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# Phylogenetic relationships in *Interfilum* and *Klebsormidium* (Klebsormidiophyceae, Streptophyta)

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ABSTRACT

Members of the *genus Klebsormidium* have cosmopolitan distribution and occur in a very wide range of freshwater and terrestrial habitats. Due to its simple filamentous morphology, this genus represents a taxonomically and systematically complex taxon in which phylogenetic relationships are still poorly understood. The phylogeny of *Klebsormidium* and closely related taxa was investigated using new ITS rRNA and *rbc*L sequences generated from 75 strains (isolated from field samples or obtained from culture collections). These sequences were analyzed both as single-marker datasets and in a concatenated dataset. Seven main superclades were observed in the analyses, which included sixteen well-supported clades. Some species of *Klebsormidium*, including the type species *Klebsormidium flaccidum*, were polyphyletic. *Interfilum* was recovered with high statistical support as sister taxon to a clade of *Klebsormidium* formed mainly by strains identified as *K. flaccidum*. Whereas some clades could be easily associated with described species, this was not possible for other clades. A new lineage of *Klebsormidium*, isolated from arid soils in southern Africa and comprising undescribed species, was discovered. Several morphological characters traditionally used for taxonomic purposes were found to have no phylogenetic significance and in some cases showed intra-clade variation. The capacity to form packet-like aggregates (typical of *Interfilum*), features of the morphology of the chloroplast and the type of habitat were the main phylogenetically relevant characters. Overall, *Klebsormidium* and *Interfilum* formed a more diverse algal group than was previously appreciated, with some lineages apparently undergoing active evolutionary radiation; in these lineages the genetic variation observed did not match the morphological and ecological diversity.



## 1. Introduction

Green algae with a thallus formed by uniseriate filaments are among the most widespread and ecologically versatile photosynthetic eukaryotes. Organisms with this type of morphology occur in almost all aquatic and terrestrial environments and belong to five major green algal lineages (Chlorophyceae, Klebsormidiophyceae, Trebouxiophyceae, Ulvophyceae and Zygnemophyceae; Lewis and McCourt, 2004).

*Klebsormidium* P.C. Silva, Mattox et Blackwell is a cosmopolitan genus of filamentous green algae to which 20 species are currently ascribed (Guiry and Guiry, 2010). Species of *Klebsormidium* are common in the microalgal vegetation of many terrestrial and freshwater environments, occurring in habitats as diverse as streams and rivers (Printz, 1964; John, 2002), margin of lakes (Lokhorst, 1996), bogs (John, 2002), soil (Ettl and Gärtner, 1995), natural rocks in plains and mountainous areas (Mikhailyuk et al., 2003), tree bark (Handa et al., 1991), acidic post-mining sites and water bodies (Lukešová, 2001; Sabater et al., 2003), golf courses (Baldwin and Whitton, 1992), sand dunes (Smith et al., 2004), biotic crusts of hot deserts (Lewis, 2007), bases of urban walls (Rindi and Guiry, 2004) and building façades (Barberousse et al., 2006). Species of *Klebsormidium* consist of uniseriate filaments devoid of differentiated or specialized cells. Each cell has a parietal chloroplast containing a pyrenoid and encircling half to 3/4 of the cell wall; reproduction takes place by biflagellate asymmetrical zoospores devoid of stigma that are produced singly in unspecialized cells, from which they escape through a pore (Silva et al., 1972; Lokhorst, 1991; Sluiman et al., 2008). The distinctness of *Klebsormidium* from similar genera of green algae was highlighted by TEM observations of cell division and zoospore ultrastructure

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(Floyd et al., 1972; Marchant et al., 1973; Lokhorst and Star, 1985). Stewart and Mattox (1975) established the placement of *Klebsormidium* in the Charophyceae, noting the presence of ultrastructural characters typical of this class (a persistent interzonal mitotic spindle, two laterally inserted flagella, a single broad band of microtubules associated with the flagellar basal bodies). These authors also based on *Klebsormidium* the new family Klebsormidiaceae and the new order Klebsormidiales, using characters specific to this taxon (centripetal cleavage furrow, little change in chromosome to spindle pole distance during anaphase, persistent chromosomal microtubules at telophase, and absence of plasmodesmata). The circumscription of the Klebsormidiales has changed over time as new data became available (Lokhorst, 1991; Van den Hoek et al., 1995; Sluiman et al., 2008) and is not yet fully clarified; it has been established that species of two other filamentous genera, *Entransia* Hughes and *Hormidiella* Iyengar et Khantamma, are the closest known relatives of *Klebsormidium* (Karol et al., 2001; Sluiman et al., 2008). Molecular data have confirmed conclusions based on ultrastructure and have robustly established the position of *Klebsormidium* and related taxa in the streptophytan lineage (Kranz et al., 1995; Karol et al., 2001; Turmel et al., 2002; Qiu et al., 2006). In more recent treatments this group has been regarded as an independent class, the Klebsormidiophyceae (Van den Hoek et al., 1995; Lewis and McCourt, 2004; Becker and Marin, 2009).

The reconstruction of the phylogenetic relationships in *Klebsormidium* and closely related taxa is problematic. Until recently the taxonomy of these algae was based on morphological data, which offer only a limited set of characters. Width and length of filaments, shape of cells, texture of the cell wall, presence/absence of a mucilaginous envelope, presence/absence of cell doublets or false branches, formation of H-shaped pieces, shape of the chloroplast and shape and size of the pyrenoid are the characters most commonly used for identification of field-collected specimens of *Klebsormidium* (Hazen, 1902; Printz, 1964; Ramanathan, 1964; Ettl and Gärtner, 1995; Lokhorst, 1996; Rifón-Lastra and Noguerol-Seoane, 2001; John, 2002). Some authors (Lokhorst, 1996) have attached more taxonomic significance to characters observable in liquid cultures, such as: presence/absence of a superficial hydrorepellent layer; tendency to fragment into short unattached filaments; type of reproduction (zoosporogenesis only or zoosporogenesis in combination with aplanosporogenesis); ease of inducibility of release of zoospores; shape of release aperture in lateral wall of zoosporangial cell (large and distinct or small and indistinct); and germination pattern of zoospores (unipolar and bipolar or unipolar only). It is known, however, that several characters exhibit great phenotypic plasticity and may vary dramatically in relation to environmental conditions and physiological state of the specimens examined (Lokhorst, 1996; Dřímalová and Poulíčková, 2003; Škaloud, 2006; Rindi et al., 2008). It is presently unclear how morphological characters have evolved and which characters must be considered phylogenetically significant. In this regard, unfortunately, our understanding of the evolutionary history of the group is too limited to be of any assistance. No fossil record is available for *Klebsormidium* (a situation that is general to the green algae, with the exception of a few groups provided with calcified cell walls, e.g. McCourt et al., 1996; Verbruggen et al., 2009). Therefore, it is unknown what was the morphology of the ancestral klebsormidialean alga and what character states should be considered ancestral.

Molecular studies focused on phylogeny at genus and species level have become available recently and have led to several unexpected discoveries (Novis, 2006; Mikhailyuk et al., 2008; Rindi et al., 2008; Sluiman et al., 2008). Sluiman et al. (2008) demonstrated that *Entransia fimbriata* Hughes and *Hormidiella attenuata* Lokhorst are the closest relatives to *Klebsormidium* currently known. The topology of the ITS rRNA tree presented by these authors is in disagreement with the morphological parsimony trees presented by Lokhorst (1996). Rindi et al. (2008), whose analyses included the largest taxon sampling of *Klebsormidium* strains currently available, found great difficulties in mapping morphological characters with phylogenetic significance on their *rbc*L trees. Recently, Mikhailyuk et al. (2008) showed that *Interfilum* Chodat et Topali is closely related to *Klebsormidium*, but they were not able to clarify the relationships between these genera. Species of *Interfilum* consist of unicells, packet-like aggregates or filaments of cells united by thin mucilaginous envelopes or strands of cell wall material (Chodat and Topali, 1922; John, 2002; Mikhailyuk et al., 2008). This finding revolutionised the concept of morphological evolution in *Klebsormidium* and close relatives, revealing that this group encompasses a wider range of morphological forms than previously appreciated. Overall, these studies indicate that our understanding of the genetic diversity and phylogeny of this algal group is still very incomplete and that new data are required.

In the present study, 29 new *rbc*L sequences and 66 new ITS rRNA sequences of *Interfilum* and *Klebsormidium* are added to the body of molecular data previously available. Sequences of these markers are also analyzed for the first time in a concatenated dataset. Our results extend considerably the taxon sampling and provide robust phylogenies that advance substantially our understanding of the relationships of these algae. These data will be of fundamental importance for future reassessments of the classification of the order Klebsormidiales.

## 2. Materials and methods

### 2.1. Collections, taxon sampling and vouchering

Samples of *Klebsormidium* and *Interfilum* were isolated by the authors or external collaborators or obtained from culture collections (Supplementary Table 1). For the new samples isolated in the course of this study, the following morphological characters were examined in freshly isolated cultures: habit of filaments; tendency to fragmentation; width and length of cells; shape of cells; formation of H-shaped pieces; thickness and texture of the cell wall; formation of a mucilaginous envelope; shape of the chloroplast; shape and size of pyrenoid; macroscopic habit on agar; macroscopic habit in liquid culture. For strains isolated in previous studies and included in the phylogenetic analyses, information on the morphology is available in Lokhorst (1996), Novis (2006), Mikhailyuk et al. (2008), Rindi et al. (2008).

### 2.2. Culture conditions and microscopic observations on new strains

Cultures of strains isolated in this study were maintained on agar slants with Bold' Basal Medium with vitamins and triple nitrate (Starr and Zeikus, 1987) at 18 °C under a 14:10 h L:D regime at a photon irradiance of about 25 μmol photons $m^{-2}$ $s^{-1}$ from white fluorescent bulbs. Microscopic observation was performed using an Olympus BX60 microscope (Olympus Europa Holding, Hamburg, Germany) with Nomarski DIC optics or with a Mikmed-2 bright field microscope on cultures not older than 5 weeks. Micrographs were taken with a ColorView III camera (Soft Imaging System GmbH, Münster, Germany) in connection with the Cell^D imaging software (Soft Imaging System GmbH, Münster, Germany).

### 2.3. DNA extraction, PCR and sequencing

DNA was extracted from strains of *Klebsormidium* and *Interfilum* as detailed in Supplementary Table 1. PCR and sequencing of the *rbc*L gene were performed at the University of Alabama following

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the methods of Rindi et al. (2008). PCR and sequencing of the ITS rRNA were performed at the Georg-August University Göttingen following the methods of Mikhailyuk et al. (2008).

### 2.4. Sequence alignment and phylogenetic analyses

Phylogenetic analyses were performed separately on the two markers sequenced and on a concatenated ITS rRNA-*rbc*L dataset. Besides the new sequences produced in this study, most of the *rbc*L and ITS sequences available in GenBank were included in the alignments and used for the analyses. The *rbc*L alignment was constructed by eye using MacClade 4.05 (Maddison and Maddison, 2002); it consisted of 1149 basepairs, corresponding to the positions 61-1209 of the complete sequence of *Chlorella vulgaris* AB001684 (Wakasugi et al., 1997). No indels were created in the alignment. For *rbc*L, several preliminary analyses were performed in order to establish the best rooting method, following the recommendations of Verbruggen and Theriot (2008). Overall, these analyses showed a limited effect of the choice of the outgroup, providing identical topologies and very similar support values in all cases (including midpoint rooted trees devoid of designated outgroups). We present here trees in which species of *Klebsormidium* and *Interfilum* were treated as ingroup taxa, rooted using as outgroups *Chlorokybus atmophyticus* AY823706, *E. fimbriata* AF203496 (sequence obtained from strain UTEX2353) and a newly produced sequence of *H. attenuata* CCAP329/1 (HQ613235). Based on the information currently available (Karol et al., 2001; Turmel et al., 2002; Sluiman et al., 2008) these taxa are the closest known relatives of *Klebsormidium*, and therefore represent the most logical choice as outgroups. The ITS alignment consisted of 517 basepairs of the ITS-1 and ITS-2 regions that could be aligned unambiguously. To guide the alignment of the sequences, we used the secondary structure models of Mikhailyuk et al. (2008) (which were in turn based on those of Sluiman et al., 2008) and the corresponding alignment used by these authors. The newly generated sequences for the present study did not necessitate modification of the secondary structure models developed by Mikhailyuk et al. (2008). For the ITS dataset, it was chosen to perform the analyses only on *Klebsormidium* and *Interfilum* using midpoint rooting. The reason was that, as noted previously by Sluiman et al. (2008), alignment of complete ITS-1 and ITS-2 regions of all klebsormidialean taxa including *E. fimbriata* UTEX2353 and *H. attenuata* CCAP329/1 proved to be problematic due to the high level of divergence of the unpaired inter-helix domains of these two species; it was therefore chosen not to use them. Despite different rooting methods, the analyses performed separately on the two markers showed a congruent phylogenetic signal and recovered equivalent topologies. In consideration of this, it was decided to join in a concatenated dataset 42 taxa for which both ITS and *rbc*L could be sequenced successfully. The concatenated dataset *rbc*L-ITS consisted of 1665 basepairs.

Phylogenetic inference was based on Neighbor Joining (NJ), Maximum-Likelihood (ML) and Bayesian (BI) analyses. NJ was performed using PAUP* 4.0b10 (Swofford, 1998). For the *rbc*L and ITS rRNA datasets, NJ analyses were based on Maximum-Likelihood corrected distances. The parameters used to set the distances were obtained with ModelTest 3.7 (Posada and Crandall, 1998) under the Akaike Information Criterion (AIC). For the combined *rbc*L-ITS dataset, the analysis was based on uncorrected *p*-distances. For *rbc*L and the combined *rbc*L-ITS dataset, the model-based analyses (ML and BI) were performed on partitioned datasets, applying separate models to each partition. Three partitions were used for *rbc*L (first, second and third codon position); four partitions were used for the combined dataset (the three codon positions of *rbc*L and the whole ITS). Recent studies in which partitioning strategies were selected after tests based on the Bayesian Information Criterion (Verbruggen et al., 2010) indicated that the partition of protein-coding organellar genes in first, second and third codon position is a recommendable strategy, and we therefore decided to adopt it for our *rbc*L dataset. ML was performed using Treefinder (version October 2008; Jobb, 2008). The models and parameters selected by Treefinder under the corrected Akaike Information Criterion (AICc) were applied in the analysis. For NJ and ML, nodal support was assessed by non-parametric bootstrap analysis (Felsenstein, 1985) with 1000 resamplings.

BI was performed using MrBayes 3.04 (Huelsenbeck and Ronquist, 2001). Two parallel runs of four Monte Carlo Markov Chains (one cold and three incrementally heated) were conducted for 2000,000 generations, with tree sampling every 100 generations. Priors and probability proposals set as default in MrBayes were used. The stationary distribution of the runs was verified before to stop the analysis; it was assumed that the stationary distribution was reached when the average standard deviations of split frequencies between the two runs was lower than 0.01. The burn-in phase was assessed by plotting the number of generations against the likelihood scores and determining where the analysis reached stationarity; 100,000–400,000 generations were discarded as burn-in, and the remaining samples were used to construct the majority-rule consensus trees. In the *rbc*L and *rbc*L-ITS analyses, separate models were applied to each partition by setting number of substitution types, gamma shape parameter and proportion of invariable sites in accordance with the results of ModelTest. The parameters were unlinked and allowed to vary across partitions.

## 3. Results

Details of the characteristics of the datasets analyzed are presented in Table 1. For each dataset analyzed, analyses performed with different methods of inference provided similar topologies, usually with limited differences in nodal support. The *rbc*L gene and the ITS rRNA showed congruent phylogenetic signals and identical groups were recovered in the two phylogenies. However, most internal nodes did not receive significant statistical support in any analysis for any dataset.

The results of the ITS and *rbc*L analyses are shown in Figs. 1 and 2, respectively; the results of the analyses on the concatenated dataset are shown in Fig. 3. Seven main superclades (indicated

**Table 1**
Summary of the characteristics of the datasets analyzed.

| | ITS rRNA | *rbc*L |
|---|---|---|
| Parsimony-informative characters | 114 (22%) | 296 (26%) |
| Parsimony-uninformative characters | 42 (8%) | 40 (3.5%) |
| Constant characters | 361 (70%) | 813 (71%) |
| Average uncorrected *p*-distance | 5% | 8.5% |
| Range of uncorrected *p*-distance | 0.2–10.9% | 0.2–16% |
| Taxa with maximal uncorrected *p*-distance | *Interfilum terricola* LUK305 *Klebsormidium* sp. 14613.5e | *Klebsormidium* cf. *flaccidum* Lira7 *Klebsormidium* sp. 14621.6 |

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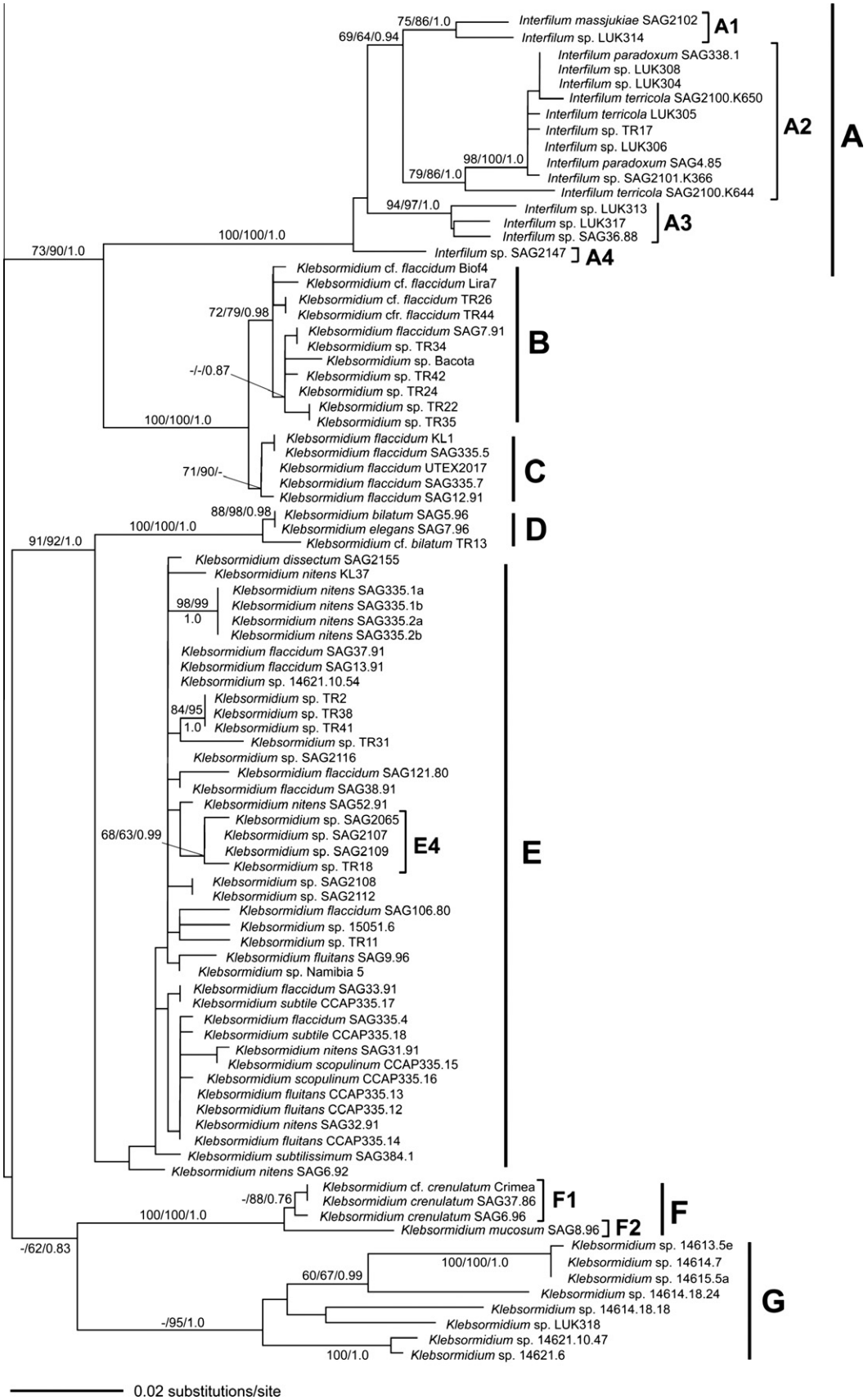


**Fig. 1.** Phylogram inferred from Maximum-Likelihood analysis of the ITS rRNA in the Klebsormidiales, with bootstrap support (BP) and Bayesian Posterior Probabilities (PP) indicated at the nodes. From left to right and from top to bottom the support values correspond to Neighbor Joining BP, Maximum-Likelihood BP and Bayesian PP. BP values lower than 60% and PP lower than 0.8 are not shown.

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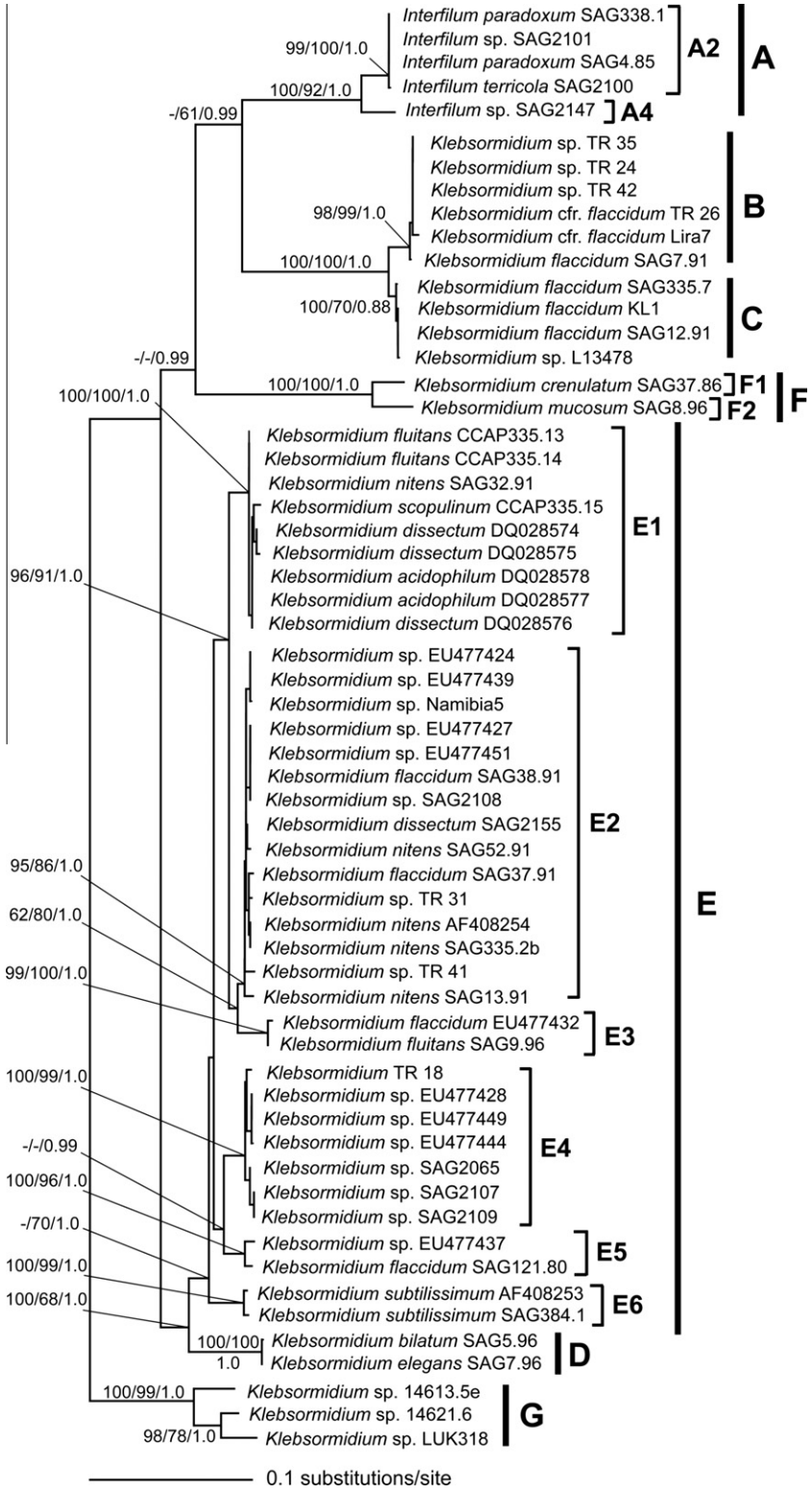


**Fig. 2.** Phylogram inferred from Maximum-Likelihood analysis of the *rbc*L gene in the Klebsormidiales, with bootstrap support (BP) and Bayesian Posterior Probabilities (PP) indicated at the nodes. From left to right and from top to bottom the support values correspond to Neighbor Joining BP, Maximum-Likelihood BP and Bayesian PP. BP values lower than 60% and PP lower than 0.8 are not reported. The tree was rooted using the sequences of *Hormidiella attenuata*, *Entransia fimbriata* and *Chlorokybus atmophyticus* specified in the Section 2.

with letters: A, B, C, D, E, F, G) were recovered in all phylogenies with moderate to high support. Additionally, in the ITS phylogeny four highly supported clades were identified within superclade A (indicated as A1, A2, A3 and A4 in Fig. 1) and in the *rbc*L phylogeny six highly supported clades were recognized within clade E (indicated as E1, E2, E3, E4, E5 and E6 in Fig. 2). Two clades were also observed within the superclade F. All strains for which both markers could be sequenced occurred in corresponding superclades in

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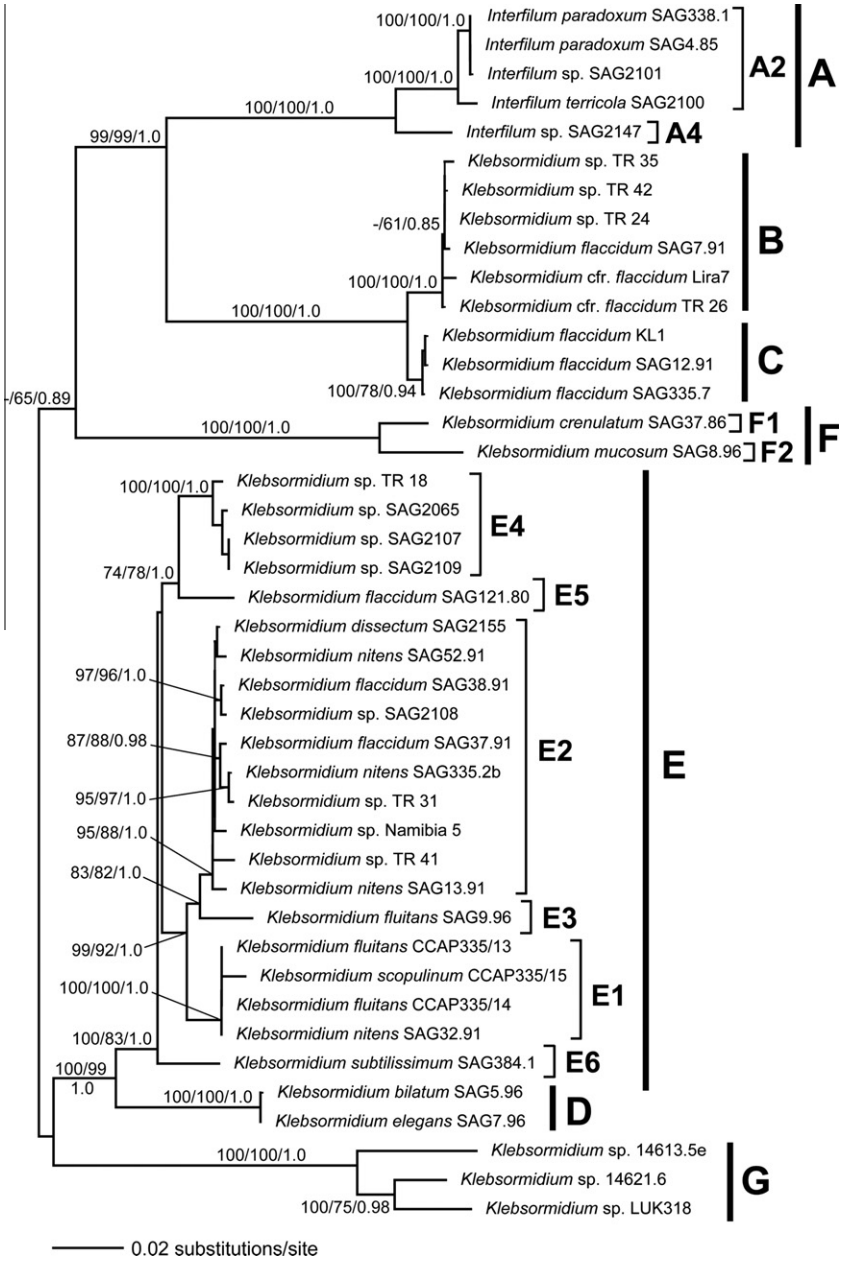


**Fig. 3.** Phylogram inferred from Maximum-Likelihood analysis of the concatenated dataset *rbc*L-ITS rRNA in the Klebsormidiales, with bootstrap support (BP) and Bayesian Posterior Probabilities (PP) indicated at the nodes. From left to right and from top to bottom the support values correspond to Neighbor Joining BP, Maximum-Likelihood BP and Bayesian PP. BP values lower than 60% and PP lower than 0.8 are not reported.

the two phylogenies. Morphological data of the strains belonging to these superclades/clades and habitat information are summarized in Table 2, and micrographs of a selection of the strains can be found in Figs. 4 and 5.

Superclade A corresponded taxonomically to the genus *Interfilum* and was recovered with high support in both phylogenies. More strains could be sequenced for ITS than for *rbc*L, and within this superclade ITS showed generally higher resolution. Four well-supported clades were found in the ITS phylogeny. The two most derived ones were referable to described morphological species (A1 to *I. massjukiae* Mikhailyuk et al., and A2 to *I. paradoxum* Chodat & Topali/*I. terricola* (J.B. Petersen) Mikhailyuk et al.). A3 and A4, the two earliest-diverging clades, could not be identified unambiguously, and may represent undescribed taxa. The separation of these four clades was supported by morphological differences (Table 2; see also Mikhailyuk et al., 2008).

Superclade A was sister with moderate support to a lineage formed by superclades B and C. A sister relationship between superclades B and C was recovered with very high support in both the ITS and *rbc*L analyses. Superclade B was composed of unidentified *Klebsormidium* strains isolated mainly from terrestrial habitats in eastern Europe (Ukraine and Russia). These strains showed morphological similarity to *K. flaccidum* (Kützing) P.C. Silva, Mattox & Blackwell, but they differed from it in some characters (in particular the morphology of the chloroplast) and could not be identified unambiguously. Superclade C included strains identified as *K. flaccidum* obtained from culture collections, isolated from different types of habitats mainly in western Europe.

Superclades D and E were recovered as sister taxa with moderate to high support in both phylogenies. Superclade D was formed by strains identified as (or morphologically resembling) *K. bilatum* Lokhorst and *K. elegans* Lokhorst. The strains *K. bilatum* SAG5.96

**Table 2**
Synopsis of the morphological features of the clades of Klebsormidiales recovered in the phylogenetic analyses. For some clades, the information is partially or primarily based on data published in other studies (Lokhorst, 1996; Novis, 2006; Mikhailyuk et al., 2008; Rindi et al., 2008).

| Clade | Taxon with nomenclatural priority | Other taxa belonging to clade | Habitat | Morphology |
|---|---|---|---|---|
| A1 | *Interfilum massjukiae* | | Cracks in surface of natural rocks and soil; so far known only from eastern Europe | Cells aggregated in groups of 2–4, sometimes forming large multicellular cubic aggregations, uni- and bi-seriate branched filaments. Cells widely ellipsoid to rounded or hemispherical, (6) 7–11 μm long, (5) 6.4–9 μm wide; terminal cells in branched filaments 10–15 μm long; cell walls thick, without mucilage. Chloroplast parietal, plate-shaped, dissected in 5–8 lobes. Pyrenoid surrounded by one to several layers of starch. Cell division in three planes. Remnants of mother cell wall (if observed) with cap-like habit. Reproduction exclusively by fragmentation |
| A2 | *Interfilum paradoxum* | *Interfilum terricola* | Soil and terrestrial habitats, rarely freshwater | Cells single or in pairs, sometimes forming short filaments or chains of cells. Cells ellipsoid, widely ellipsoid, oviform to rounded or hemispherical, (6) 7–10 (11) μm long, (4.5) 5.5–7 (7.5) μm wide, surrounded by clear mucilage envelope with striation. Chloroplast and pyrenoid with morphology similar to *I. massjukiae*. Remnants of mother cell wall well-developed, cap-like or forming threads between cells. Reproduction exclusively by fragmentation |
| A3 | *Interfilum* sp. | | Soil | Cells single or in pairs, sometimes forming short filaments. Cells ellipsoid, stretch-oviform to cylindrical, sometimes curved, (7) 9–13 (18) μm long, (4) 5–6 (7) μm wide. Chloroplast and pyrenoid with similar morphology to other species of *Interfilum*. Habit of mucilage envelope similar to *I. paradoxum* and *I. terricola*. Remnants of mother wall well-developed, cap-like. Reproduction exclusively by fragmentation. |
| A4 | *Interfilum* sp. | | Soil | Cells aggregated in strong groups of 4–16 and more cells. Cells widely ellipsoid to rounded or hemispherical, (5) 6–7.5 (9) μm long, (3) 4.5–6 (7) μm wide; cell walls thick, without mucilage. Chloroplast and pyrenoid with the same morphology as for the clades A1–A3. Cell division in three planes. Reproduction exclusively by fragmentation. |
| B | *Klebsormidium* sp. | Possibly *Klebsormidium dissectum*? | Mainly natural rocks and soil, but also freshwater; so far recorded from eastern Europe. | Filaments long or easily disintegrated, (6) 6.8–8 (12) μm wide, not or slightly constricted; some strains showing fast and vigorous fragmentation; cells cylindrical, sometimes slightly swollen, (0.7)1.5–1.8 times as long as wide; cell doublets present in some strains; cell wall thin to moderately thickened; H-pieces absent or present; chloroplast covering 1/2–2/3 of the cell wall, with margins crenulated or irregularly dissected; pyrenoid large, surrounded by several layers of starch. In liquid media forming superficial hydrorepellent layer and submerged tufts; on agar forming waved colonies or colonies with homogeneous margins; reproduction appears to take place only by filament fragmentation |
| C | *Klebsormidium flaccidum* | | Soil and freshwater habitats; so far recorded mainly from western Europe | Filaments long, 6–8 μm wide, not or slightly constricted; cells cylindrical, 1–1.4(2.4) times as long as wide; cell doublets absent; cell wall thin to moderately thickened; H-pieces absent or present (if present, not clear and located in the middle parts of the filaments); chloroplast covering 1/2–2/3 of the cell wall, with margins usually smooth, pyrenoid large, surrounded by several layers of starch. In liquid media forming superficial hydrorepellent layer and submerged tufts; on agar forming waved colonies; asexual reproduction by zoosporogenesis, easily inducible; release aperture small and indistinct; germination unipolar and bipolar |
| D | *Klebsormidium bilatum*/ *Klebsormidium elegans* | | Humid soils, particularly at margin of water bodies; bark at the base of trees, natural rocks. | Filaments long, generally robust but sometimes easily dissociating when old, 7–9(10.2) μm wide, sometimes growing in rope-like aggregates; cells cylindrical to barrel-shaped, 1–1.5(2.2) times as long as wide; cell doublets may be present or not; cell wall thin to moderately thickened; H-pieces present, prominent; chloroplast covering half to 3/4 of the cell wall, with a median incision in the margin, dissected in four or more lobes; pyrenoid large, surrounded by several layers of starch. In liquid media forming submerged tufts; the superficial layer may be present or not; on agar, forming wavy colonies with small waves; asexual reproduction absent or present, if present it takes place by zoosporogenesis and aplanosporogenesis; zoosporogenesis not easily inducible; release aperture small, not easily visible; germination unipolar |
| E1 | *Klebsormidium acidophilum* | Probably *Klebsormidium scopulinum* and *Klebsormidium subtile* | Freshwater, including low pH habitats; rivers, streams. | Filaments long, either easily dissociating or not, 5–7(9) μm wide, without constrictions; cells stretch-cylindrical to cylindrical and slightly swollen, 1–3 times as long as wide; cell walls thin to moderately thickened; H-pieces absent or present; chloroplast covering approximately half of the cell wall, with smooth or slightly lobed margins; pyrenoid medium-sized, surrounded by a layer of starch. In liquid media forming a mixture of long and short filaments; on agar forming homogeneous growths with rough surface; reproduction only by filament fragmentation. |
| E2 | *Klebsormidium nitens* | Probably | Soil, freshwater, | Filaments either long or short, (4)5–6.5(9) μm wide; tendency to |

(*continued on next page*)

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**Table 2** (*continued*)

| Clade | Taxon with nomenclatural priority | Other taxa belonging to clade | Habitat | Morphology |
|---|---|---|---|---|
| | | *Klebsormidium klebsii* | natural rocks, artificial surfaces including bases of urban walls. | fragmentation variable between strains, but usually strong; cells cylindrical, constricted, (0.5)1–1.5(3) times as long as wide; cell wall thin to moderately thickened; H-pieces usually absent, but present in some strains; chloroplast covering 1/2–2/3 of the cell wall, with smooth margins, delicately lobed in some strains; pyrenoid small, usually surrounded by a layer of starch. In liquid culture forming submerged tufts and superficial layer, but the development of the superficial layer varies considerably between strains; on agar forming smooth colonies or wavy growths with homogeneous margin; asexual reproduction by zoosporogenesis, either easily inducible or not; release aperture large, well discernible; germination unipolar |
| E3 | *Klebsormidium fluitans* | | Freshwater and margin of freshwater bodies; humid soil | Filaments long, not easily dissociating, 7–8.5(10.2) μm wide; cells cylindrical to square or slightly swollen, 1–1.5 times as long as wide; cell wall thin, thickened in old cells; H-pieces present; chloroplast covering 1/2–3/4 of the cell wall, with margins smooth, sometimes slightly lobed; pyrenoid medium-sized, surrounded by several layers of starch.In liquid culture, submerged tufts present, superficial layer either present or absent; on agar, forming growths with rough surface; zoosporogenesis easily inducible; release aperture small, not easily visible; germination either unipolar or bipolar |
| E4 | *Klebsormidium* sp. | | Primarily epilithic; particularly common at the bases of urban walls | Filaments long in field specimens and young cultures, sometimes with slight contrictions, 5–9 μm wide; in mature cultures they may either remain long or disintegrate to short filaments and unicells, 5.5–8 μm wide, with cells cylindrical, more or less swollen or ellipsoid, 0.5–2 times as long as wide; cell doublets present in some strains; cell wall thin to moderately thickened; H-pieces absent; chloroplast covering 1/2–2/3 of the cell wall, either entire with smooth margins or lobed; pyrenoid medium-sized, surrounded by a layer of starch. In liquid culture forming submerged tufts, which may become completely fragmented; superficial layer absent in most strains but present in some; on agar, forming homogeneous colonies with smooth surface; reproduction only by filament fragmentation. |
| E5 | *Klebsormidium* sp. | | Tree bark and wooden surfaces | Filaments long, easily dissociating or not dissociating, 6–7 μm wide; cells cylindrical, 1–2 times as long as wide; cell doublets absent; cell wall medium- thickened; H-pieces absent; chloroplast covering 1/2–2/3 of the cell wall, with smooth margins; pyrenoid surrounded by a layer of starch.In liquid culture, forming submerged tufts and superficial layer; submerged filaments may become more or less fragmented; on agar, forming homogeneous growths with rough surface; reproduction only by filament fragmentation |
| E6 | *Klebsormidium subtilissimum* | | Not known in detail; the specimens sequenced were originally isolated from snow in Alaska | Filaments long, but easy disintegrated, curved, 5–6 μm wide, slightly constricted; cells cylindrical, (1)1.5–1.7 times as long as wide; cell wall moderately thickened; H-pieces present; chloroplast covering 1/2–2/3 of the cell wall, with smooth margins; pyrenoid small, surrounded by a layer of starch. In liquid media forming a mixture of long and short filaments; on agar forming wavy colonies with homogeneous margin. |
| F1 | *Klebsormidium crenulatum* | Possibly *Klebsormidium lamellosum* and *Klebsormidium montanum* | Soil, especially sandy; margin of water bodies; base of tree trunks; natural rocks. | Filaments long, strong, thick, (9)13–15(18) μm wide, sometimes growing in rope-like aggregates; cells cylindrical, becoming barrel-shaped and narrow-square in old filaments, 0.5–1(1.5) times as long as wide; cell doublets occasionally present; cell wall initially thin, becoming thick and corrugated in old filaments; H-pieces common, prominent; chloroplast girdle-shaped, almost ring-like, covering most of the cell wall, with longitudinal margins smooth or slightly lobed; pyrenoid large, surrounded by several layers of small starch grains.In liquid media forming only submerged tufts, on agar forming rough, waved colonies with moss-like habit; asexual reproduction absent or not easily inducible; if present, it takes place by either zoosporogenesis or aplanosporogenesis; release aperture irregular and not easily observable; germination unipolar. |
| F2 | *Klebsormidium mucosum* | | Soil, margin of water bodies | Morphology very similar to *K. crenulatum*, but with thicker filaments, (13)15–20(23) μm wide. In liquid media forming only submerged tufts; on agar forming rough, waved colonies with moss-like habit; reproduction only by filament fragmentation. |
| G | *Klebsormidium* sp. | | Arid soils, mainly in biotic crusts of warm desertic areas | Long filaments with tendency to disintegration, becoming completely disintegrated in some strains, curved, strongly constricted, bead-like, 4.5–8 μm wide; cells short or square, elongated in old filaments, 0.5–1 (3) times as long as wide; cell wall thin to moderately thickened; H-pieces present or absent; chloroplast with smooth margin, often four-lobed; pyrenoid small, surrounded by a few starch grains. In liquid media forming mixture of short and long filaments; on agar forming cluster- and knot-like colonies. Reproduction only by filament fragmentation |

and *K. elegans* SAG7.96 were originally isolated by Lokhorst (1996) and therefore represent authentic cultures for these two species.

Superclade E was an heterogeneous assemblage of strains with different morphologies, collected from many different habitats and

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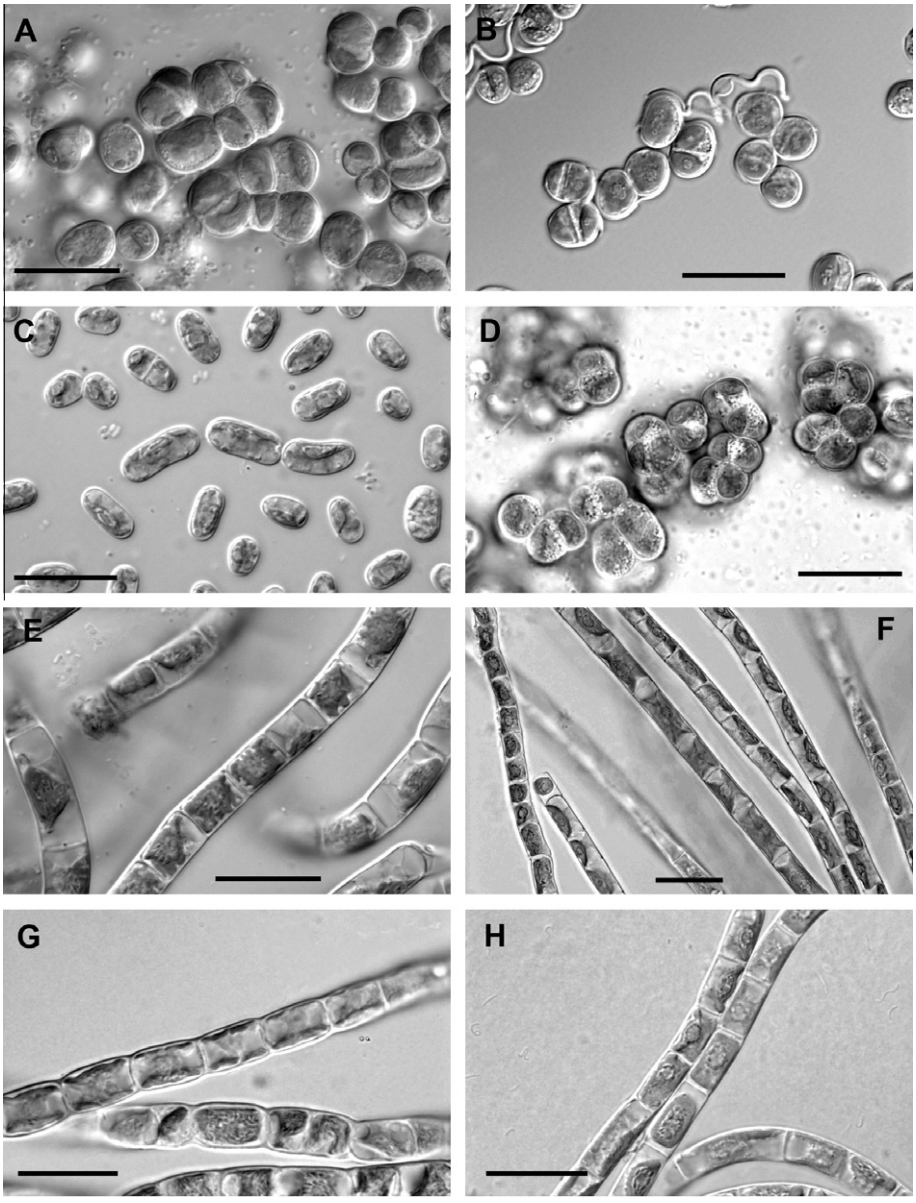


**Fig. 4.** Morphology of strains of Klebsormidiales belonging to the superclades A–E. (A) *Interfilum massjukiae* SAG2102 (clade A1). (B) *Interfilum paradoxum* SAG338.1 (clade A2). (C) *Interfilum* sp. LUK317 (clade A3). (D) *Interfilum* sp. SAG 2147 (clade A4). (E) *Klebsormidium* sp. TR42 (superclade B). (F) *Klebsormidium flaccidum* KL1 (superclade C). (G) *Klebsormidium bilatum* SAG5.96 (superclade D). (H) *Klebsormidium fluitans* CCAP335.13 (clade E1). Scale bar = 20 μm.

geographical regions. This superclade had no statistical support and very limited resolution in the ITS analyses, but a better resolution was obtained in the *rbc*L analyses in which six well-supported clades (E1, E2, E3, E4, E5, E6) were recovered (Fig. 2). Clade E1 was formed by strains from culture collections identified as *K. fluitans* (F. Gay) Lokhorst, *K. nitens* (Meneghini) Lokhorst and *K. scopulinum* (Hazen) Ettl & Gärtner, and GenBank sequences of specimens from New Zealand identified by Novis (2006) as *Klebsormidium acidophilum* Novis and *K. dissectum* (F. Gay) Ettl & Gärtner. Clade E2 consisted of a morphologically heterogeneous group of specimens, identified as *Klebsormidium nitens*, *Klebsormidium flaccidum* or not identified at species level. This clade included also *Klebsormidium dissectum* SAG2155; although not explicitly designated as neotype, this culture was isolated by Lokhorst (1996) from the locality that this author designated as neotype locality (Col du Bussang, France). A small clade (E3) was sister to clade E2 with high support; it consisted of *Klebsormidium fluitans* SAG9.96 (neotype culture of this species, Lokhorst, 1996) and an epilithic urban strain identified by Rindi et al. (2008) as *K. flaccidum*. For three other clades, the relative positions were not resolved. One of these (E4) was formed by strains obtained from bases of urban walls by Rindi et al. (2008) and strains of unknown origin deposited in the SAG culture collection; this was the only clade that was recovered with high support in the ITS phylogeny too (Fig. 1). Another clade (E5) was formed by a strain of *K. flaccidum* from SAG (121.80) and an unidentified strain collected from a wooden building in Florida (described by Rindi et al., 2008). Sequences of *K. subtilissimum* (Rabenhorst) P.C. Silva, Mattox et Blackwell obtained from culture collections formed an additional clade (E6), whose relationships with the other lineages were not resolved.

Superclade F was formed by strains referable to two well-characterized morphological species, *Klebsormidium crenulatum* (Kützing) Lokhorst and *K. mucosum* (Boye Petersen) Lokhorst; its position could not be determined with certainty. In the ITS phylogeny, it was sister to superclade G (Fig. 1); in the *rbc*L phylogeny it was sister to the lineage formed by superclades A–C (Fig. 2). However, the statistical support of these relationships was low (Figs. 1 and 2) and the two topologies cannot be considered to be in conflict.

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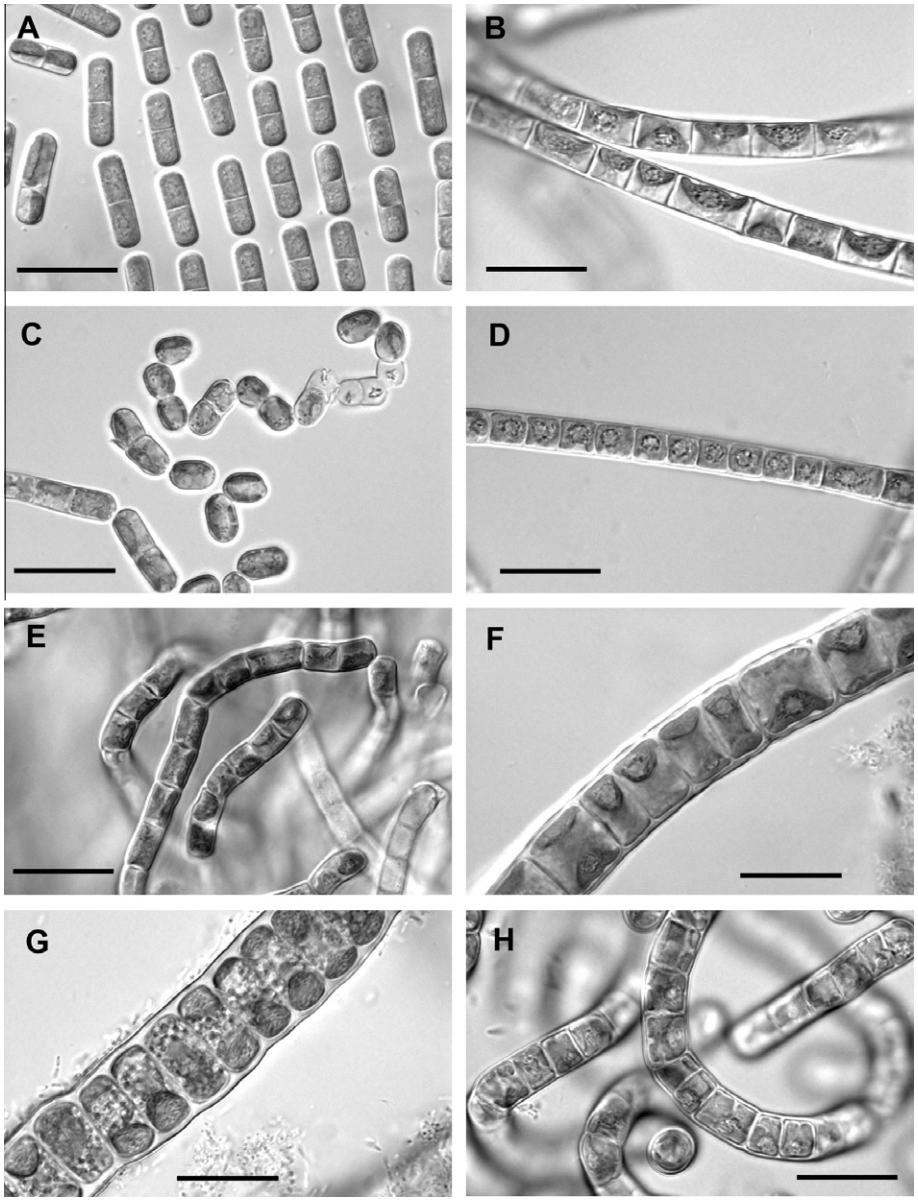


**Fig. 5.** Morphology of strains of Klebsormidiales belonging to the superclades E–G. (A) *Klebsormidium dissectum* SAG 2155 (clade E2). (B) *Klebsormidium fluitans* SAG9.96 (clade E3). (C) *Klebsormidium* sp. SAG2065 (clade E4). (D) *Klebsormidium* sp. SAG121.80 (clade E5). (E) *Klebsormidium subtilissimum* SAG384.1 (clade E6). (F) *Klebsormidium crenulatum* SAG37.86 (clade F1). (G) *Klebsormidium mucosum* SAG8.96 (clade F2). (H) *Klebsormidium* sp. 14614.18.18 (superclade G). Scale bar = 20 μm.

Finally, a new and previously unknown lineage (superclade G) was revealed in our analyses. This lineage appeared relatively isolated from all other klebsormidialean taxa and no clear sister group was identified. It was formed by strains of *Klebsormidium* isolated mainly from biotic crusts of arid soils in South Africa. Morphologically, the strains belonging to this group were mainly characterized by the thin filaments and the four-lobed chloroplasts (Table 2).

The results obtained for the concatenated dataset (Fig. 3) reflected the topology of the *rbc*L analyses and provided stronger support for all relationships that had been recovered in the single-marker analyses. The same seven superclades were recovered with higher support; in particular, the sister relationship between superclade A and the lineage formed by superclades B and C showed a considerable increase in support. The most internal nodes, however, could not yet be resolved. The positions of superclades F and G remained unresolved.

## 4. Discussion

### 4.1. Characteristics of the datasets analyzed

The larger taxon sampling of the phylogenies presented here resulted in an increased statistical support and better resolution in comparison with previous investigations. This is also the first study on *Klebsormidium* and *Interfilum* in which two different molecular markers are combined and analyzed in a concatenated dataset. Not unexpectedly, in the combined dataset the nodal support is higher than in single-marker analyses; it is well established that an increase in the number of genes or characters improves the quality of phylogenetic inference (Sanderson and Shaffer, 2002). The phylogenetic signals of *rbc*L and ITS show excellent correspondence, recovering similar topologies and retrieving identical superclades and clades. These markers have comparable substitutions rates and are among the most popular for phylogenetic

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inference at the species and genus level. When used in combination, they have been very effective and have contributed to the solving of major taxonomic problems (Hayden et al., 2003); it is in fact surprising that relatively few studies on freshwater and terrestrial green algae have used them in conjunction (e.g., Sakayama et al., 2005; Yamada et al., 2008; Xue et al., 2009). In terms of topology, the position of superclade F (sister to superclade G for ITS, sister to a lineage formed by superclades A–C for *rbc*L) is the only difference observed between the two phylogenies. However, the relationships of this superclade are poorly resolved by both *rbc*L and ITS, and the analyses on the concatenated dataset do not represent a significant improvement. Therefore, the position of superclade F is presently considered uncertain, and clarification requires further studies incorporating a larger number of molecular markers. Interestingly, even though the ITS and *rbc*L phylogenies are congruent, for certain superclades some differences in terms of substitution rate and phylogenetic resolution were observed between these markers. For superclade E, in particular, *rbc*L showed better resolution than ITS, recovering six different well-supported clades (E1, E2, E3, E4, E5, E6) that were not resolved by ITS (with the exception of E4). The combined analyses reflected the *rbc*L results and resolved the six clades with increased support, presumably because the higher length of *rbc*L (1149 bp versus 517 bp of ITS) makes the phylogenetic signal of this marker prevailing. The limited resolution of ITS was due to the low substitution rate in this superclade. Only 22 of 513 characters (equivalent to 4%) were parsimony informative, whereas 460 (=89%) were constant; uncorrected *p*-distances ranged between 0.1% and 2.5% (*Klebsormidium* sp. 15051.6 vs *K. nitens* SAG 31.91), with an overall average of 1.1%. It should be noted that *rbc*L showed also a low substitution rate in this lineage (highest uncorrected p-divergence was 5%, between *Klebsormidium fluitans* SAG9.96 and *K. subtilissimum* SAG384.1; overall average 2.3%). These results indicate that superclade E is a morphologically and ecologically dynamic group of relatively recent evolutionary origin, in which the genetic variation does not yet match morphological and ecological diversity. This group appears to be still in the process of active radiation and species boundaries are not yet well delineated. A direct implication emerging from these results is that in fast-evolving lineages of green microalgae phylogenetic inference and taxonomic conclusions should not be based on a single molecular marker. For the Klebsormidiales, the addition of sequences of further molecular markers will be necessary to clarify the relationships that could not be resolved by ITS and *rbc*L, especially in the basal nodes. Due to their proximity to land plants, we suggest that the Klebsormidiales are also a suitable group to test the usefulness of several chloroplast markers that have been recently used for DNA barcoding in vascular plants (Kress et al., 2005; Lahaye et al., 2008; Fazekas et al., 2008; Seberg and Petersen, 2009).

### 4.2. Phylogenetic relationships and taxonomic implications

Our analyses revealed new lineages and provided stronger support for several relationships observed in previous studies. In particular, the paraphyly of *Klebsormidium* caused by *Interfilum* (which was recovered in the ITS phylogeny of Mikhailyuk et al., 2008, but without statistical support) was established with robust support. Two lineages of particular interest are superclade B and superclade G.

For superclade B, *Klebsormidium* sp. SAG7.91 was the only strain sequenced in previous investigations (Mikhailyuk et al., 2008; Rindi et al., 2008). Morphologically, the strains of *Klebsormidium* forming this group show considerable similarity to *K. flaccidum* and *K. dissectum* (as characterized by Lokhorst, 1996), but they are set apart by the morphology of the chloroplast with margins crenulated or irregularly dissected. The combination of morphological characters observed for these strains does not correspond with any known species of *Klebsormidium* and will require the description of one or more new species. Interestingly, from a biogeographic point of view this clade shows a marked association with eastern Europe (all strains sequenced were isolated from Ukraine and Russia, mainly from granite outcrops). Biogeography of small-sized organisms is a hotly debated topic (Finlay et al., 2006; Foissner, 2008), and it is extremely difficult to demonstrate endemicity or restricted distribution in terrestrial microalgae. This is particularly true for this study, because about 80% of the strains used have been isolated from Europe and the taxon sampling from other continents is currently too limited to draw strong generalizations. However, the fact that all the 11 strains belonging to superclade B originate from the same region is possibly not a coincidence.

The strains of *Klebsormidium* forming superclade G represent an unknown evolutionary lineage revealed in this study. These strains are characterized by a combination of morphological characters not found in any known species of *Klebsormidium* (filaments curved and strongly constricted, cells bead-like, chloroplast four-lobed with a small pyrenoid surrounded by a few starch grains, formation of cluster- and knot-like colonies on agar). This lineage includes several undescribed species, for which we are planning to propose formal descriptions in a forthcoming paper. These strains are unique in terms of habitat, since they are mostly associated with biotic crusts of arid soils in subdesertic regions of southern Africa (described in detail by Jürgens et al., 2010). It would be interesting to clarify their relationships with strains isolated from biotic crusts of North American deserts (Lewis and Flechtner, 2002; Lewis, 2007), in order to understand how many times the physiological attributes required to colonize arid habitats have evolved in *Klebsormidium*. Unfortunately our analyses do not allow clarification of the position of superclade G in relation to the other superclades; this lineage, however, appears relatively distant from the others (as is also suggested by the high uncorrected *p*-distances from all other strains of *Interfilum* and *Klebsormidium* sequenced). If future studies support the position of superclade G as the earliest-diverging lineage in the order, a very interesting evolutionary scenario would emerge. This would suggest a split of two early *Klebsormidium* lineages that colonized habitats with different characteristics: arid desertic and subdesertic habitats (the superclade G) and more or less humid habitats (a lineage that has diversified and developed into all other taxa).

The phylogenetic relationships revealed in this study have major implications for the taxonomy and classification of the Klebsormidiales. A taxonomomic reassessment and development of a species concept are not among the aims of this study, as we are planning to address these questions in future investigations; it seems inevitable, however, that a major taxonomic rearrangement will be required. The most important problem is the correct characterization of *Klebsormidium flaccidum*, the type species of *Klebsormidium*, as its definition affects the circumscription of the whole genus. In our phylogenies, strains identified morphologically as *K. flaccidum* are polyphyletic and occur in five different clades/superclades (B, C, E2, E3, E5). At present it is impossible to assign the type specimen to any of these clades, as the original description (Kützing, 1849) does not provide sufficient information to link unambiguously the species with any of them. The ideal solution would be to sequence the type specimen, but unfortunately this is not possible (the type material consists of only few filaments embedded in a drop of mud; Willem Prud'homme van Reine, personal communication). Therefore, the designation of an epitype specimen based on a subjective choice will almost certainly be necessary. Due to the paraphyly of *Klebsormidium* caused by *Interfilum*, the choice of the new type will affect the classification at genus level and will either require the separation of one or more new genera or the reduction of *Interfilum* to a subgenus of *Klebsormidium*.

Linnaean names should be attached unambiguously to all clades and superclades recovered in our analyses. This appears straight-

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**Table 3**
Summary of morphological characters that represent shared derived characters associated with individual clades or superclades.

| Character | Superclade/clade | Species |
|---|---|---|
| Unicellular or few-celled morphology with tendency to formation of more or less complex aggregations | A | Species of *Interfilum* |
| Mucilage envelope with striations and cap-like or thread-like remnants of mother cell wall | A | *Interfilum* spp. (some exclusions) |
| Thallus forming packet-like aggregations and bi-seriate branched filaments that may occasionally disintegrate | A1 | *Interfilum massjukiae* |
| Thallus forming strong, not easily disintegrated packet-like aggregations | A4 | *Interfilum* sp. |
| Filaments thick (mostly > 10 μm, up to 20–23 μm in old filaments) with walls rough and corrugated in old specimens | F | *Klebsormidium crenulatum*, *Klebsormidium mucosum* |
| Chloroplast dissected on 5–8 (sometimes more) clear lobes | A | Species of *Interfilum* |
| Chloroplast with margins crenulated or irregularly dissected | B | *Klebsormidium* sp. |
| Chloroplast with a median incision, divided in several lobes | D | *Klebsormidium bilatum*, *Klebsormidium elegans* |
| Chloroplast girdle-shaped (*Ulothrix*-like), encircling most of the cell wall | F | *Klebsormidium crenulatum*, *Klebsormidium mucosum* |
| Chloroplast four-lobed | G | *Klebsormidium* sp. |
| Pyrenoid small and surrounded by a few starch grains | G | *Klebsormidium* sp. |
| Distribution in freshwater, including low pH habitats | E1 | *Klebsormidium acidophilum* |
| Distribution primarily in biotic crusts of hot subdesertic areas | G | *Klebsormidium* sp. |
| Distribution associated primarily with eastern Europe | B | *Klebsormidium* sp. |

forward for some clades but it might prove complicated for others, and it will require the description of some new species (certainly for the superclades B and G, possibly for the clades E4 and E5). A number of species of *Klebsormidium* were unavailable for this study (*K. fragile* (Kützing) Wagner & Zaneveld, *K. drouetii* Wagner & Zaneveld, *K. klebsii* (G.M. Smith) P.C. Silva, Mattox & Blackwell, *K. lamellosum* Wei & Hu, *K. montanum* (Hansgirg) S. Watanabe, *K. pseudostichococcus* (Heering) Ettl & Gärtner, *K. subtile* (Kützing) Tracanna ex Tell and *K. tribonematoideum* (Skuja) Hindák). A comprehensive revision of the classification of the Klebsormidiales will only be possible after sequences of these species have become available.

### 4.3. Evolution of morphological characters

Most green algal lineages have a long and complex evolutionary history, in which many morphological characters were gained and lost multiple times. For some taxa, this may make it problematic to detect morphological characters with phylogenetic significance. This problem is exacerbated in taxa with a simple morphology, especially when strong morphological plasticity is evident (Leliaert et al., 2009; Rindi et al., 2009). Our results document this type of difficulty for *Klebsormidium*. Many morphological characters, including some considered of great taxonomic value, have evolved separately in different clades/superclades and therefore are phylogenetically irrelevant; in fact, some of them show different characters states even between different strains of the same clades. Examples of such characters include tendency to fragmentation, presence of H-shaped cell wall pieces, presence of water-repellent superficial layer of filaments in liquid culture and germination pattern of zoospores. However, our results suggest also a set of characters that appear to have phylogenetic significance; the characters that represent unique synapomorphies associated with particular clades/superclades are summarized in the Table 3. Characters related to the gross morphology represent the most obvious, since the capacity to form threedimensional aggregations with packet-like habit is limited to species of *Interfilum* (superclade A). Characters related to the morphology of the chloroplast seem also to be phylogenetically valuable. In *Klebsormidium* the chloroplast is usually a parietal plate with smooth margins, encircling a half to 2/3 of the cell wall and containing one pyrenoid surrounded by a variable number of starch grains (Printz, 1964; Ettl and Gärtner, 1995; Lokhorst, 1996). This is the habit observed in most of the strains that were sequenced in this study. However, variously lobed or dissected chloroplasts are found in several strains, and it is possible to observe variations that are unique to individual superclades (A, B, D, F, G; Table 2). Further, the distinctly lobed chloroplast in *E. fimbriata*, one of the closest relatives of *Klebsormidium,* indicates that this is a feature that was already present in the common ancestor of *Entransia* and *Klebsormidium*. The type of habitat may also be a character that deserves more attention than it has been so far appreciated, since some clades seem to be associated with certain habitats. For example, in our phylogenies strains of *Klebsormidium* isolated from biotic crusts in subdesertic areas of southern Africa were clustered in superclade G. In the same way, clade E1 consists of freshwater strains, some of which were isolated from low pH habitats (*K. acidophilum*; Novis, 2006). Species of *Klebsormidium* have been reported from low pH habitats in many regions (Lukešová, 2001; Verb and Vis, 2001; Sabater et al., 2003; Lear et al., 2009; Urrea-Clos and Sabater, 2009) and it would be interesting to verify whether acidophilic strains from different regions are closely related or not. To answer these and other questions an extended sampling that was not possible for this study will be required.

### 4.4. General aspects and conclusions

Our results highlight some points of general relevance with regard to diversity and evolution of terrestrial green algae. They show that the Klebsormidiales span a broader range of genetic, morphological and ecological diversity than previously believed. The demonstration of the paraphyly of *Klebsormidium* caused by *Interfilum* expands the morphological concept of this algal group, which so far was considered to include only uniseriate filamentous forms. *Interfilum* represents a further example of how widespread the sarcinoid or packet-like growth habit is among terrestrial green algae. It is clear that this habit is particularly suited to an aeroterrestrial lifestyle, since it is characteristic of some of the most widespread species (López-Bautista et al., 2007) and has evolved separately in several lineages of green algae (Friedl and O'Kelly, 2002; Watanabe et al., 2006; Lemieux et al., 2007; Rindi et al., 2007; Mikhailyuk et al., 2008), but it is not clear what features make it so advantageous. In consideration of this, it cannot be ruled out that new lineages of Klebsormidiales with morphologies currently unknown for this order (e.g., unicellular, or branched filamentous) will be discovered in the future. The discovery of

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superclades G and B indicates that the Klebsormidiales have still major surprises in store and that the diversity and distribution of this order are still poorly understood. The complex of species currently belonging to *Klebsormidium* are among the most widespread and earliest-described terrestrial algae (Kützing, 1849). The fact that even such a common group still offers the opportunity of unexpected discoveries suggests that our knowledge of the diversity of terrestrial green algae is far from complete; therefore, the fundamental importance of new surveys of natural history combining morphological and molecular data cannot be stressed enough. Special attention should be devoted to unusual and extreme habitats or little-explored geographical regions, where new, hiterto unknwon evolutionary lineages may be discovered. We suggest that for the Klebsormidiales acidic water bodies, sites affected by several types of chemical pollution, high mountain habitats, polar soils and rocks and tropical regions offer the best promises for the discovery of new taxa.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.11.030.

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