. This analysis also included newly determined sequences for *Geminella* and *Desmococcus* as well as other trebouxiophyte green algae (Table S2). The selection of sequences was based on a phylogenetic tree comprising an expanded sample of >1,500 rDNA sequences from green algae and embryophytes, which is available in the 18S rDNA sequence database maintained in the ARB program (version 05.05.26, Ludwig et al. 2004, http://www.arb-home.de). This database was updated with all currently available 18S rDNA sequences from streptophyte green algae and Chlorophyta. The newly determined sequences were added to the database using the parsimony interactive tool in ARB. The alignment was refined by comparing the sequences with their next relatives from the ARB database based on their pairing across a helix using secondary structure models as implemented in ARB. This program generates an MP tree from all sequences and all positions in the database as its reference tree, using a filter based on 50% base frequency across all species. A subset of these sequences comprising a total of 55 representatives of streptophyte green algae, embryophytes (8 sequences), prasinophyte green algae, Trebouxiophyceae, and two glaucophytes (as outgroup taxa) were then downloaded from the ARB database for further analyses using the 50% base frequency filter. The sequence alignment was 1,711 nucleotides long, of which 678 positions were variable and 474 parsimony informative. Because in the 18S rDNA phylogeny the sarcinoid isolates, *G. terricola* SAG 2100 and strains of *Interfilum paradoxum* were nested within a clade otherwise comprising strains and species of *Klebsormidium* and no resolution was found within that clade due to only minor sequence differences, their ITS-1–5.8S–ITS-2 rDNAs were compared with corresponding sequences of *Klebsormidium* and phylogenetically analyzed. For the ITS rDNA data set, secondary structure models for ITS-1 and ITS-2 of the new sequences were constructed. Using these models, the new sequences were then aligned to the alignment of *Klebsormidium* ITS rDNAs of Sluiman et al. (2008) (EMBL, http://www.ebi.ac.uk, accession no. ALIGN_001122). The ITS rDNA data set also included four sequences newly determined for *K. flaccidum* and an unidentified *Klebsormidium* strain (Table S2). This data set comprised the ITS-1 and ITS-2 rDNA regions for a total of 24 taxa and was 551 nucleotides long, of which 121 positions were variable and 78 parsimony informative. The SSU and ITS alignments used in this study are available from EMBL Align (http://www.ebi.ac.uk), accession numbers ALIGN 001255 and ALIGN 001256.

Phylogenetic analyses. The 18S and ITS rDNA sequence data sets were subjected to minimum-evolution (ME), maximum-parsimony (MP), and maximum-likelihood (ML) approaches with the programmes PAUP* version 4.0b10 (Swofford 2001) or MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). For the model-based approaches ME and ML, a likelihood ratio test using MODELTEST version 3.7 (Posada and Crandall 1998; Akaike information criterion [AIC]) was performed to determine the model that fits the observed data best. For the 18S rDNA data set, the selected model was Tamura and Nei (TrN+I+G; Tamura and Nei 1993) with estimations of nucleotide frequencies (A = 0.2525, C = 0.2205, G = 0.2759, T = 0.2511), a rate matrix with six different substitution types, assuming a heterogeneous rate of substitutions with a gamma distribution of variable sites (number of rate categories = 4, shape parameter $\alpha = 0.5801$) and a proportion of invariable sites (pinvar) of 0.3734. For the ITS-1,2 rDNA data set, the selected model was a general-time-reversible model (GTR+G; Tavaré 1986) with estimations of nucleotide frequencies (A = 0.2005, C = 0.3066, G = 0.3048, T = 0.1881), a rate matrix with six different substitution types, assuming a heterogeneous rate of substitutions with a gamma distribution of variable sites (number of rate categories = 4, shape parameter $\alpha = 0.3402$) without a proportion of invariable sites (pinvar = 0). For the distance analyses, two different methods were used, that is, NJ (Saitou and Nei 1987) in connection with the ''HKY85 model'' (Hasegawa et al. 1985) and ME (Rzhetsky and Nei 1992). ME distance trees were constructed with DNA distances set to ML, and a heuristic search procedure with 10 random input orders and tree-bisection-reconnection branch swapping (TBR) were employed to find the best tree. Best scoring trees were held at each step. In MP analyses, the sites were weighted (rescaled consistency index [RI] over an interval of 1–1,000). The heuristic search for the best tree was the same as in ME analyses. Bootstrap resampling was performed on NJ trees with 2,000, for ME (only ITS rDNA data set) and MP with 1,000 replications. Due to CPU time constrains, bootstrapping in ME analyses was not possible for the 18S rDNA data set. For the ML method, two approaches were used. The ML trees shown in Figures 6 and 7 (with -ln likelihoods of 12755.4585 and 1733.9832) were calculated with PAUP using best-fit models as determined above and used as input for three additional rounds of ML analyses to search for trees with smaller -ln likelihoods, but trees with better likelihood scores were not obtained. The other approach was Bayesian analysis performed in MrBayes with a GTR+I+G model (rate matrix with six different substitution types, number of rate categories = 4, and with the nucleotide frequencies, shape parameter α, and pinvar estimated from the data). Four Markov chains and 2,000,000 generations sampling every 100 generations were used with the first 25% of the sampled trees discarded, leaving 15,000 trees. Posterior probabilities were then calculated from two

independent runs using the 50% majority-rule consensus of the kept trees.

RESULTS

Morphology and ultrastructure. Isolates SAG 2101, SAG 2102, and SAG 2147 were characterized by the ability to form cell packages (Figs. 1, 2). In strains SAG 2102 and SAG 2147, large sarcinoid aggregations as well as long uni- and biseriate filaments were expressed prominently, particularly in cultures older than 3 weeks (Figs. 1, e, f, h; 2g). Strain SAG 2101, however, only rarely formed single packets and short biseriate filaments (Fig. 2, a, b, f) as unicells (Fig. 2, b, d, e) or short uniseriate filaments (Fig. 2c) prevailed. Vegetative cells of the sarcinoid strains had a very similar size and morphology (Figs. 1, 2). A single parietal, plate-shaped chloroplast was positioned to one side of the cell and had lateral incisions resulting in 5–8 lobes (Figs. 1, c, d, g; 2, a, b, f, g). The center of the chloroplast contained one pyrenoid, surrounded by 8–10 small elongated starch grains, which were oriented parallel to the longitudinal axis of the cell and were sometimes arranged in layers (Figs. 1, a, c, d, g; 2, b, f). Cells contained a single nucleus situated opposite the pyrenoid (Fig. 1c). Various stages of cell division and the formation of cell packages were observed in cultures of different ages. Briefly, the protoplast divided into two parts, each forming a new cell wall within the mother cell wall. Then the latter ruptured in the middle (aequatorially) and split into two equal halves. The mother cell halves became separated during expansion of the daughter cells. As a result, the daughter cells were capped by the mother cell wall ''halves'' (Figs. 1b; 2, c–e). Sometimes the mother cell wall halves further disintegrated due to the continuous growth of the daughter cells, resulting in a ring-like structure around the daughter cell (Fig. 3d). Sarcinoid packages were formed when this process was repeated during subsequent division cycles and successive division planes were perpendicular to the previous ones. In cell packages, the remains of the mother cell walls mostly remained closely associated with the daughter cell walls, resulting in an integrated wall structure without intervening spaces and in which no remains of the mother cell wall were visible (Fig. 1, c and d). Only in cases when the cell packages started to disintegrate were wall remains visible as tiny wedges (Fig. 1b), especially after methylene blue staining (Fig. 2, c–e). In strain SAG 2101, the remains of mother cell walls appeared to gelatinize because methylene blue stained the mucilaginous remains of the mother cell wall more intensely than the thin mucilage envelope of the daughter cells (Fig. 2, c–e). The layer of mucilage on daughter cells was ~0.3–0.5 μm thick and had a pattern of radiating striations (Fig. 2, c–e). No mucilage was discernable in strains SAG 2102 and SAG 2147.

In *G. terricola* strain SAG 2100 vegetative cells had a morphology that was virtually identical to that observed in the three sarcinoid isolates (Fig. 3). However, the cells were sometimes more elongated, oviform, or slightly cylindrical (Fig. 3, a, b, c, g). In long cells, sometimes two prominent terminal vacuoles were visible, with the nucleus situated in a cytoplasmatic bridge located between the vacuoles (Fig. 3g). The cells had a thin (~1–3 μm) mucilaginous envelope in which, as in SAG 2101, a pattern of radial striations was visible (Fig. 3, c, d, f). Cells formed uniseriate short filaments that readily disintegrated into diads and unicells (Fig. 3). The process of cell division appeared to be the same as in the sarcinoid strains except that division occurred only in one plane. Mother and daughter cell walls adhered so closely to one another that the cell wall appeared as a single integrated structure (Fig. 3, a and b). Remains of mother cell walls appeared as small wedges at the sides of cell (Fig. 3, a and b) or after methylene blue staining (Fig. 3, c–f, h). The mucilaginous material formed after gelatinization of these walls was striated (Fig. 3, c, d, f).

I. paradoxum strains SAG 338-1 and SAG 4.85 had a cellular morphology that was virtually identical to that of the sarcinoid isolates SAG 2101, SAG 2102, and SAG 2147 and *G. terricola* SAG 2100 (Fig. 4). As in *G. terricola,* cells of *I. paradoxum* divided in one plane only, forming short uniseriate filaments that often disintegrated into diads or unicells (Fig. 4). However, a unique feature of these strains was that the remains of the mother cell wall did not adhere closely to the daughter cells. Instead, remains of mother cell walls were often separated from the daughter cells, giving rise to anastomosing structures that connected daughter cells (Fig. 4, b–d, f–h), or were simply discarded (Fig. 4, a, e, j). Cells of *I. paradoxum* were surrounded by a thin (~0.5–1 μm) mucilaginous envelope with a radial structure after staining with methylene blue (Fig. 4, e and i). There were only minor differences between *I. paradoxum* strains SAG 338-1 and SAG 4.85, that is, in the size and shape of the cells (Fig. 4f).

EM. The ultrastructure of vegetative cells of the two sarcinoid strains SAG 2101 and SAG 2102, and the weakly filamentous *G. terricola* SAG 2100 and *I. paradoxum* SAG 338-1 were observed to be virtually identical (Fig. 5). The chloroplast was located at one side of the cell, covering half of the cell's volume (Fig. 5, a and f). The pyrenoid matrix was traversed by an array of parallel thylakoid bands, which sometimes formed loops (Fig. 5, a, d, g). Between the nucleus and chloroplast, a microbody (peroxisome) was located that had a slightly curved bacilliform shape in cross-sections (Fig. 5, b, d, f, g). The mitochondrion was single, curved, and slightly branched. It was associated with the chloroplast, the nuclear envelope, and perhaps the microbody (Fig. 5, d, f). The strains differed slightly in the cell wall ultrastructure: vegetative cells of strain SAG

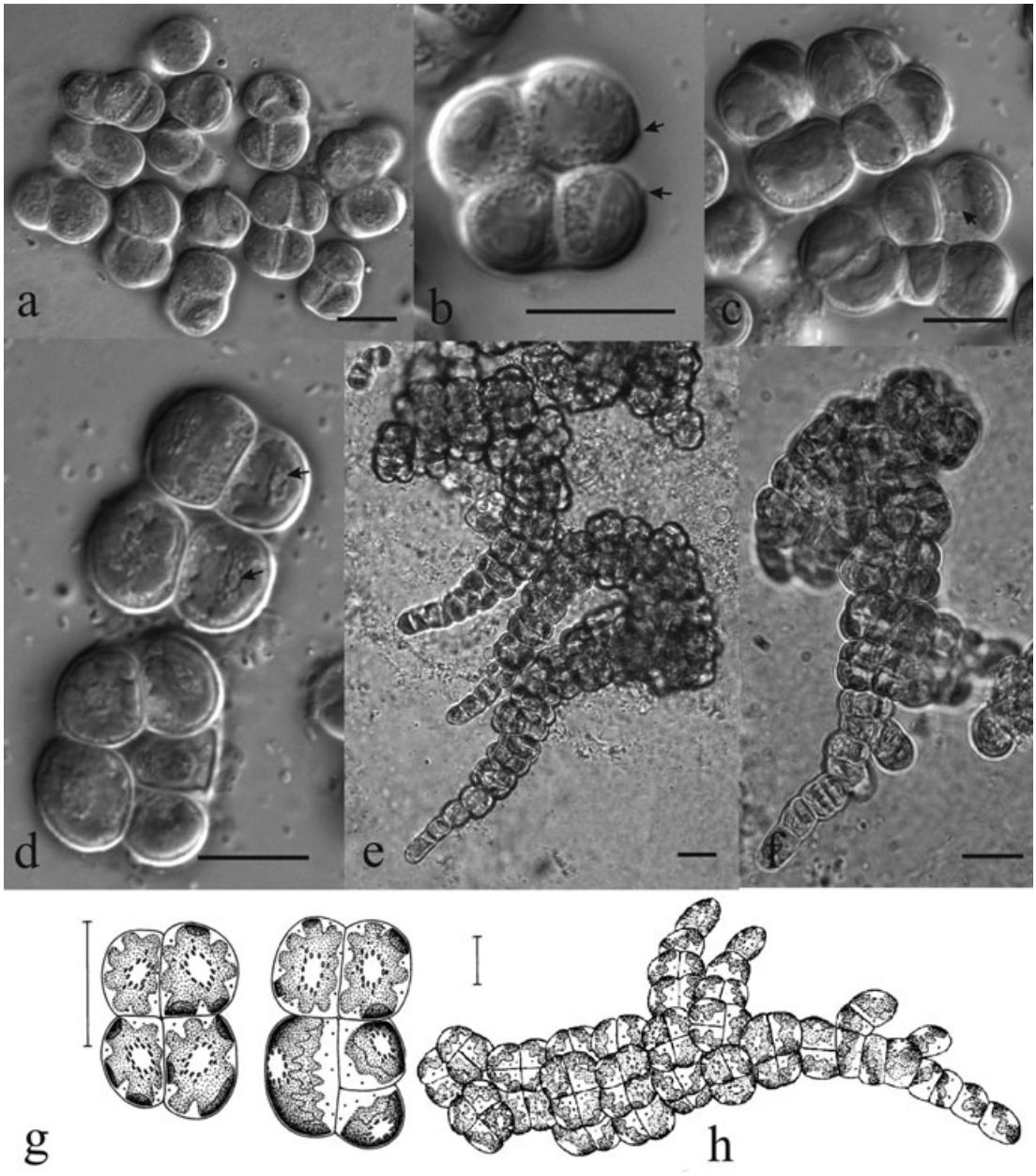

FIG. 1. Morphology of strain SAG 2102 (*Interfilum massjukiae* sp. nov.). (a) Cell packets in young culture. (b) Disintegrating cell package with remains of the mother cell wall (arrows). (c, d) Cell packages with details of cell morphology showing location of nucleus directly opposite the pyrenoid (arrows in c) and lobed chloroplast with pyrenoid surrounded by numerous starch grains (arrows in d). (e, f) Large sarcinoid aggregations and uni- and biseriate filaments in a 3-week-old culture. (g, h) Interpretative drawings showing cell packages and chloroplast morphology in a young culture (g), and sarcinoid and filamentous aggregation of cells (h). Scale bars, 10 μm.

2102 had a thick cell wall with a unique layered-lacunar structure without a mucilage envelope (Fig. 5, a and c), whereas the cell walls of the other strains had a homogeneous structure and were surrounded by a clear mucilage envelope with radial striations (Fig. 5, e and f).

Phylogenetic analysis of rDNA sequences. Comparisons of 18S rDNA sequences suggested a close relationship of the three new sarcinoid isolates and *G. terricola* SAG 2100 with strains of *Interfilum* and *Klebsormidium*. The 18S rDNA sequences of strain SAG 2101, *G. terricola* SAG 2100, *I. paradoxum* SAG 338-1 and SAG 4.85 were identical, apart from the presence/absence of a group I intron in some sequences (see below). Comparison of this invariant exon region with the 18S rDNA of the sarcinoid strains SAG 2102 and SAG 2147 revealed two and five differences, respectively. All six strains had no more than 3–7 base differences when compared with the 18S rDNA regions of *Klebsormidium* spp.

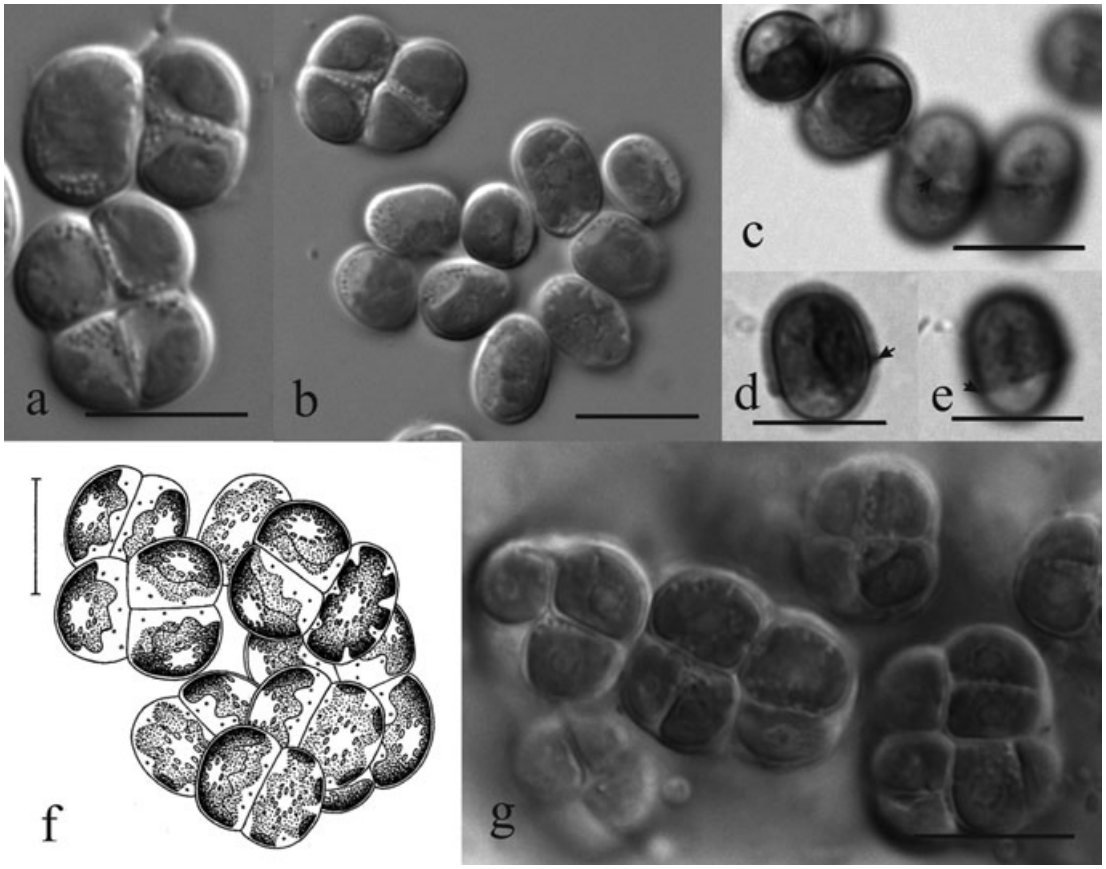

FIG. 2. Morphology of strains SAG 2101 and SAG 2147 (*Interfilum* sp.). (a–f) SAG 2101. (g) SAG 2147. (a, b) Cell packets and unicells in a 3-week-old culture. Note chloroplast with lobes and central pyrenoid (b, right half). (c–e) Diads and unicells in different optical sections after staining with methylene blue showing cap-like remains of mother cell wall closely associated with daughter cells (arrows) and gelatinized cell walls with striations. (f) Interpretative drawing of cell packages in a young culture showing chloroplast morphology. (g) Sarcinoid packets and cubic aggregations in a 3-week-old culture. Note lobed chloroplast with central pyrenoid. Scale bars, 10 μm.

Interestingly, there were no more than one or three synapomorphic sequence differences between the six strains and *Klebsormidium* spp. In the six strains, an A was located in a loop in the V5 region of the 18S rRNA secondary structure model (Wuyts et al. 2000, Ueno et al. 2007), that is, at pos. 1619 of the SAG 2102 18S rDNA sequence, corresponding with a G in all *Klebsormidium* spp. Strains of *Klebsormidium* other than *K. flaccidum* were characterized by a double-sided compensatory base substitution (CBS) in stem E32-1 of the V4 region (at pos. 1068 and 1097 of the SAG 2102 18S rDNA sequence) where a C-G pair was exchanged for a U-A.

Phylogenetic analyses of the 18S rDNA sequences (Fig. 6) showed that two of the three sarcinoid isolates, SAG 2102 and SAG 2147, and *I. paradoxum* SAG 338-1 (representing a group of identical exon sequences, that is, from strains SAG 2100, SAG 2101, and SAG 4.85) formed a clade together with strains of *Klebsormidium* in the Streptophyta. The clade received high support in bootstrap and Bayesian analyses, and *Entransia fimbriata* and *Hormidiella attenuata* were resolved as closest relatives. Two strains of *Desmococcocus* (SAG 1.92, SAG 25.92), which have a similar growth habit as the three sarcinoid isolates, were positioned within the Trebouxiophyceae. The *Desmococcus* strains shared a single origin and were a sister-group of two strains of *Stichococcus bacillaris*, SAG 335-3 and SAG 379-1b. In contrast to strain SAG 2100, identified as *G. terricola,* which was a member of Streptophyta, all other *Geminella* strains including two that represented *G. interrupta,* the type species of the genus (SAG 9.91 and SAG 9.97), were placed within a lineage in the Trebouxiophyceae. This lineage also contained strain SAG 20.91, previously assigned to *G. terricola.* A sequence accessioned in GenBank as AF387153 reportedly representing ''*Microspora stagnorum*'' SAG 51.86 was also placed within the trebouxiophyte clade representing *Geminella*; it was almost identical to our newly determined sequence of *Geminella* sp. SAG 20.84 (GenBank accession EU434025). Interestingly, the latter strain had been sequenced previously (GenBank accession AF387157), but that sequence was positioned firmly within the *Klebsormidium* clade of the Streptophyta (Fig. 6). Microscopic

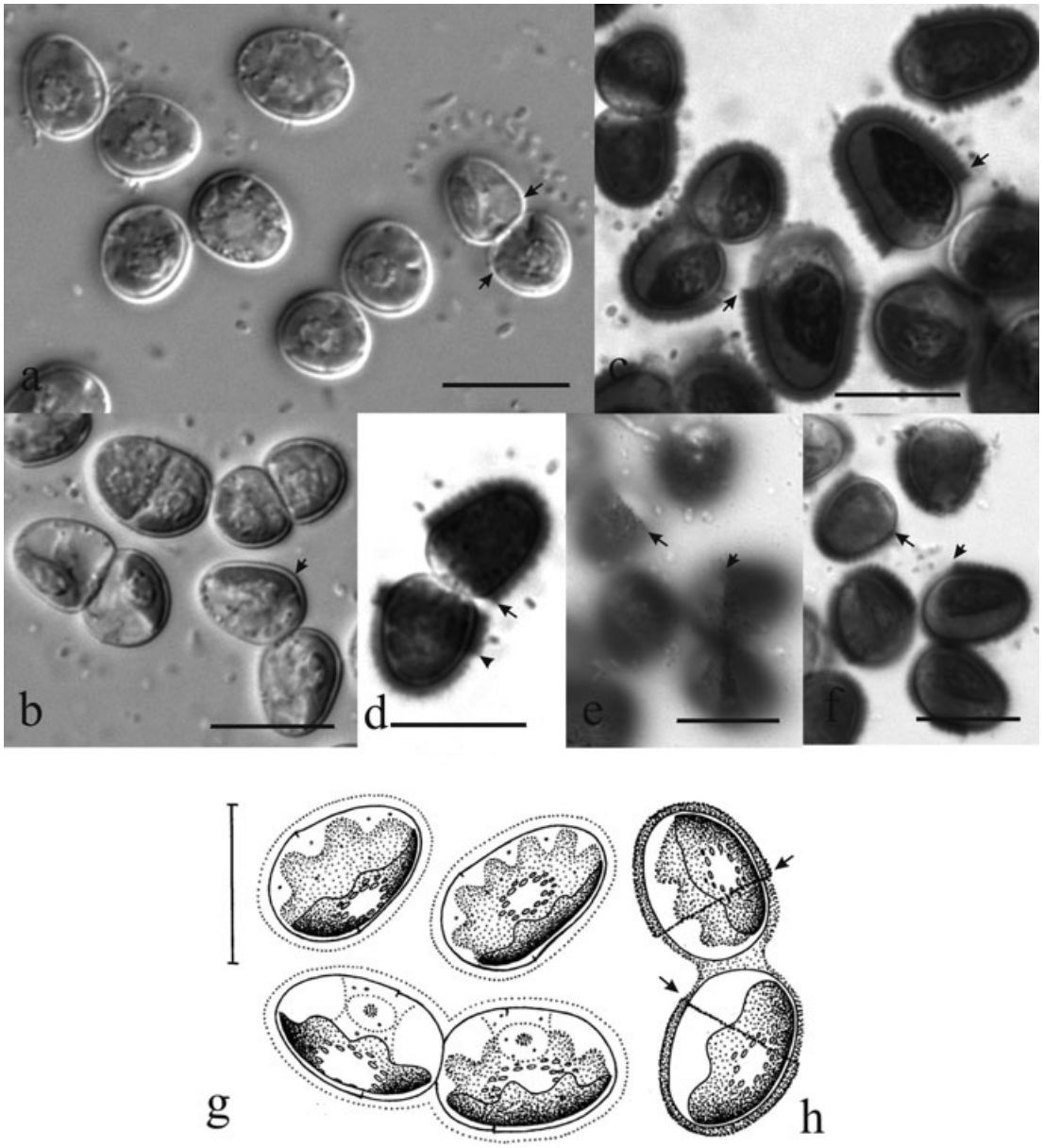

FIG. 3. Morphology of *Geminella terricola* (*Interfilum terricola* comb. nov.) strain SAG 2100. (a–b) Single vegetative cells and diads with ellipsoidal-ovoid shape. Note lobed chloroplast with central pyrenoid and remains of mother cell walls as projections at the sides of cell (arrows). (c–f) Diads and unicells after staining with methylene blue in different optical sections. Cap-like (arrows) and ring-like (d, arrowhead) gelatinized and striated remains of mother cell walls are closely associated with daughter cells. (g–h) Interpretative drawings. (g) Single vegetative cells and details of cellular morphology. (h) Diad of cells after methylene blue staining revealing the cap-like remains of the mother cell wall (arrows). Scale bars, 10 μm.

analysis of strain SAG 51.86 revealed features that are characteristic for *Klebsormidium* (e.g., the chloroplast shape and parallel arrangement of starch grains around the pyrenoid), which is at variance with the position of its sequence within the Trebouxiophyceae. We therefore resequenced strain SAG 51.86, and a comparison of its 18S rDNA sequence (GenBank accession EU434028) confirmed a close relationship with *Klebsormidium* spp. (Fig. 6). This finding suggested that the earlier sequences, AF387157 and AF387153, had been wrongly assigned to SAG 20.84 and SAG 51.86.

Group I introns at *E. coli* position 516 were present in the SSU rDNAs of all three sarcinoid strains, SAG 2101, SAG 2102, SAG 2147, and *I. paradoxum* SAG 338-1 but were absent in *G. terricola* SAG 2100 and *I. paradoxum* SAG 4.85. The 516 intron sequence of SAG 2101 was identical with that of *I. paradoxum* SAG 338-1. The corresponding intron of strain SAG 2102, however, was longer (432 bp) than

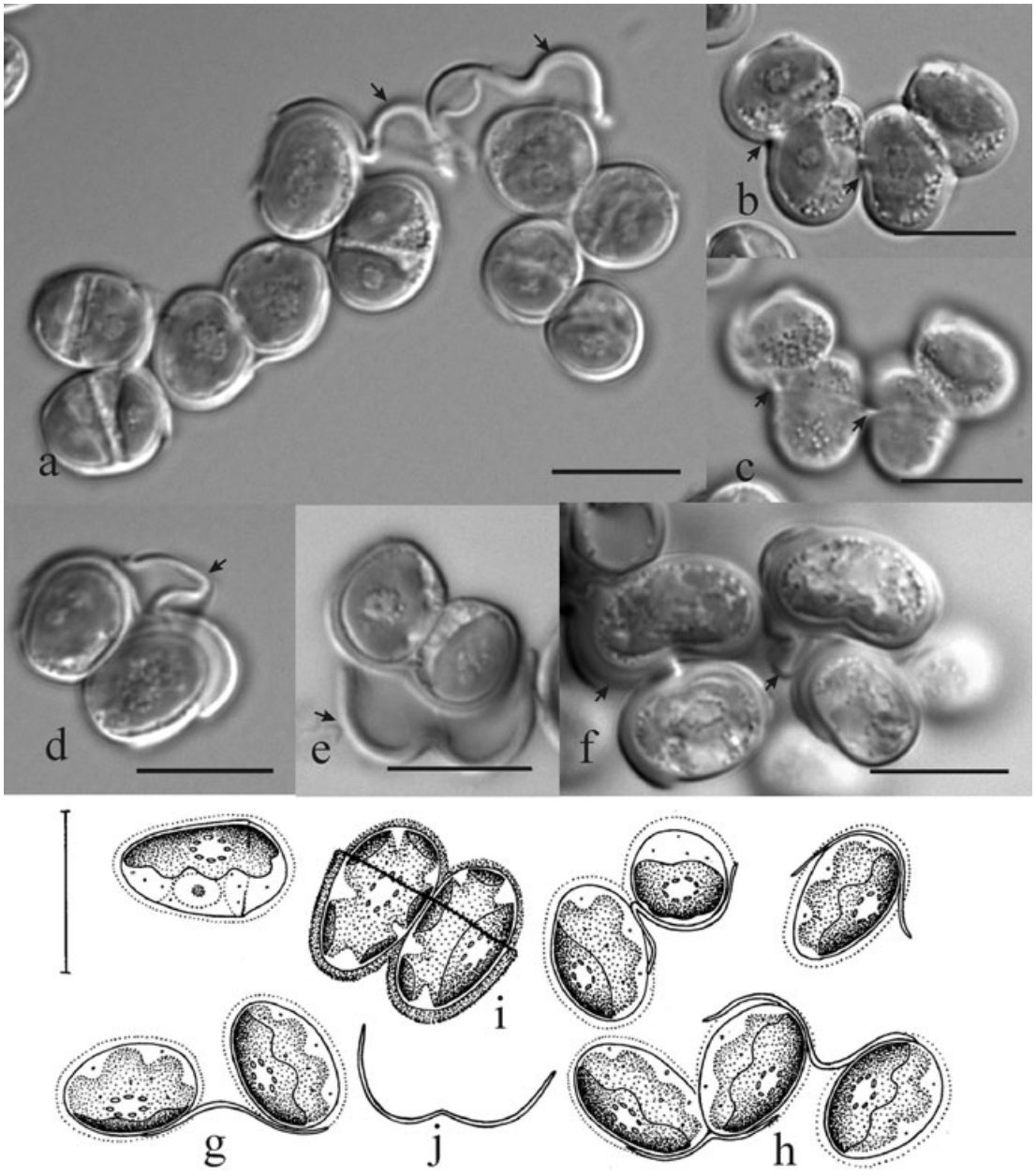

FIG. 4. Morphology of *Interfilum paradoxum* strains SAG 338-1 and SAG 4.85. (a–e, g–j) SAG 338-1. (f) SAG 4.85. (a) Unicells and diads with exfoliated mother cell walls (arrows) and details of cellular morphology. (b, c) Cells connected by remains of mother cell walls (arrows) in a four-celled filament in different optical sections. (d) Diad of cells with exfoliated remains of mother cell wall (arrow). (e) Diad of cells stained with methylene blue. Note remains of mother cell wall (arrow). (f) Elongated vegetative cells connected to each other by mother cell wall remains (arrows). (g–j) Interpretative drawings of single vegetative cells, diads connected by mother cell wall remains, details of cellular morphology (g, h, i), and a discarded remain of the mother cell wall (j). Cells shown at (i) were stained with methylene blue. Scale bars, 10 μm.

in SAG 2101/SAG 338-1 and SAG 2147 (401 bp and 378 bp). This was due to different lengths in the terminal loops of the P2, P5, P8, and P9.2 regions of the secondary structure model (Cech et al. 1994). Interestingly, the SAG 2102 intron was almost identical in its primary structure with that of the corresponding intron from *K. nitens* SAG 335-2b (AM490844), except for length differences located in the P2 and P9.2 regions. The results of a phylogenetic analysis of green algal 516 introns will be published separately, but preliminary studies showed that the introns occurring in strains SAG 2101, SAG 2102 and *I. paradoxum* SAG 338-1 formed a robust clade together with the position 516 introns in *Klebsormidium* (H. J. Sluiman, unpublished). The 516 introns found elsewhere in the green algae, that

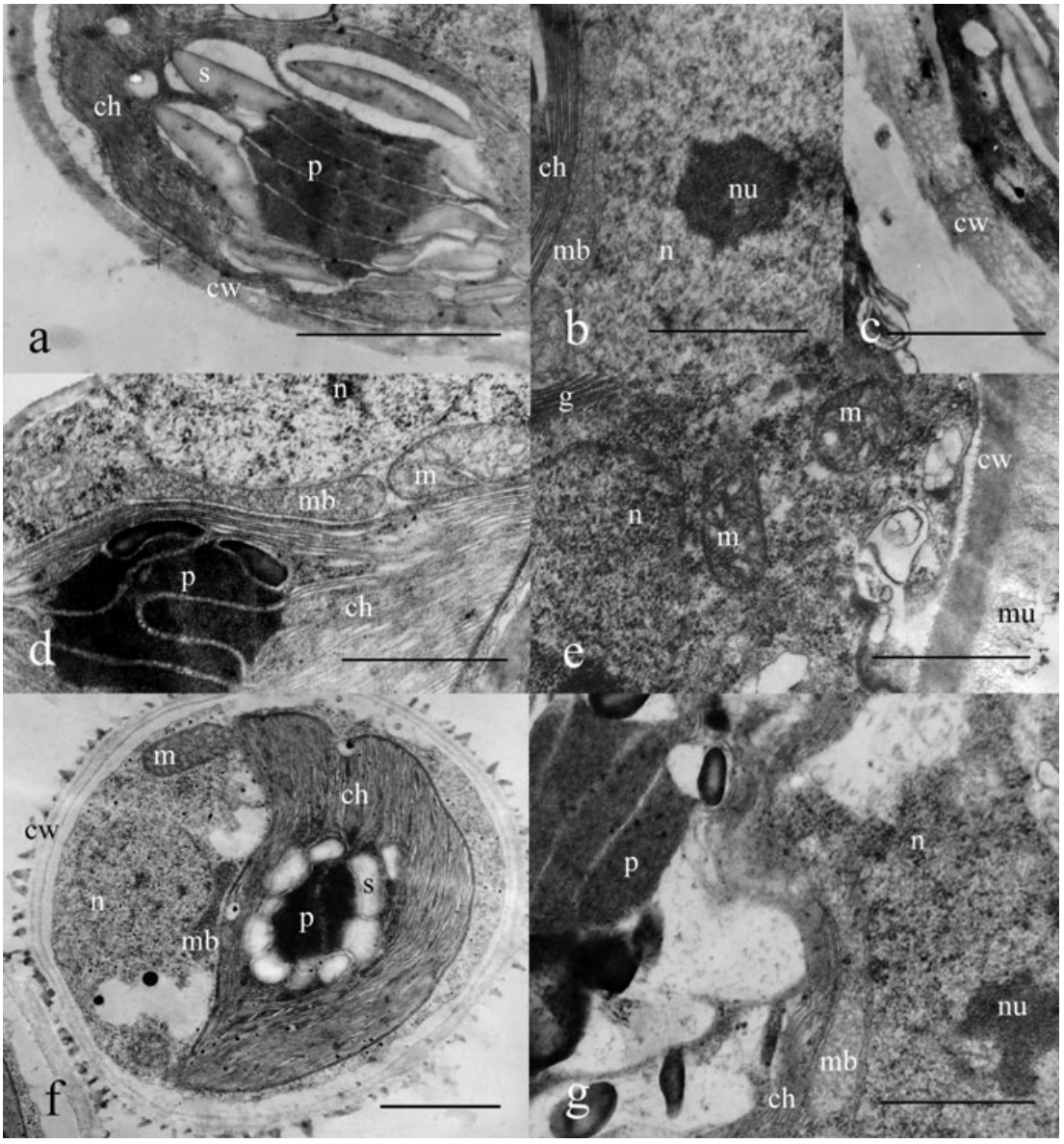

FIG. 5. Transmission electron micrographs of vegetative cells of strains *Interfilum massjukiae* sp. nov. SAG 2102, *Interfilum* sp. SAG 2101, *Interfilum terricola* comb. nov. SAG 2100, and *I. paradoxum* SAG 338-1. (a–c) SAG 2102. (a) Portion of cell showing the pyrenoid whose matrix is traversed by parallel thylakoids and associated starch grains. Note the absence of extracellular mucilage. (b) Section showing a microbody situated between the chloroplast and the nucleus. (c) Section showing a cell wall without mucilage and exhibiting a layered-lacunar structure. (d, e) SAG 2101. (d) Section showing a chloroplast containing a pyrenoid whose matrix is traversed by parallel thylakoid bands and loops and the chloroplast-nucleus-microbody-mitochondrion (CMNM) complex. (e) Cell wall with mucilage (right) and portion of protoplast with nucleus, Golgi body, and mitochondrial profiles. (f) SAG 338-1. Overview of vegetative cell in cross-section showing pyrenoid, CMNM complex, and cell wall with mucilaginous envelope. (g) The CMNM complex in strain SAG 2100. ch, chloroplast; s, starch grains; p, pyrenoid; m, mitochondrion; mb, microbody; n, nucleus; nu, nucleolus; cw, cell wall; mu, mucilage envelope; g, Golgi body. Scale bar, 1 μm (b–e, g) or 2 μm (a, f).

is, in certain Trebouxiophyceae and Chlorophyceae, were relatively unrelated.

Phylogenetic analyses of the ITS-1 and ITS-2 rDNAs revealed five distinct clades (Fig. 7). The ''*Interfilum* clade'' was formed by the three sarcinoid isolates (SAG 2101, SAG 2102, SAG 2147), *G. terricola* SAG 2100, and strains representing *I. paradoxum* and *Interfilum* sp. The other clades were formed by strains assigned to *K. flaccidum*, *K. mucosum/crenulatum*, *K. bilatum/K. elegans*, and a larger clade comprising all other remaining species of *Klebsormidium*. Interestingly, in the unrooted phylogeny, *K. flaccidum* did not group with other *Klebsormidium* spp., but with the *Interfilum* clade. In *G. terricola* SAG 2100 and the sarcinoid isolate SAG 2101, intragenomic variation in the ITS-1 and ITS-2 rDNA regions was detected.

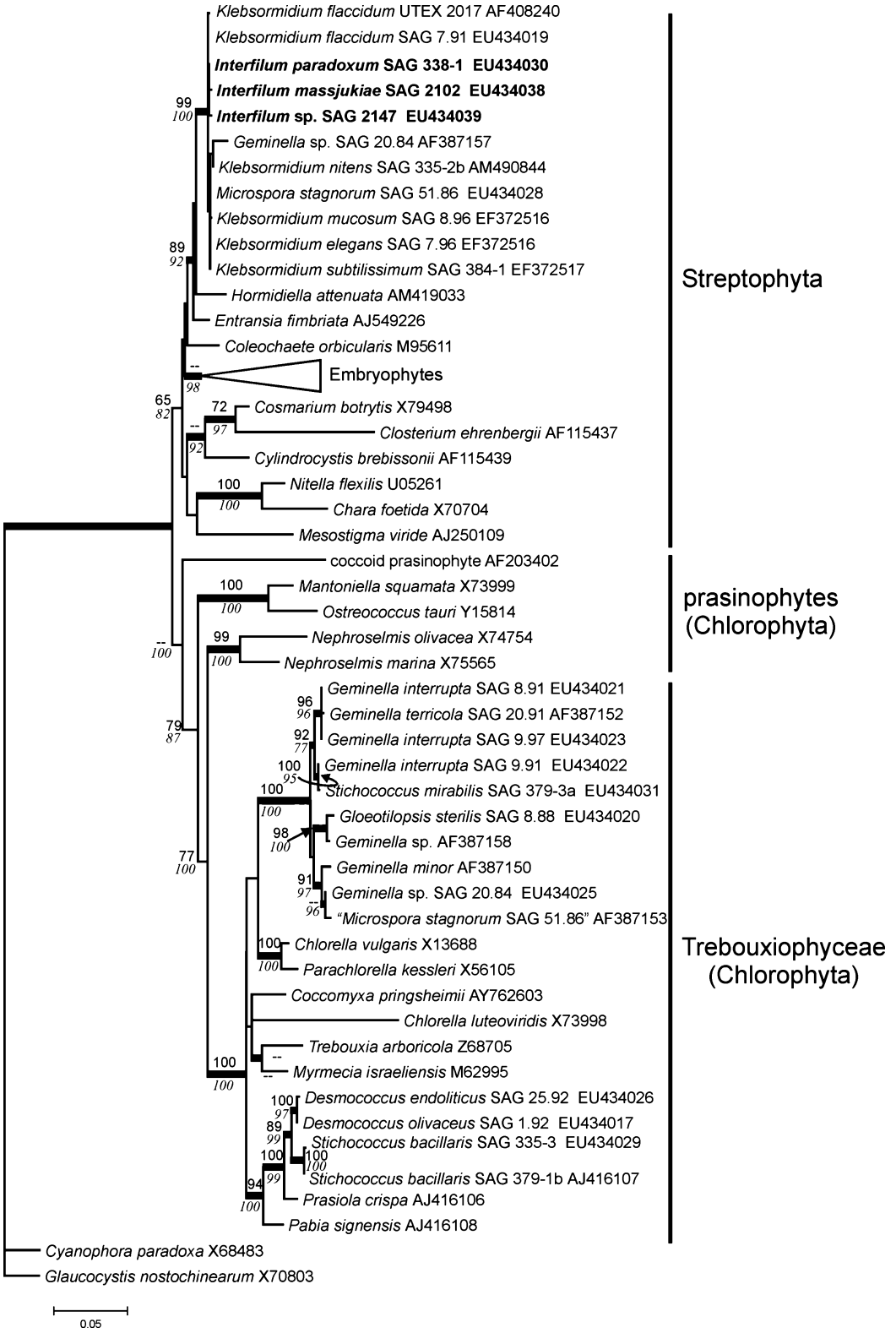

FIG. 6. Maximum-likelihood phylogeny of 18S rDNA sequences of species of *Interfilum* (shown in bold), *Klebsormidium,* other representatives of streptophyte green algae, embryophytes, *Geminella, Desmococcus,* other members of the Trebouxiophyceae, and some prasinophyte green algae. Thick lines mark internal branches that were supported by Bayesian analysis with posterior probabilities >0.95. Numbers above and below (italicized) branches represent bootstrap support values (if >70%) from neighbor-joining distance and maximum-parsimony analyses, respectively. Sequences representing the embryophytes included *Cucurbita pepo* AF206895, *Cyperus albostriatus* AF168838, *Ginkgo biloba* D16448, *Haplomitrium hookeri* U18504, *Pinus wallichiana* X75080, *Sphagnum palustre* Y11370, and *Welwitschia mirabilis* D85299. The tree is rooted with the glaucophytes *C. paradoxa* and *G. nostochinearum.*

As we failed to obtain unambiguous sequences by direct sequencing, we cloned the ITS-1, 5.8S, and ITS-2 region PCR products of both strains. For the sarcinoid strain SAG 2101, three types of sequences (ITS variants, named K366, K370, and K371) were detected, which exhibited differences at only three positions. However, in *G. terricola* SAG 2100, the two detected, ITS variants (named K644 and K650) differed considerably, that is, by 15 sequence positions. The ITS-1, 5.8S, and ITS-2 region sequences among the sarcinoid isolate SAG 2101, *G. terricola* SAG 2100 K650, and the two *I. paradoxum* strains had no more than six positions that were different. Consequently, in the phylogenetic analyses, these sequences formed a distinct subclade within the *Interfilum* clade, which, however, received only moderate bootstrap support, but which was distant from the other sequences of the *Interfilum* clade in all analyses. ITS variant K644 of *G. terricola* SAG 2100 branched off as next sister to that clade, followed by the sarcinoid strains SAG 2102, *I.* sp. SAG 36.88, and SAG 2147. The branching order of these three strains was the same in all different analyses, although it was not supported by boostrap tests and in Bayesian analyses; only the ME distance analysis showed strain SAG 2102 instead of *G. terricola* SAG 2100 K644 as the next sister with the *Interfilum* subclade. The basal position in the *Interfilum* clade of strain SAG 2147 was well supported in all analyses. The *Klebsormidium* sequences formed two separate clades, that is, one by the four *K. flaccidum* sequences with only little within-group variation, and a well-supported clade comprising the remaining *Klebsormidium* strains. Within the latter, three well-supported subclades were resolved: a clade formed by *K. crenulatum* and *K. mucosum;* another by the species pair *K. bilatum-K. elegans*; and an unresolved group comprising six sequences representing *K. dissectum, K. fluitans, K. nitens, K. subtilissimum,* and one unidentified isolate. The topology of the *Klebsormidium* part of the ITS phylogeny was essentially the same as reported by Sluiman et al. (2008). The absolute genetic distances among sequences of the *Klebsormidium* spp. clade ranged from 1 to 44; those between the four *K. flaccidum* strains, from 1 to 7; and the range of differences observed within the *Interfilum* clade, from 1 to 20.

DISCUSSION

This study has provided microscopical and molecular evidence that the three new sarcinoid isolates, the weakly filamentous *G. terricola* SAG 2100, as well as culture strains assigned to the genus *Interfilum* are all closely related and are members of the streptophycean lineage in the green plants, with a clear affinity to the Klebsormidiales. This finding is in concordance with characteristic ultrastructural features that the four strains that have been studied at the TEM level so far (SAG 2100, SAG 2101, SAG 2102, and SAG 338-1) share with various streptophycean algae, namely, the presence of a large single microbody in vegetative cells typically located between the nucleus and the chloroplast, and a branched mitochondrion closely associated with the chloroplast and the microbody. This chloroplast-mitochondrion-nucleus-microbody (CMNM) complex is characteristic of vegetative cells of various streptophycean algae (Kostikov et al. 2001, Massalski 2002, Massalski and Kostikov 2005), including *Klebsormidium* (e.g., Stewart et al. 1972, Silverberg 1975, Lokhorst and Starr 1985, Morison and Sheath 1985), Mesostigmatophyceae (Melkonian 1989), Chlorokybophyceae (Rogers et al. 1980), Coleochaetophyceae (Stewart et al. 1972, Sluiman 1985, Van den Hoek et al. 1995), Charophyceae (Silverberg and Sawa 1973), Zygnemaphyceae (Tourte 1972), and embryophytes (e.g., Raven et al. 2005). Thus, the CMNM complex appears to be a reliable character at the cellular level to identify streptophyte green algae. In addition, there was also a complex of certain light microscopical features of the protoplasts seen in the new sarcinoid isolates, *G. terricola* SAG 2100, and the studied strains of *Interfilum* that are shared with *Klebsormidium* spp. (see below); its phylogenetic significance is supported by the ITS rDNA analyses that show a very close relationship with species of *Klebsormidium*.

Definition and phylogeny of the genus Interfilum. Isolates SAG 2101 and 2102 were initially identified as species of *Desmococcus* (Mikhailyuk et al. 2003), but the position of this genus (represented by strains SAG 1.92 and SAG 25.82 in our 18S rDNA analysis) is clearly in the Trebouxiophyceae. This necessitates the identification of a suitable genus in the Streptophyta to which isolates SAG 2101, SAG 2102, and SAG 2147 could be assigned. While the 18S rDNA analyses failed to provide the resolution necessary to clarify relationships with specific strains of *Interfilum*, analyses of the more variable ITS rDNA regions have revealed the relationships of the three sarcinoid isolates and *G. terricola* SAG 2100 with strains of *Interfilum*. The seven studied strains formed a well-supported clade that is considered to represent the genus *Interfilum*, albeit in a broader sense than originally described by Chodat and Topali (1922). The oldest validly described taxon and type species in this clade is *I. paradoxum*, which predates Petersen's *G. terricola* (Petersen 1932), represented here by strain SAG 2100, by 10 years. Our phylogenetic analyses of 18S rDNA sequences have established that the genus *Geminella* is not a taxon of the Streptophyta because strains assigned to the type species, *G. interrupta*, are clearly placed in the Trebouxiophyceae. Consequently, strain SAG 2100 cannot be accepted as a species of *Geminella*. Several morphological characters are shared between the newly isolated strains and the type of *Interfilum, I. paradoxum*, namely, an almost identical chloroplast and pyrenoid structure, and a peculiar type of

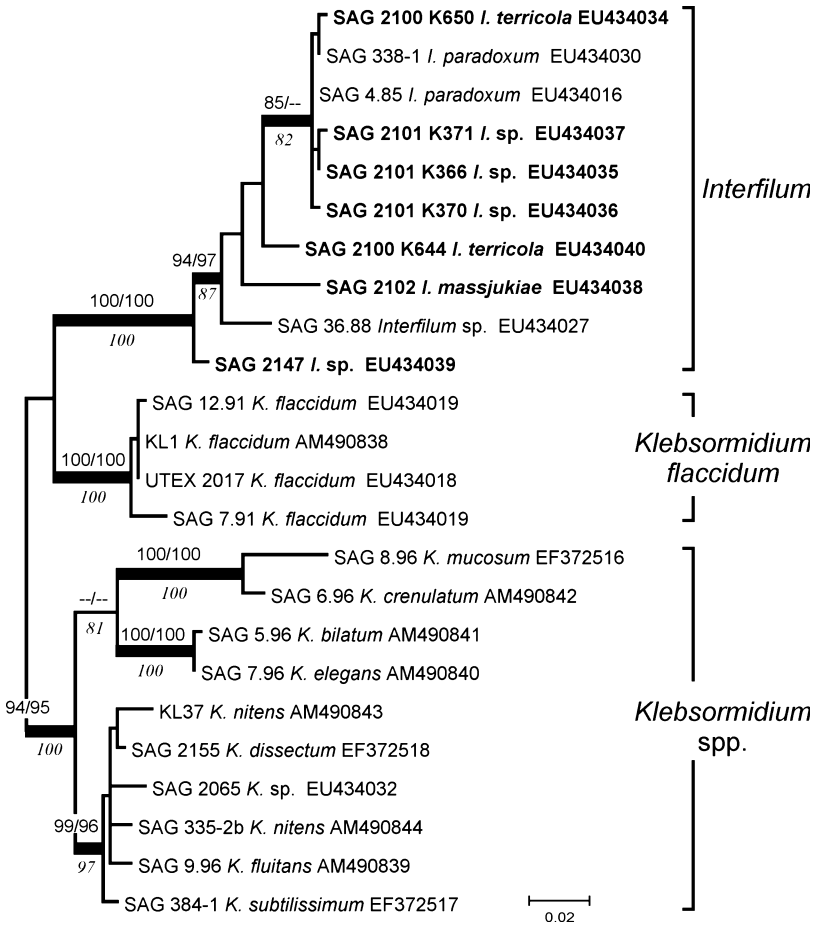

FIG. 7. Unrooted maximum-likelihood phylogeny of ITS-1,2 rDNA sequences of strains and species of *Interfilum* and *Klebsormidium*. The three studied sarcinoid isolates, *I. terricola* comb. nov., and *I. massjukiae* sp. nov. are shown in bold. Thick lines mark internal branches that were supported by Bayesian analysis with posterior probabilities >0.95. Numbers above lines are bootstrap values from neighbor-joining (left) and minimum-evolution (right) distance analyses; numbers below lines (italicized) are from maximum-parsimony analyses. Only bootstrap values >70% have been recorded.

formation of daughter cells within the mother cell wall that typically results in cap- or ring-like structures (Figs. 2, c–e; 3, c–f; 4, a, d–f). In addition, the general ultrastructural features of all these strains are very similar. We therefore assign the three sarcinoid isolates, SAG 2101, SAG 2102, and SAG 2147, as well as the weakly filamentous SAG 2100 to the genus *Interfilum* notwithstanding the fact that there are certain morphological differences with the type species *I. paradoxum*, namely, the sarcinoid growth habit and the absence of exfoliated mother cell walls. The monotypic genus *Interfilum* as described by Chodat and Topali (1922) includes usually unbranched or slightly branched uniseriate filamentous green algae composed of chains of nearly spherical cells that have bipartite cell walls, are connected by thin threads, and are surrounded by a mucilaginous sheath that displays radial striations upon staining with methylene blue. The validity of Chodat and Topali's description of *I. paradoxum* was confirmed in a later study by Fritsch and John (1942) who found it in liquid cultures derived from soil, and by our observations on strains SAG 338-1 and SAG 4.85. In the latter strains, it was observed that the remains of mother cell walls, which initially formed hemispherical ''caps'' closely associated with daughter cells, became gradually detached and sometimes formed a short, thin structure that connected the daughter cells for some time. There is little doubt that these structures are the same as those described as ''threads'' by previous authors. Fritsch and John (1942) described the interconnecting threads as mucilaginous, but it seems likely that mother cell wall material also contributes to the formation of these structures. Contrary to the previous reports, we only observed unbranched chains of cells. We obtained clear evidence of the bipartite nature of the cell walls, as shown in Chodat and Topali's figure 2(B).4, and Fritsch and John's figure 4, F–J. However, this is different from ''true'' bipartite cell walls in algae where the cell wall consists of two halves with one half formed by the mother cell and the other by the daughter cell. In *Interfilum*, the daughter cell walls are formed within

and closely associated with the mother cell wall. After cell division and upon daughter cell release, the mother cell wall splits in two halves; thereby each daughter cell receives one half of the mother cell wall, which results in cap- or ring-like structures on the daughter cells. We suggest naming this type of cell wall ''pseudobipartite.'' On the basis of the information currently available, we propose an emended diagnosis of the genus *Interfilum*.

Interfilum Chodat and Topali (1922) emend. Mikhailyuk, Sluiman, A. Massalski, Mudimu, Demchenko, T. Friedl et, S. Y. Kondr.

Thalli cellulis unis, cellulis paribus, cellularum laxis seriebus vel sarcinoidalibus aggregationibus compositi. Cellulae ellipsoideae, oviformes, hemisphaericae vel sphaericae. Parietes cellulares crassae, cum vagina mucilaginosa in speciebus nonnullis. Cellulae filis subtilibus, desquamatione cellulae matris parietis orientibus, connexae. Chloroplastus unicus, parietalis, laminaris, lateralibus incisuris lobatus, dimidium cellulae complens. Pyrenoides unicum, ellipticum, in chloroplasti centro locatum, parvis amylaceis granis, vulgo parallelis, circumcinctum. Nucleus unicus, centralis, pyrenoidi oppositus. Cellulae filiae in cellulae matris pariete productae. Paries divisione cellulari in dimidiis fracta; cellulae filiae ambae dimidia parietis, pileata vel annulata, accipientes. Fragmentatio sola reproductio cognita.

Type species: *I. paradoxum* Chodat and Topali (1922), p. 66, Figs. 1A, 2B.

Epitype: The strain SAG 338-1 permanently preserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in the Sammlung für Algenkulturen der Universität Göttingen (SAG), Göttingen, Nikolausberger Weg 18, Germany.

Taxonomic assessment of G. terricola. The morphological features of strain SAG 2100 are in full agreement with Petersen's diagnosis of *G. terricola* (Petersen 1932), and hence there is little doubt that this strain represents this species. Petersen (1932) commented that *G. terricola* is morphologically similar to other *Geminella* species. Their common characters include the formation of uniseriate unbranched filaments surrounded by a common mucilage envelope and a plate-shaped chloroplast with one pyrenoid. Three years later, however, Petersen (1935) stated (without presenting supporting reasons) that *G. terricola* might actually represent a form of *Klebsormidium (''Hormidium'') flaccidum.* Although we do not know what led to this change of mind, it is interesting that in retrospect Petersen's second thoughts were justified. Additional features of *G. terricola* observed here were the structure of starch envelope of the pyrenoid, the chloroplast morphology, and the pseudobipartite nature of the cell wall. Similar cap-like structures were recently found in a strain assigned to *G. terricola* that was isolated from Belgian forest soil (Hoffmann et al. 2007) and are a feature that unites this species with *I. paradoxum*. The close affiliation of *G. terricola* SAG 2100 and *I. paradoxum* as members of the Streptophyta is further supported by cytological and rDNA sequence data (this study). In contrast, the type species of the genus *Geminella, G. interrupta,* represented in our analyses by strains SAG 8.91, SAG 9.91, and SAG 9.97, forms an independent lineage within the Trebouxiophyceae, together with a number of other *Geminella* strains, including SAG 20.91, which previously was assigned to *G. terricola* (Fig. 6). The latter strain, however, does not exhibit the species characteristic features that are displayed by strain SAG 2100; hence, it is concluded that the correct identity of SAG 20.91 is not *G. terricola*. Also, in the studied strains assigned to *G. interrupta* as well as in SAG 20.91, no cap- or ring-like structures are seen.

It is proposed that *G. terricola* is transferred to the genus *Interfilum* as *I. terricola* comb. nov.

Interfilum terricola (B. Petersen) Mikhailyuk, Sluiman, A. Massalski, Mudimu, Demchenko, T. Friedl et, S. Y. Kondr.

Basionym: *G. terricola* Petersen (1932), p. 39, Fig. 21.

Epitype: The strain SAG 2100 permanently preserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in SAG, Göttingen, Nikolausberger Weg 18, Germany.

An attempt to resolve the affinities of *I. terricola* with species *I. paradoxum* by comparing ITS-1,2 rDNA sequences proved inconclusive due to intragenomic polymorphisms. Two ITS rDNA variants, K650 and K644, were detected in the same strain SAG 2100, and the differences between the two variants resulted in different positions in the ITS phylogeny. The sequence of variant K650 and those of *I. paradoxum* SAG 338-1 and SAG 4.85 were virtually indistinguishable, while variant K644 of the same strain was clearly very distinct (Fig. 7). The level of intragenomic rDNA variation within a species is normally low, due to a homogenization process known as concerted evolution (Elder and Turner 1995). The efficiency of concerted evolution is influenced by the degree to which sexual reproduction occurs. Sexual processes have not been observed in any member of the Klebsormidiales, which therefore are assumed to propagate and multiply solely by asexual (vegetative) means. The high intragenomic variation demonstrated for *I. terricola* strain SAG 2100 may be attributed to the absence of sexual recombination, possibly in combination with a low growth rate. Despite the virtual identity of one variant of *I. terricola* SAG 2100 with strains of *I. paradoxum,* there is a morphological character that distinguishes both species, namely, the absence in *I. terricola* of any signs of exfoliated cell walls in the vegetative cells, thread-like connections (anastomoses) between cells in short chains, and discarded cell wall halves. We therefore regard *I. terricola* SAG 2100 as representing a species clearly distinct from *I. paradoxum*.

Taxonomy of sarcinoid strains of Interfilum. Strains SAG 2102 and SAG 2147 share the ability to form sarcinoid cell packages with strain SAG 2101, but it is less pronounced in the latter. The ability to form sarcinoid stages seems to be correlated with the absence of mucilage that envelopes cell walls: in strain SAG 2101 as well as *I. paradoxum* and *I. terricola*, mucilage is present around the cell walls, and cell packages are absent or poorly developed. Conversely, mucilage is lacking in SAG 2102 and SAG 2147, strains that form prominent sarcinoid stages. Strain SAG 2102 is characterized by the formation of large cubic packages, a phenomenon that is far more pronounced than in SAG 2101. This finding is congruent with strain SAG 2102 being phylogenetically separated from *I. paradoxum*, *I. terricola,* and strain SAG 2147 by the ITS rDNA sequence analyses and 2–5 sequence positions in the 18S rDNA. As this prominent sarcinoid growth habit is a feature that uniquely distinguishes SAG 2102 from *I. paradoxum* and *I. terricola,* it is proposed to designate this strain to a new species of *Interfilum, I. massjukiae.*

Interfilum massjukiae Mikhailyuk, Sluiman, A. Massalski, Mudimu, Demchenko, T. Friedl et, S. Y. Kondr.

Cellulae latiellipsoideae, rotundae vel hemisphaericae, (6.0) 8.5–9.8 (11.9) μm longae, (5.1) 6.8–8.1 (9.8) μm latae; cellulae apicales in filamentis ramosis 10.2–14.5 μm longae. Cellulae binae, trinae quaternaeque aggregatae. Thalli latis cubicis multicellularibus aggregationibus, filamentis uniseriatis vel filamenti biseriatis compositi. Paries cellularis crassa, sine vagina mucilaginosa. Chloroplastus unicus, parietalis, laminaris, in latere parietis locatus, cum 5–8 lobis. Pyrenoides unicum, in chloroplasti centro locatum, 8–10 parvis elongatis amylaceis granis, ad longitudinem cellulae parallelis, circumcinctum. Nucleus unicus, pyrenoidi oppositus. Cellularum divisio in directiones tres. Cellulae filiae in cellula matre orientes, ambae dimidia parietis accipientes; dimidia parietis pileata residua producentia. Reproduction thallorum fragmentatione; reproductio sexualis incognita.

Cells widely ellipsoid to rounded or hemispherical, length (6.4)–6.8–10.7 μm, width (5.1)–6.4–8.6 μm, terminal cells in branched filaments up to 10.2–14.5 μm long; cells aggregated in groups of 2–4, sometimes forming large multicellular cubic aggregations as well as uni- and biseriate branched filaments. Cell walls thick, without mucilage. Chloroplast single, parietal, plate-shaped, positioned at one side of the cell wall, dissected in 5–8 lobes. Pyrenoid single, located in the center of the chloroplast, surrounded by 8–10 or more small elongated starch grains orientated parallel to the longitudinal axis of the cell. Nucleus single, situated opposite the pyrenoid. Cell division in three planes, resulting in daughter cells being formed within the mother cell wall and each receiving one half of the mother cell wall, which remains closely associated with the daughter cell wall (resulting in cap-like remains of mother cell wall). Reproduction by fragmentation of thalli; sexual reproduction not observed.

Holotype: A fixed sample of strain SAG 2102 has been deposited at the Herbarium of University of Göttingen (GOET) as No. SAG 2102 (GOET) and is designated as type material. Figures 1, a–h, illustrate this material.

Epitype: Strain SAG 2102 permanently preserved in a metabolic inactive state (cryopreserved in liquid nitrogen) in SAG, Nikolausberger Weg 18, Germany.

Iconotype: Figures 1, d, f–h.

Type locality: Cracks and surface of pyroclastic outcrops, mountain range ''Karagach,'' Karadag Nature Reserve (Crimea, Ukraine).

Etymology: The species is named after renowned Ukrainian phycologist Prof. Dr. Sc. Nadiya Prokhorivna Massjuk in recognition of her contributions to the taxonomy of various algal groups, as well as the education of a generation of students in Ukrainian cryptogamic plants.

Distribution: Presently known only from the type locality.

I. massjukiae SAG 2102 differs morphologically from SAG 2101 and SAG 2147 in the absence of a mucilaginous envelope and in a tendency to form biseriate branched filaments. Preliminary TEM studies have revealed differences in the structure of the cell walls; that is, in strain SAG 2102 a sublayer with a lacunar or alveolate substructure that may support the formation of sarcinoid cell packages is present, whereas SAG 2101 (with a reduced ability to form prominent cell packages) has a homogenous cell wall structure, like *I. paradoxum* and *I. terricola.* It is expected that a more detailed morphological and ultrastructural study that also should include strains SAG 2147 and SAG 36.88 will reveal additional diagnostic features of the three sarcinoid isolates. Strains SAG 2147 and SAG 36.88 may represent separate new species of *Interfilum* distinct from *I. massjukiae* and *I. terricola/I. paradoxum.* Preliminary investigations have shown that SAG 36.88 stands apart by its elongated and almost cylindric cells, differences in chloroplast structure, and lack of sarcinoid stages.

Cell division in Interfilum. A common feature of all species of *Interfilum* is that the daughter cell walls are formed within the mother cell wall followed by the formation of a cap- or ring-like structure associated with the daughter cells. This feature is reminiscent of a type of cell division known as sporulation (as variously defined, e.g., by Sluiman et al. 1989, Massjuk 1993, 1997, Massjuk and Demchenko 2001, Yamamoto et al. 2007), although there may be differences regarding the function and structure of the division products, and sequence of division stages (Ettl 1988a,b, Massjuk 1993). This type of cell division in *Interfilum* may be seen as transitional between typical ''sporulation'' and ''vegetative cell

division'' (in the sense of Sluiman et al. 1989 and Massjuk 1993). The term ''desmoschisis'' as proposed by Massjuk (1993, 1997) may not be appropriate because ''desmoschisis'' is also used to describe a process known as ''vegetative cell division'' (*Zellteilung* = *Cytotomie* = desmoschisis, Ettl 1988a,b, Sluiman et al. 1989). When daughter cells form within a persisting mother cell, their growth requires the mother cell walls to be at least partially eliminated or to be transformed in a similar way as during sporulation. In green algae this transformation can be achieved in different ways: gelatinization of walls, which gives rise to the formation of mucilage envelopes, or widening and retention of the mother cell wall, which, after successive division cycles, leads to thick multiple-layered cell walls (Starmach 1972, Hindák 1996). In *Interfilum* two ways of transformation of mother cell walls appear to be realized (Fig. 8: 1): gelatinization and rupture (Fig. 8a) or preservation and rupture (Fig. 8b). The first process leads to short filaments and easily disintegrating packets, and single cells, and is exemplified by *I. paradoxum, I. terricola,* as well as strains SAG 2101 and SAG 36.88. These have a mucilage envelope, and their cell walls appear homogenous at the ultrastructural level. The second process (Fig. 8b) leads to packets, that is, a tight cubic aggregation of closely adhering cells, and to biseriate filaments, and is exemplified by *I. massjukiae* and strain SAG 2147. There is no mucilage envelope and, in *I. massjukiae*, the ultrastructure of the cell wall reveals a layered-lacunar texture. The latter may have originated from many generations of mother cell walls that were compressed during successive cell division cycles, as also visible on TEM micrographs of the related genus *Klebsormidium* (Lokhorst 1996, figs. 251–253). *Interfilum* shares with *Klebsormidium* a tendency that cell division can take place in three planes, leading to the development of sarcinoid cell packages. Biseriate filaments or sarcinoid packages have been observed in *K. montanum*, *K. crenulatum,* and *K. mucosum* (Ettl and Gärtner 1995, Lokhorst 1996, T. I. Mikhailyuk unpublished). Our interpretation is that the type of cell division in these cases is fundamentally similar between both genera, namely, protoplast division and daughter cell wall formation taking place within the mother cell wall (Fig. 8: 2). As shown for *Interfilum* here, in *Klebsormidium* the nonelastic mother cell wall ruptures in the central region during the process of growth of daughter cells, but it remains closely associated with the daughter cell walls (Fig. 8: 2). This may lead to either a layered-lacunar dense cell wall consisting of many generations of mother cell walls (visible at the ultrastructural level; Lokhorst 1996) or one resembling the pseudobipartite cell walls that are formed in *Interfilum*. Rupture of the mother cell wall leads to H-pieces, which often can be observed at the ends of filaments in many species of *Klebsormidium* (Fig. 8c). Similar H-pieces are also known for other

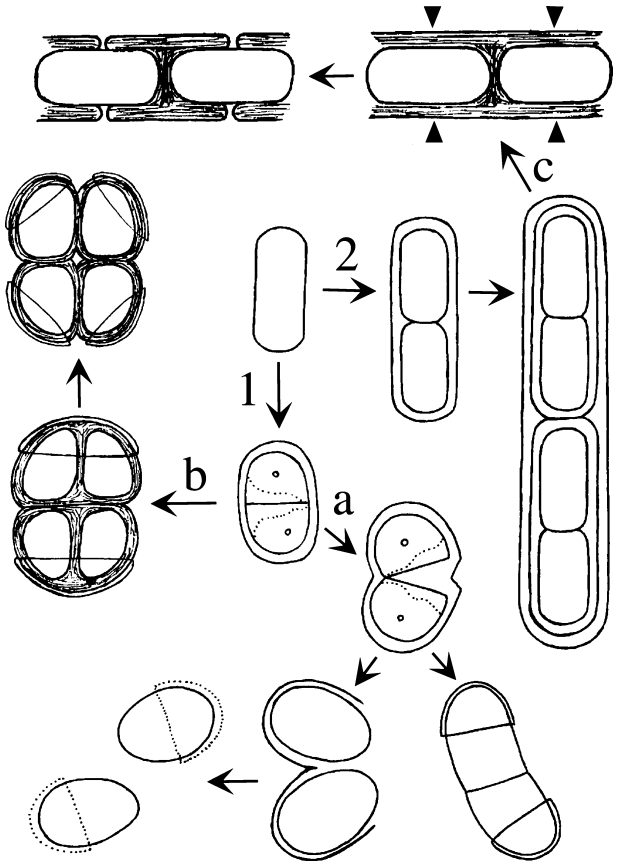

FIG. 8. Diagrammatic summary of the types of cell division characteristic for *Interfilum* and *Klebsormidium* and their distinct developmental pathways. (1) Formation of short disintegrated filaments and cell packet formation (*Interfilum*). (2) Formation of long filaments (*Klebsormidium*). (a) Rupture and later gelatinization of mother cell walls while remaining closely associated with daughter cells. (b) Rupture and preservation of the mother cell wall while remaining closely associated with daughter cells. (c) Close association of preserved mother cell walls with daughter cells followed by their rupture at the equatorial plane of the daughter cells resulting in H-shaped wall pieces. Arrowheads mark sites of rupture.

representatives of streptophycean algae, namely, *Entransia* (Cook 2004) and the sarcinoid streptophyte alga *Chlorokybus atmophyticus* (Rieth 1972).

Relationship of Interfilum *and* Klebsormidium. Certain morphological characters that are shared among the various strains of *Interfilum* appear to be characteristic of members of the Klebsormidiophyceae and even other streptophycean algae. These characters include a plate-shaped chloroplast occupying half of the cell volume, a starch envelope surrounding the pyrenoid consisting of numerous single grains arranged almost in parallel, and a position of the nucleus opposite to pyrenoid. The same morphological features apply to ''*Microspora stagnorum*'' strain SAG 51.86 (T. I. Mikhailyuk, unpublished observation), which corroborates its placement in the *Interfilum/Klebsormidium* clade in the 18S rDNA phylogeny (Fig. 6). Therefore, it is concluded that

strain SAG 51.86 has been wrongly identified as *"Microspora stagnorum"*; it actually represents a species of *Klebsormidium*. Interestingly, it represents a species isolated from freshwater. We anticipate further ITS rDNA sequence analyses to reveal its phylogenetic position relative to aeroterrestrial members of the genus.

In the ITS rDNA phylogenies, the four sarcinoid isolates, *I. terricola* SAG 2100, and *I. paradoxum* appeared to be more closely related with *K. flaccidum* than with any other species of *Klebsormidium* (Fig. 7). In an attempt to further clarify the relationship between *Interfilum* and *Klebsormidium*, and in particular to determine whether the two genera are monophyletic or paraphyletic, we included *Entransia fimbriata* and *Hormidiella attenuata* in the analyses. A previous study (Sluiman et al. 2008) has shown that these taxa are the closest known relatives of *Klebsormidium*, and that, by virtue of a certain degree of similarity between their ITS rDNA sequences, they can serve as outgroups to resolve the phylogeny of *Klebsormidium*. The ITS rDNAs were aligned according to the alignment of Sluiman et al. (2008), which only included putative preserved secondary structure elements, resulting in a data set of 272 aligned bases. Phylogenetic analyses recovered a tree topology (not shown), which, when rooted with *E. fimbriata* and *H. attenuata* as outgroups, showed again the sister group relationship of *K. flaccidum* and *Interfilum*, and the distribution of *Klebsormidium* species over the same four clades as reported by Sluiman et al. (2008) and shown in Figure 7, albeit with low support for internal branches. A number of alternative topologies were tested with the Paired Sites test suite as implemented in the program Treefinder (Jobb 2008) (results not shown). These included trees in which (a) *Interfilum* was sister to all *Klebsormidium* sequences (nonrejection of which would support the monophyletic status of each genus), (b) *Interfilum* was sister to *K. flaccidum* only (nonrejection of which would indicate that *Klebsormidium* is paraphyletic), and (c) some changes in the branching order within the *Klebsormidium* clade. None of these alternative trees was rejected by these tests, which leads to the conclusion that the currently available ITS rDNA data set is not able to resolve unequivocally whether *Klebsormidium* is paraphyletic and *Interfilum* is monophyletic, or whether both genera represent two closely related but distinct monophyletic entities. The taxonomic and phylogenetic relationships of these genera and of Klebsormidiophyceae in general may become clearer when a broader range of samples, both geographically and taxonomically, and information from other genetic markers will become available for analysis.

Our sincere thanks to Dr. Thomas Pröschold for discussion on taxonomical aspects and Dr. Fabio Rindi for provision of the Latin diagnoses. We thank Elke Zufall-Roth for her skillful sequencing work as well as for purification and maintenance of cultures, Dr. K. Mohr for assistance with preparing the sequence submissions, Professor Igor Kostikov for providing an isolate of *Interfilum terricola* (as "*Geminella terricola*"), Dr. Pavel Škaloud for providing an isolate of *Interfilum* sp., and Dr. Jiři Neustupa for helpful discussions. Parts of this work were supported by the German Science Foundation (DFG) by a grant extended to T. F. (Fr 905/14-1,2) and by the German Federal Ministry of Education and Research, BMBF (AlgaTerra project, grant 01 LC 0026) within the BIOLOG program. T. M. gratefully acknowledges the support through a Grant for Young Scientist Fellowship extended to her by INTAS (Ref. No. 06-1000014-6216).

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Supplementary Material

The following supplementary material is available for this article:

Table S1. The strains and species of *Interfilum* used in this study, their origin and GenBank sequence accession numbers.

Table S2. rDNA sequences and their corresponding GenBank accession numbers for strains and species of *Klebsormidium*, *Geminella, Desmococcous,* and additional green algae (Trebouxiophyceae) that have been determined in this study.

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