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DISTRIBUTION, MORPHOLOGY, AND PHYLOGENY OF *KLEBSORMIDIUM* (KLEBSORMIDIALES, CHAROPHYCEAE) IN URBAN ENVIRONMENTS IN EUROPE[1]

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Klebsormidium **is a cosmopolitan genus of green algae, widespread in terrestrial and freshwater habitats. The classification of** ***Klebsormidium*** **is entirely based on morphological characters, and very little is understood about its phylogeny at the species level. We investigated the diversity and phylogenetic relationships of** ***Klebsormidium*** **in urban habitats in Europe by a combination of approaches including examination of field-collected material, culture experiments conducted in many different combinations of factors, and phylogenetic analyses of the** ***rbc*****L gene.** ***Klebsormidium*** **in European cities mainly occurs at the base of old walls, where it may produce green belts up to several meters in extent. Specimens from different cities showed a great morphological uniformity, consisting of long filaments 6–9 μm in width, with thin-walled cylindrical cells and smooth wall, devoid of false branches, H-shaped pieces, and biseriate parts. Conversely, the** ***rbc*****L phylogeny showed a higher genetic diversity than expected from morphology. The strains were separated in four different clades supported by high bootstrap values and posterior probabilities. In culture, these clades differed in several characters, such as production of a superficial hydro-repellent layer, tendency to break into short fragments, and inducibility of zoosporulation. On the basis of the taxonomic information available in the literature, most strains could not be identified unambiguously at the species level. The** ***rbc*****L phylogeny showed no correspondence with classification based on morphology and suggested that the identity of many species, in particular the type species** ***K. flaccidum*** **(kütz.) P.C. Silva, Mattox et W. H. Blackw., needs critical reassessment.**

Key index words: **Charophyceae; Europe;** ***Klebsormidium*****; morphology; phylogeny;** ***rbc*****L gene**

Abbreviations: **BBM, Bold's basal medium; BI, Bayesian analysis; JM, Jaworski's medium; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining;** ***rbc*****L, large subunit of RUBISCO**

The genus *Klebsormidium* P. C. Silva, Mattox et W. H. Blackw. (Silva et al. 1972) comprises filamentous uniseriate green algae characterized by cells having a parietal chloroplast with a single pyrenoid, asexual reproduction by biflagellate zoospores, sporelings germinating into new thalli without production of differentiated holdfasts, and flagellar apparatus with unilateral construction (Silva et al. 1972, Ettl and Gärtner 1995, Hoek et al. 1995). Its position in the streptophytan lineage, in close phylogenetic proximity to stoneworts and land plants, is robustly supported by ultrastructural and molecular data (Marchant et al. 1973, Sluiman and Guihal 1999, Karol et al. 2001, Lewis and McCourt 2004, McCourt et al. 2004, Sluiman et al. 2008). *Klebsormidium* is one of the most widespread taxa of microchlorophytes in the world, ranging in distribution from polar to tropical regions (Ramanathan 1964, Lee and Wee 1982, Broady 1996, Lokhorst 1996, John 2002, 2003) and occurring in a wide range of terrestrial and freshwater habitats. Algae of this genus have been reported from streams and rivers (Morison and Sheath 1985, Necchi et al. 1991), bogs (John 2002), bare soil (Deason 1969), sand dunes (Smith et al. 2004), acidic post-mining sites (Lukešová 2001), golf courses (Baldwin and Whitton 1992), tree bark (Handa et al. 1991, Nakano et al. 1991), exposed rocks in plains and mountainous areas (Frémy 1925), seepage rocks (Fjerdingstad 1965), stone monuments (Uher et al. 2005, Barberousse et al. 2006), bases of urban walls (Rindi

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and Guiry 2003, 2004), and iron railings (Schlichting 1975).

Like many other filamentous algae, *Klebsormidium* has a complicated nomenclatural and taxonomic history. The name *Klebsormidium* was proposed by Silva et al. (1972) to resolve major taxonomic confusion concerning a group of algae [originally described by Kützing (1843)] for which the name *Hormidium*, until then in common use in the literature, had been misapplied (Lokhorst 1996). As presently delimited, the genus includes some 22 species (Guiry and Guiry 2007). This number, however, should be considered a provisional estimate, since for many species and subspecific taxa, the circumscription is still very uncertain. It has long been a matter of dispute whether several taxa should be regarded as independent species or varieties or forms of other species (Ettl and Gärtner 1995, Lokhorst 1996, John 2002, Škaloud 2006). To date, in most cases, there is no generally accepted solution to these difficulties.

Such taxonomic confusion is due to several reasons. First of all, the morphology of *Klebsormidium* is extremely simple and offers a very limited set of characters that can be satisfactorily used for species identification. Width of filaments, type of growth of filaments, tendency to fragmentation, shape of cells, texture of the cell wall, formation of H-shaped pieces, shape of the chloroplast, and shape of the pyrenoid are the characters most commonly used in identification keys available in the literature (Printz 1964, Ramanathan 1964, Ettl and Gärtner 1995, Rifón-Lastra and Noguerol-Seoane 2001, John 2002). To these, Lokhorst (1996) added some new characters based on observations of cultured material, such as presence of a superficial hydro-repellent layer in liquid cultures, shape of the release aperture of zoospores in the lateral walls, and germination pattern of zoospores. In practice, however, it is quite common that different keys lead to different identifications, or that it proves impossible to obtain any credible identification at all. For quantitative characters (width of filaments, in particular), there is a large overlap between different species, and some features of taxonomic importance are known to show some variation depending on the age and the physiological conditions of the specimens examined (e.g., texture of the cell wall: Lokhorst 1996). Reproductive characters are usually not observable in field collections, and in some species, the production of zoospores has never been documented (Lokhorst 1996). Treatments of the same species provided in different studies are sometimes contradictory and usually do not refer to the original descriptions. Very few exceptions to this situation are available, such as the investigations of Lokhorst (1996) and Novis (2006); the study of Lokhorst (1996), in particular, although limited to central Europe, had the great merit of examining type specimens and designating lectotypes and neotypes for several species.

Another major problem is that no substantial use of molecular data has been made so far to clarify systematics and species delimitation in *Klebsormidium*. Most of the molecular data available for this genus have been produced in studies focused on higher-level classification, aimed primarily at assessing which algal groups are the closest living relatives of land plants (Karol et al. 2001, Turmel et al. 2002, Qiu et al. 2006). Two recent studies by Novis (2006) and Sluiman et al. (2008) have produced useful new data in this regard. The survey of Novis (2006), focused on strains from acidic streams in New Zealand, is the only investigation in which a detailed field-based study has been combined with molecular data (*rbc*L sequences); on the basis of his results, Novis (2006) described the new species *Klebsormidium acidophilum* Novis. Sluiman et al. (2008) sequenced ITS in 10 strains of *Klebsormidium* obtained mostly from culture collections. The data presented in these studies lack sufficient taxon sampling to draw strong conclusions on the evolutionary patterns in the genus, and Novis (2006) himself concluded that further molecular data are necessary to obtain better resolution in the evolution of *Klebsormidium*. Overall, the molecular phylogeny of *Klebsormidium* at the species level is still far from a substantial clarification, and it is presently impossible to infer which morphological characters should be considered phylogenetically relevant.

In recent years, two of us (F. R. and M. D. G.) have made detailed observations on the subaerial algal flora of urban environments in Europe. In the course of these surveys, we noticed that species of *Klebsormidium* are widespread in urban habitats (Rindi and Guiry 2004). These algae are a common occurrence at the base of old walls and on the concrete between paving stones and produce large green patches at sites characterized by high humidity, for example, around the discharge mouth of rain pipes (Rindi and Guiry 2004, Rindi 2007). In spite of detailed examination of numerous collections, it turned out to be impossible to characterize and identify unambiguously most field-collected specimens on the basis of the literature available. We therefore extended our investigations to include molecular data (*rbc*L gene sequences) and culture experiments conducted in many different combinations of factors. The results, which are presented here, provide major insights into the diversity of these algae, suggesting that considerable genetic diversity is hidden behind the simple morphology of *Klebsormidium*. Overall, the new data produced here suggest that the taxonomy of *Klebsormidium* will require a substantial rearrangement and represent a basis of information of critical importance for all systematic studies that will consider this genus in the future.

MATERIALS AND METHODS

Collections and morphological studies. The strains of *Klebsormidium* used for this study were primarily collected from

European cities in the years 2002–2005 (Table S1 in the supplementary material). The number of samples obtained varied from city to city. Some collections were made in the course of fieldtrips specifically aimed at collecting subaerial algae, during which 20–25 samples were collected (Bordeaux, Copenhagen, Manchester, Marseilles, and Pisa; see Rindi and Guiry 2004). Other collections were occasional or provided from local colleagues, and no more than one or a few samples could be obtained. The material was collected using a knife or a screwdriver at sites where green patches produced by *Klebsormidium* were visible with the unaided eye. The collecting tool used was cleaned and sterilized with alcohol between each collection. After collection, the samples were conserved dry in sealed plastic bags until examination in the laboratory. When conserved in this way, samples of *Klebsormidium* remain viable for several weeks; the morphological characters considered of taxonomic importance are not severely affected by this treatment and remain observable for 2–3 weeks. In the laboratory, each sample was examined by light microscope (Nikon Optiphot-2, Nikon UK Ltd., Surrey, UK), and the following characters were observed: (1) width of filaments (calculated from at least 20 replicates); (2) length of filaments (short: up to 10 cells; medium sized: 11–50 cells; long: more than 50 cells); (3) habit of filaments (straight or forming knee-shaped bends); (4) presence/absence of false branches; (5) presence/absence of constrictions between adjacent cells; (6) texture of cell wall (smooth and thin or rough and thickened); (7) cell shape (cells cylindrical or more or less barrel-shaped; cells straight or more or less curved); (8) chloroplast shape (chloroplast with smooth margin or with differentiated margin, e.g., incised or with equatorial constriction); (9) length of the chloroplast relative to the length of the cell (covering most of the length of the cell or forming a ring that covers 50% or less of the cell length); (10) shape of the pyrenoid in front view (spherical or more or less elongate); (11) presence/absence of H-shaped pieces; (12) occasional presence/absence of biseriate parts; (13) presence/absence of intercalary globular structures or mucilaginous balls; and (14) presence/absence of a thick glistening sheath. Voucher specimens were deposited in the phycological herbarium of the National University of Ireland, Galway (GALW) and in the herbarium of the University of Alabama (UNA).

Culture studies. For each city, one to three samples of *Klebsormidium* were isolated into unialgal cultures using Jaworski's medium (JM; Tompkins et al. 1995). Stock cultures were maintained in glass dishes containing ~400 mL of medium, at 15°C, 16:8 light:dark (L:D), 20–30 μmol photons $\cdot m^{-2} \cdot s^{-1}$. The morphology of strains of *Klebsormidium* from 17 cities (marked with an asterisk in Table S1) was examined in a series of culture experiments conducted under different combinations of factors. Two strains were used for Bergen, Bordeaux, Copenhagen, Manchester, Marseilles, and Pisa; one strain was used for the other cities. Multiple strains from the same city were originally collected from separate sites located at least 100 m from each other. The experiments were carried out in walk-in constant temperature rooms. The factors tested were the following: culture medium [liquid cultures, two levels: JM and Bold's basal medium (BBM, Bold and Wynne 1978)]; temperature (3 levels: 10, 15, and 20°C); and photon irradiance (two levels: 15–20 and 45–50 μmol photons $\cdot m^{-2} \cdot s^{-1}$). The material initially used for each experiment was a fragment 50–100 cells long; this was obtained from a culture that had been previously maintained in the combination of factors to be tested for at least 2 weeks. At least three replicates were used for each experiment. Unfortunately, for some strains (Koper, London, and Porto), it was not possible to complete the series of experiments. In July 2005, due to a malfunction, the temperature in the room in which the stock cultures were maintained rose up to 61°C for a night, which caused the loss of these strains. The culture experiments were carried out in plastic petri dishes containing ~30 mL of medium (Bibby Sterilin®, Stone, UK). The culture medium was replaced every 10 d, to avoid depletion of nutrients. The duration of each experiment was 8–12 weeks (10 weeks for most strains). The same characters considered for the field-collected material were observed; additionally, the following characters were also noted: (1) growth habit (long filaments more or less robustly attached to the bottom of the dish; alga fragmented into short unattached filaments, with the habit of a green soup; or a mixture of these two growth forms); (2) presence/absence of a superficial layer of hydro-repellent filaments; (3) shape of release aperture in lateral wall of zoosporangial cell (large and distinct or small and indistinct); (4) germination pattern of zoospores (unipolar and bipolar or unipolar only); and (5) facility of induction of release of zoospores (easily inducible or not). The last character was assessed by short-term experiments based on procedures used in previous studies (Mattox 1971): release of zoospores (or presence of settled sporelings on the bottom of the culture vessels) was checked after keeping the strains in darkness for 24 h at 15°C and 20°C in BBM, JM, and sterile distilled water. If no release of zoospores took place in any of the conditions tested, the experiment was repeated with the same media and temperatures, keeping the strains in darkness for a week. If no release of zoospores took place even in this situation, release of zoospores was considered not easily inducible.

Molecular studies. DNA was extracted from 32 strains of *Klebsormidium* (Table S1) using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). These included 17 strains from European cities, two additional strains from Florida and South Africa, and 12 strains obtained from the Sammlung von Algenkulturen, Universität Göttingen, Germany (SAG). The choice of the strains from SAG was dictated by two important reasons: (1) Among the main culture collections, SAG offers the best taxon sampling of *Klebsormidium* species; and (2) several strains deposited in SAG were personally identified and deposited by Dr. Gijsbert Lokhorst. Since Lokhorst (1996) examined original collections and designated lectotypes and neotypes for several species, the strains of SAG represent the best choice to sequence material referable with certainty or high probability to the original species descriptions [for *Klebsormidium dissectum* (F. Gay) H. Ettl et G. Gärtner, *K. elegans* Lokhorst, and *K. fluitans* Lokhorst, the SAG strains are the type cultures].

For PCR amplification of the *rbc*L gene, a GeneAmp PCR 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) was used. PCR was performed as two overlapping fragments using primers obtained from the literature and primers designed by JMLB using Oligo6.89 (Molecular Biology Insights, Cascade, CO, USA; Table S2 in the supplementary material). Each 13 μL reaction contained: 4.4 μL of water; 1.25 μL of 10× reaction buffer; 1.25 μL of MgCl2 (25 mM); 1.25 μL of dATP, dCTP, dGTP, and dTTP (8 mM); 0.625 μL of each primer (10 mM); 0.1 μL of *Taq* (New England BioLabs, Ipswich, MA, USA); 2.5 μL of betaine (5M); and 1.0 μL of total genomic DNA. The normal PCR protocol consisted of an initial denaturing phase of 10 s at 96°C, followed by 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min, with a final extension of 8 min at 72°C. For some samples, however, an annealing temperature of 55°C was necessary. The PCR products were examined for correct length, yield, and purity under UV light on 1.5% agarose gels stained with ethidium bromide, and they were subsequently purified using the Qiagen MinElute Gel Extraction Kit (Qiagen). The amount of DNA in PCR products was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). PCR products were sequenced using the Big Dye chemistry (Applied Biosystems), and sequence readings were obtained using an ABI 3100 automated sequencer

(Applied Biosystems). Sequence chromatograms were aligned and edited using Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA).

Sequence alignment and phylogenetic analyses. An alignment of 1,275 base pairs (bp) was constructed by eye using MacClade 4.05 (Maddison and Maddison 2002). Besides the newly sequenced strains, all *rbc*L sequences of *Klebsormidium* currently available in GenBank were included in the ingroup: *Klebsormidium acidophilum* DQ028577 and DQ028578; *K. dissectum* DQ028574, DQ028575 and DQ028576; *K. nitens* (Menegh.) Lokhorst AF408254; *K. subtilissimum* (Rabenh.) P. C. Silva, Mattox et W. H. Blackw. AF408253; and *K.* sp. L13478. Phylogenetic trees were rooted using the following sequences as outgroup: *Chlorokybus atmophyticus* AY823706; *Coleochaete scutata* AY082329; *Entransia fimbriata* AY823705; and *Spirogyra gracilis* DQ015937. These taxa were chosen because in previous studies, *Entransia* was resolved as sister taxon to *Klebsormidium* (Karol et al. 2001), and the other genera are known to be closely related to it (Karol et al. 2001, Turmel et al. 2002). The aligned data set was analyzed for neighbor joining (NJ) and maximum parsimony (MP) using PAUP* 4.0b10 (Swofford 1998), and for maximum likelihood (ML) using GARLI (Zwickl 2006; http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html). Bayesian analysis (BI) was performed using MrBayes 3.04 (Huelsenbeck and Ronquist 2001). The MP analysis was performed as a heuristic search with random addition (100 replicates), tree-bisection-reconnection, branch swapping, steepest descent and Multrees option enabled. The evolutionary model for ML and BI was determined using ModelTest 3.7 (Posada and Crandall 1998). The model selected for ML under the Akaike Information Criterion was GTR + I + G, in which the following parameters were specified: proportion of invariable sites I = 0.4575; gamma distribution shape parameter = 1.1935; base frequencies A = 0.2767, C = 0.1755, G = 0.2135, and T = 0.3343; rate matrix [A-C] = 2.5049, [AG] = 6.0673, [A-T] = 5.4206, [C-G] = 1.1232, [C-T] = 17.7081, and [G-T] = 1.0000. The model selected for BI under the Bayesian Information Criterion was again GTR + I + G, with identical parameters. The robustness of the tree topologies was assessed by bootstrapping the data set (Felsenstein 1985) with 1,000 resamplings for NJ, MP, and ML. The BI analysis was performed using the priors set as default in MrBayes. Four Monte Carlo Markov chains were used; 3×10^6 generations were run, with sampling every 100 generations and discarding the first 200,000 generations as burn-in.

RESULTS

Distribution and habitats occupied. In European cities, the base of walls is the sort of habitat most frequently occupied by *Klebsormidium*. Most samples examined for this study were collected from bases of old walls or in immediate vicinity of such structures (no more than 0.5 m distance; Fig. 1a illustrates the most common situation). Exceptions to this situation were the samples collected in Pavia (Fig. 1b) and Siena, and two samples collected in Stockholm. In these cases, *Klebsormidium* occurred on surfaces not immediately close to walls (several meters distance); the sites in which these specimens occurred were surrounded by tall buildings, which produced shaded, weather-sheltered conditions. The largest populations were observed at sites with conditions of shade and high humidity, where *Klebsormidium* produced on the walls green belts several meters long (Fig. 1c). The preference of *Klebsormidium* for concrete surfaces, especially horizontal, was obvious in all cities visited personally by the authors (Fig. 1, a–d). These

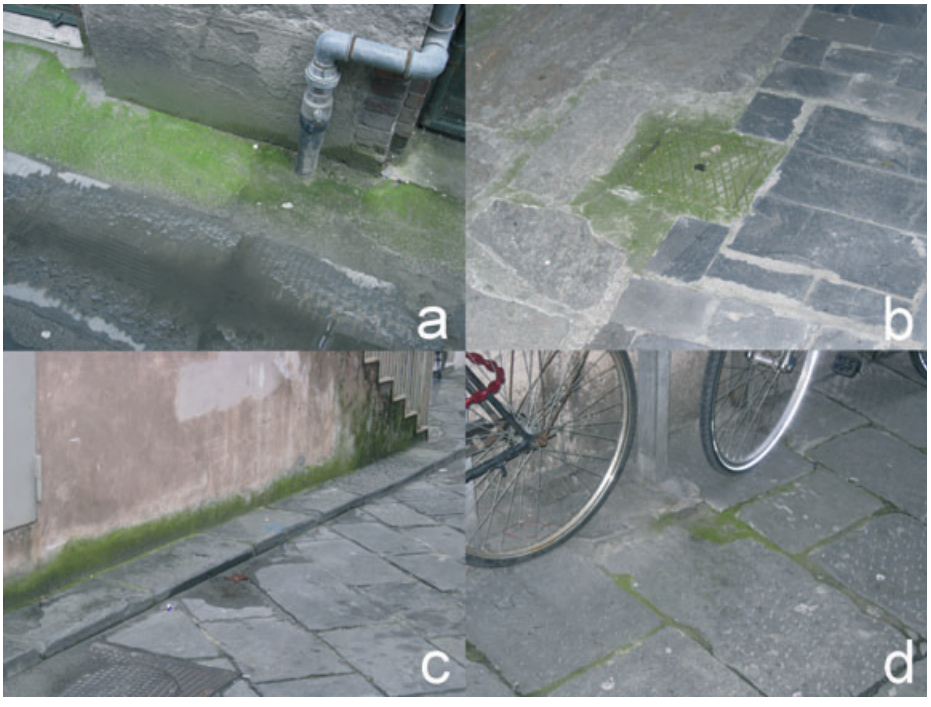

FIG. 1. Examples of populations of *Klebsormidium* in urban habitats in Europe. (a) Details of a population forming a large green patch at the base of a wall (Pisa). (b) Population sampled in Pavia, growing on concrete surface. (c) Population forming a belt several meters long at the base of wall in Pisa. (d) Population growing on concrete surface between paving stones near the base of wall (Pisa).

algae were rarely found on stones, bricks, tar, or other types of solid substratum. It was, however, not uncommon to find *Klebsormidium* on the soil produced by the dust and detritus accumulated between adjacent stones of old pavements. This was the type of habitat from which the samples from Galway, La Valletta, and Siena were collected; this was also the most common situation in Copenhagen. When collected from this habitat, *Klebsormidium* was usually mixed with large amounts of sand and debris, which did not seem to cause any harm to the alga. In all samples examined, *Klebsormidium* was quantitatively dominant, producing dense velvety mats formed by many entangled filaments. Other subaerial algae and cyanobacteria (coccalean greens, *Desmococcus*, diatoms, and *Phormidium autumnale*) were occasionally present, but usually in much smaller amounts.

Morphology of field-collected material. Overall, the morphology of the strains of *Klebsormidium* from European cities was very uniform. Very limited differences were observed among strains collected in different cities and at different sites in the same city. Filaments were typically long, flexuous, and devoid of knee-shaped bends. In several collections, medium-sized and short fragments were also present, either in smaller amounts (collections from Bergen, Konstanz, and Pisa) or in amounts similar to the long filaments (Koper, London, Pavia, and Prague). The filaments were 6–9 μm wide (mainly 6–8 μm). No false branches, H-shaped pieces, or biseriate parts were observed in any collection examined. In a collection from Pisa, some cells enlarged producing intercalary structures of uncertain identity, with a globular or elliptical shape, 8–11 μm in width. Slight constrictions between adjacent cells occurred in most samples. They did not appear to be a constant feature; parts with constrictions and parts completely devoid occurred frequently in the same filaments. The cells were cylindrical, 0.5–2 times as long as wide and devoid of sheaths. The cell wall was smooth, and corrugations were observed very rarely, only in some old filaments. The chloroplast was parietal; it extended for the whole length of the cell and covered approximately two-thirds of the cell circumference. The margin of the chloroplast was smooth. Incisions or denticulations were observed in some cells in the strains from Plymouth, Porto, and Stockholm, but their presence was the exception rather than the rule. Each chloroplast contained a pyrenoid, which was surrounded by starch granules and more or less flattened in side view. Its shape in front view varied from rounded to elongated; this character, however, appeared to be taxonomically irrelevant, as its shape clearly varied from cell to cell. In cells approaching cell division (about two times as long as wide), it was elongated, whereas in cells derived from recent division (0.5–1 times as long as wide), it was rounded.

The morphological characteristics of the strains examined are reported in Table 1. On the basis of the combination of characters observed, no unambiguous identification could be obtained [with the only exception of the strain from Galway, for which the combination of characters observed in the field and in culture corresponded with *Klebsormidium flaccidum* as characterized by Lokhorst 1996]. See Discussion for further details.

Culture experiments. The results of the culture experiments are summarized in Table 2. All strains used in the experiments generally grew well in both media tested, although a few exhibited a quite clear preference: the Bergen and Hamburg strains showed faster growth in BBM, while the Siena strain grew best in JM. Morphology and growth habit of the strains cultured were relatively constant, but some differences in relation to culture medium and temperature were observed. Conversely, no evident differences between the two levels of photon irradiance used were noticed.

La Valletta, Marseilles, and Pisa showed the strongest tendency to fragmentation. After a few weeks in JM at 15°C and 20°C, these strains consisted of a green soup of short fragments, mostly two to six cells long. However, in JM at 10°C and in BBM, fragmentation was not so marked, and the cultures consisted of a mixture of short fragments and long filaments attached to the bottom of the dishes, or mostly long filaments. To a lesser extent, fragmentation was also observed in several other strains. In the strain from London, long filaments were the dominant growth form in most combinations, but at 15°C and 20°C, 15–20 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$, the cultures consisted mainly of short fragments. In Bergen, short and medium-sized fragments were present in all combinations tested, but always in lower amounts than long filaments. A similar situation was observed for Hamburg, in which, however, the submerged filaments showed a rather different morphology: instead of long, flexuous filaments, these consisted of rope-like masses, in which the filaments were frequently bent with knee-like habit. Short and medium-sized filaments were also common in Porto, but only in JM.

A superficial layer of hydro-repellent filaments occurred in four strains (Bergen, Galway, Hamburg, and Konstanz), but Bergen was the only one in which it was well developed in all combinations of factors tested. In the Galway strain, it was produced only at 20°C, both in JM and BBM. In the Hamburg strain, it was present in almost all combinations tested, but it was not well developed and did not cover the whole surface of the medium. In the Konstanz strain, it was generally well developed in JM but weakly developed or absent in BBM.

Release of zoospores was easily inducible only in the strain from Galway. After 24 h in darkness,

TABLE 1. Summary of morphological characters of *Klebsormidium* from European cities as observed in field-collected specimens.

Strain	Cell width	Length of filaments	Presence of constrictions	Cell shape	Chloroplast shape	Presence of globular structures
Klebsormidium sp. Bergen	6.9 ± 0.7	L, MS, S	+	Cylindrical	With smooth margin	−
Klebsormidium sp. Bordeaux	7.48 ± 0.31	L	+	Cylindrical	With smooth margin	−
Klebsormidium sp. Copenhagen	7.43 ± 0.45	L	+	Cylindrical	With smooth margin	−
Klebsormidium flaccidum Galway	7.45 ± 0.16	L	+	Cylindrical or swollen	With smooth margin	−
Klebsormidium sp. Hamburg	7.63 ± 0.48	L	+	Cylindrical	With smooth margin	−
Klebsormidium sp. Konstanz	8.35 ± 0.97	L, MS, S	+	Cylindrical	With smooth margin	−
Klebsormidium sp. Koper	7.54 ± 0.15	L, MS, S	+	Cylindrical	With smooth margin	−
Klebsormidium sp. La Valletta	7.57 ± 0.32	L	+	Cylindrical	With smooth margin	−
Klebsormidium sp. London	7.43 ± 0.42	L, MS, S	+	Cylindrical	With smooth margin	−
Klebsormidium sp. Manchester	7.23 ± 0.45	L	−	Cylindrical	With smooth margin	−
Klebsormidium sp. Marseilles	7.23 ± 0.47	L	−	Cylindrical or swollen	With smooth margin	−
Klebsormidium sp. Pavia	7.32 ± 0.24	L, MS, S	+	Cylindrical	With smooth margin	−
Klebsormidium sp. Pisa	7.25 ± 0.55	L, MS, S	+	Cylindrical or swollen	With smooth margin	+
Klebsormidium sp. Plymouth	7.45 ± 0.22	L	+	Cylindrical	Margin smooth, occasionally denticulated	−
Klebsormidium sp. Porto	7.71 ± 0.26	L	−	Cylindrical	Margin smooth, occasionally denticulated	−
Klebsormidium sp. Prague	7.17 ± 0.45	L, MS, S	−	Cylindrical	With smooth margin	−
Klebsormidium sp. Siena	7.67 ± 0.45	L	+	Cylindrical	With smooth margin	−
Klebsormidium sp. Stockholm	7.86 ± 0.48	L	+	Cylindrical	Margin smooth, occasionally denticulated	−

Cell width is expressed as mean ± standard deviation. For length of the filaments: L, long; MS, medium-sized; S, short.

TABLE 2. Summary of morphological characters of *Klebsormidium* from European cities in culture.

Strain	Cell width (JM)	Cell width (BBM)	Habit (JM)	Habit (BBM)	Superficial layer (JM)	Superficial layer (BBM)	Release of zoospores	Shape of release aperture	Germination pattern of zoospores
Klebsormidium sp. Bergen	6.65 ± 0.61	6.67 ± 0.62	Mix	Mix	++	++	−	N/O	N/O
Klebsormidium sp. Bordeaux	7.54 ± 0.36	7.17 ± 0.4	Fil	Fil	−	−	−	N/O	N/O
Klebsormidium sp. Copenhagen	7.47 ± 0.34	7.52 ± 0.37	Fil	Fil	−	−	−	N/O	N/O
Klebsormidium flaccidum Galway	7.2 ± 0.37	7.37 ± 0.35	Fil	Fil	+	+	Easily inducible	Indistinct	Unipolar and bipolar
Klebsormidium sp. Hamburg	6.09 ± 0.78	6.46 ± 0.62	Mix	Mix	+	+	−	N/O	N/O
Klebsormidium sp. Konstanz	7.73 ± 0.56	8 ± 0.83	Fil	Fil	++	+	−	N/O	N/O
Klebsormidium sp. Koper	7.35 ± 0.27		Fil	Fil	+		−	N/O	N/O
Klebsormidium sp. La Valletta	6.34 ± 0.86	6.32 ± 0.56	Frag	Frag	−	−	−	N/O	N/O
Klebsormidium sp. London	6.58 ± 0.62		Mix		−		−	N/O	N/O
Klebsormidium sp. Manchester	6.67 ± 0.45	7.42 ± 0.42	Fil	Fil	−	−	−	N/O	N/O
Klebsormidium sp. Marseilles	6.94 ± 0.44	7.25 ± 0.42	Frag	Mix	−	−	−	N/O	N/O
Klebsormidium sp. Pisa	6.91 ± 0.63	7.5 ± 0.53	Frag	Mix	−	−	−	N/O	N/O
Klebsormidium sp. Plymouth	6.6 ± 0.45	7.02 ± 0.34	Fil	Fil	−	−	−	N/O	N/O
Klebsormidium sp. Porto	6.78 ± 0.9	7.32 ± 0.71	Mix	Fil	−	−	−	N/O	N/O
Klebsormidium sp. Prague	5.98 ± 0.68	7.05 ± 0.4	Fil	Fil	−	−	−	N/O	N/O
Klebsormidium sp. Siena	6.48 ± 0.5	6.96 ± 0.45	Fil	Fil	−	−	−	N/O	N/O
Klebsormidium sp. Stockholm	6.19 ± 0.72	6.14 ± 0.69	Fil	Mix	−	−	−	N/O	N/O

Habit: Fil, alga consisting entirely or primarily of long filaments remaining attached to the bottom of the dishes; Frag, alga consisting of unattached short fragments; Mix, alga consisting of a mixture of long filaments and short fragments. Superficial layer: −, absent; +, present but not well developed (covering <50% of the surface of the medium); ++, well developed (covering from 50% to the totality of the surface of the medium). The characters were observed after 5 weeks since the beginning of the experiment. BBM, Bold's basal medium; JM, Jaworski's medium.

release of zoospores took place at both 15°C and 20°C and in both media. The zoospores escaped from the sporangia through a small, indistinct aperture, and, after being released in large amounts, they settled rapidly on the bottom of the dishes. A large majority of the resulting sporelings germinated with unipolar pattern; however, some with bipolar germination were also observed.

Molecular phylogeny. Figure 2 illustrates the ML tree. Of the 1,275 characters included in the analyses, 800 were invariant; 88, parsimony uninformative; and 387, parsimony informative. The topologies recovered by different methods of phylogenetic inference were largely congruent, and several well-supported clades were recovered in all analyses. Their relative position, however, could not be assessed unambiguously; some internal nodes were not resolved, as they received no support in the bootstrap analyses. In all analyses, strains of *Klebsormidium* were resolved in two main clades (Fig. 2): a small clade formed by four strains of *Klebsormidium flaccidum* from SAG and *Klebsormidium* sp. L13478 from GenBank, and a large clade including all other strains (which, however, had no bootstrap support). The only topological difference between different phylogenetic analyses consisted of the position of *K. mucosum* (Boye Petersen) Lokhorst SAG8.96. This strain was included in the

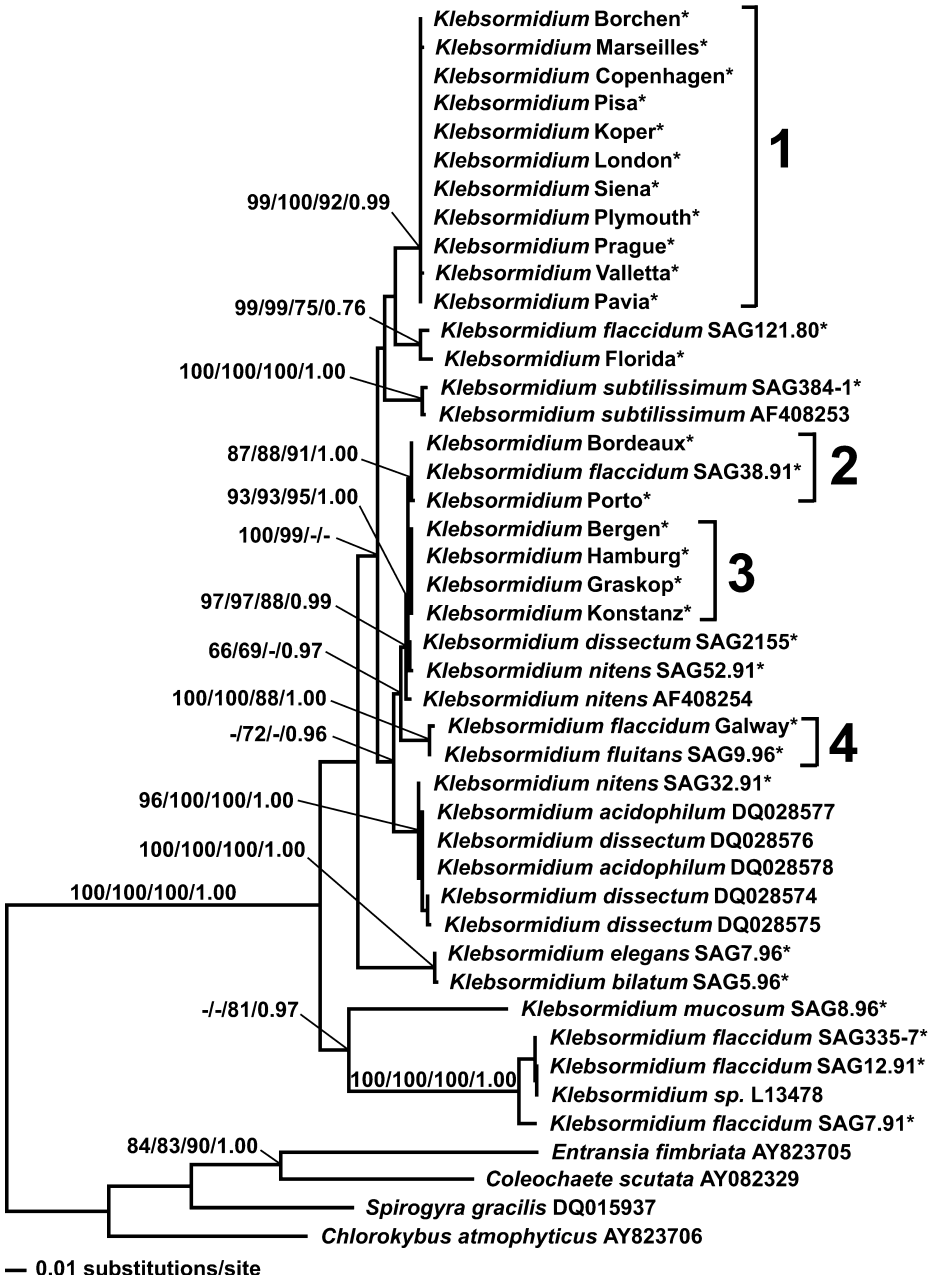

FIG. 2. Phylogram inferred from maximum-likelihood analysis of the *rbc*L gene in *Klebsormidium* and outgroup taxa, with bootstrap support (BP) and Bayesian posterior probabilities (PP) indicated at the nodes. From left to right, support values at nodes correspond to neighbor-joining BP, maximum-parsimony BP, maximum-likelihood BP, and Bayesian PP. New sequences produced in this study are marked with an asterisk. The four clades containing strains from urban environments are numbered as reported in the text.

smaller clade with moderate support in the ML tree and high support in the Bayesian consensus (Fig. 2), but it formed an intermediate separate clade, sister to the large clade, in both the NJ and strict consensus MP trees. The MP analysis recovered 16 most-parsimonious trees of 1,049 steps (consistency index = 0.599; homoplasy index = 0.401; retention index = 0.790), which differed from each other in the relative position of some strains in the terminal branches. In all analyses, the strains of *Klebsormidium* from European cities were distributed in four different clades (marked by numbers in Fig. 2), supported by high bootstrap values in all methods used. The largest clade (1) included 11 strains from cities widely scattered around the continent (from as north as Copenhagen to as south as La Valletta), which had identical or almost identical *rbc*L sequence (Marseilles and La Valletta differed by 1 bp from the other strains). These strains varied greatly in the habit in culture (filamentous in most of them, but strongly fragmented in Marseilles, Pisa, and La Valletta); none, however, produced a superficial layer. Two other groups of strains were subdivided into two separate but closely related clades, formed respectively by the strains from Bordeaux and Porto (clade 2), and the strains from Bergen, Hamburg, and Konstanz (clade 3). Clades 2 and 3 occurred as sister groups. Their sequences were very similar, and the sequences within each clade were identical; their main morphological difference was the presence of a superficial layer in clade 3 and its absence in clade 2. They were nested in a larger clade in which *K. dissectum* SAG2155 (neotype culture) and two strains of *K. nitens* from SAG were also included (Fig. 2). Finally, the fourth clade (4) contained a strain identified as *K. flaccidum* from Galway, together with *K. fluitans* (Gay) Lokhorst SAG9.96 (neotype of this species); this was sister to the larger clade in which clades 2 and 3 were nested, but with high support only under BI. Uncorrected pair-wise distances ranged from 0.46% (clade 2 vs. clade 3) to 4.7% (clade 1 vs. clade 4); the pair-wise distance between clade 1 and clade 2 was 3.7%; the pair-wise distance between clade 1 and clade 3 was 3.9%. For several species, sequences deposited in GenBank and obtained from SAG strains did not produce monophyletic groups. Strains of *K. flaccidum* from SAG occurred in three different clades and were separated from *K. flaccidum* from Galway; pair-wise distances between these strains ranged from 0.18% (SAG12.91 vs. SAG335-7) to 12.02% (SAG12.91 vs. SAG121.80). Sequences of *K. nitens* were also separated in two different clades (with up to 2.5% sequence divergence between SAG52.91 and SAG32.91). Sequences of *K. dissectum* deposited in GenBank (all obtained from New Zealand specimens by Novis 2006) occurred in a different clade from the neotype material, from which they differed by ~2.9% pair-wise divergence. *K. elegans* and *K. bilatum* from SAG had identical *rbc*L sequence and formed a well-supported clade, but their position relative to the other strains was not resolved. They formed a sister group to the large clade in the NJ, MP, and ML analyses, but they were collapsed in a polytomy with the large and the small clades in the bootstrap analyses and in the Bayesian consensus (data not shown).

DISCUSSION

The results presented here provide substantial insights into the diversity of *Klebsormidium* in Europe and, more generally, into the phylogeny of this genus.

Our phylogeny reveals that at least four different lineages of *Klebsormidium* are present in our strains obtained from urban habitats in Europe. At present, we are not able to trace clear-cut species-level delimitations among them; in fact, with the single exception of the strain from Galway, it is not possible to provide for any of them a satisfactory morphological identification. For field-collected material, the keys and descriptions available in the literature (Printz 1964, Ramanathan 1964, Ettl and Gärtner 1995, Lokhorst 1996, Rifón-Lastra and Noguerol-Seoane 2001, John 2002) usually lead us to identify all our specimens as *K. flaccidum*.

K. flaccidum is the type species of *Klebsormidium* (Silva et al.1972). It was described as *Ulothrix flaccida* by Kützing (1849: 349), using material collected by Braun in stony streets in Strasbourg, France. This taxon is considered one of the most widespread terrestrial green algae in the world (Printz 1964, Ettl and Gärtner 1995) and has been recorded in almost all regions in which subaerial algae have been systematically studied. It is generally reported as a species with filaments mostly long (>150 cells) but easily dissociating at maturity, devoid of H-shaped pieces, biseriate parts, and false branches, with thin-walled cylindrical cells, slight constrictions between adjacent cells, and chloroplast with smooth margin extending for the whole length of the cell and covering about a half of the lateral wall. Its exact delimitation, however, is not clear. *K. flaccidum* is often said to be a polymorphic species (Ramanathan 1964, Farooqui 1968, Ettl and Gärtner 1995), and it is very uncertain if some taxa described in the past (e.g., Chodat 1913) should be considered independent species or forms of *K. flaccidum* (Ettl and Gärtner 1995, Lokhorst 1996). The information reported with regard to some morphological characters, in particular the width of the filaments, is confusing (e.g., 5.5–6 μm in Farooqui 1968, 5.5–7 μm in Komáromy 1974, 1976, 5.6–7.4 μm in Lokhorst 1996, 5–8 μm in Mattox and Bold 1962, 6–9 μm in Škaloud 2006, 6–9.5 μm in Hazen 1902, and 5–14 μm in Ramanathan 1964). This uncertainty is reflected in our *rbc*L phylogeny, in which specimens identified as *K. flaccidum* and

deposited in the same culture collection (SAG) occur in at least three separate clades (Fig. 2). As Lokhorst (1996) combined detailed culture studies with examination of authentic herbarium specimens, we believe that his circumscription is the most reliable, and we base our identifications primarily on his conclusions. This author reported that in liquid cultures, *K. flaccidum* produces a superficial layer, the production of zoospores can be easily induced, the release aperture of the zoosporangium is small and indistinct, and the sporelings germinate with unipolar and bipolar pattern. The morphology of the strain from Galway and its responses in culture are thus in complete agreement with Lokhorst's (1996) characterization of *K. flaccidum*, and we therefore refer it to this species. This is not the case, however, for the other strains. The strains of clades 1 and 2 did not produce the superficial layer; the strains of clade 3 produced the superficial layer, but the zoosporulation was not easily inducible, and the release aperture and sporelings were never observed.

Klebsormidium klebsii (G. M. Smith) P. C. Silva, Mattox et W. H. Blackw. is also morphologically very close to some of our specimens. Silva et al. (1972) clarified the taxonomic and nomenclatural identity of this species, which was erected by Smith (1933: 385, as *Hormidium klebsii*) for an alga studied and reported by Klebs (1896) as ''*Hormidium nitens* Menegh.'' Klebs (1896) reported that in this species, the filaments are long, 5.5–7 μm wide, and in culture, they produce the superficial layer of hydro-repellent filaments and occasionally break into short fragments. Apart for the wider filaments, our strains from Hamburg, Konstanz, Galway, and, in particular, Bergen agree with Klebs's (1896) description. *K. klebsii* has been reported as widespread in Europe, Asia, and Africa, and more recent studies have given a cell width of 5–10 μm for it (Ramanathan 1964, Ettl and Gärtner 1995, Lokhorst 1996, John 2002). Ramanathan (1964) and John (2002) reported as distinctive of this species the presence of a glistening sheath, a character that, however, was not mentioned by either Klebs (1896) or Smith (1933). A detailed taxonomic reassessment is desirable for *K. klebsii*. However, a great obstacle to this is the fact that it will be impossible to obtain any molecular data from authentic material. No collections of this species attributable to either Klebs or Smith are available. This species is defined only on the basis of Klebs' published treatment (Silva et al. 1972).

For these reasons, currently, we prefer not to provide species-level identification for most of our specimens from European cities. We believe that it will be appropriate to do so only after the completion of a taxonomic reassessment of *Klebsormidium* based on an extensive amount of molecular work, including further sampling from type localities, sequencing of type specimens, and additional molecular data sets based on sequences of other loci.

Our results indicate that the simple morphology of *Klebsormidium* does not reflect the phylogenetic diversity of this genus. The topology of our *rbc*L tree is in basic agreement with the ITS data presented by Sluiman et al. (2008). Due to the much larger taxon sampling, however, our study provides a more complete picture and includes several evolutionary lineages missing in the study of Sluiman et al. (2008). On the basis of our results, it is impossible to identify morphological characters that can be considered good phylogenetic markers: in our *rbc*L phylogeny, none of the characters commonly used for classification and species identification are associated with well-supported monophyletic groups. This fact is also highlighted in that our *rbc*L trees show a substantially different topology from the parsimony trees based on morphological characters presented by Lokhorst (1996). The inclusion of sequences of other strains (especially from outside Europe) might bring to light the existence of new evolutionary lineages and result in a better resolution of some internal branches of our tree, which might eventually allow the identification of morphological synapomorphies. Although most of our clades are supported by high bootstrap values and posterior probabilities, the relative position of some is unclear; this is the case, for example, for *K. bilatum*, *K. elegans,* and *K. mucosum*. These species (and *K. crenulatum*, which has not been possible to include in our phylogeny) share a number of morphological traits that distinguish them from other *Klebsormidium* species, such as thicker filaments, formation of cell doublets, cell walls becoming rough and thickened in old filaments in *K. crenulatum* and *K. mucosum*, and chloroplast with incised or constricted margins in *K. bilatum* and *K. elegans*. If these species represent a monophyletic group, these features could be considered good morphological synapomorphies, but further data are necessary. In ITS rRNA-based phylogenies, these species indeed produce a monophyletic group (Sluiman et al. 2008, Thomas Friedl, personal communication), but without bootstrap support. More generally, the definition of species in this clade and in the whole genus will need to be reassessed. For example, *K. bilatum* and *K. elegans* were described by Lokhorst (1996), and the strains sequenced in this study are the type cultures. Their *rbc*L sequences are identical, and the same is the case for ITS sequences (Sluiman et al. 2008). It is therefore highly questionable whether these entities should be considered separate species.

The difficulty in identifying morphological characters of phylogenetic value is even more striking for the characters observed in liquid cultures, for which our results can be interpreted on the basis of Lokhorst's (1996) conclusions. The production in liquid culture of a superficial hydro-repellent layer is the character that Lokhorst (1996) considered the most important at the species level. In our phylogeny, this character occurs in several strains

scattered across separate lineages: clade 3 (Bergen/Hamburg/Konstanz/Graskop), the strain from Florida (which formed a moderately supported clade with *K. flaccidum* SAG121.80), the type of *K. dissectum* (SAG2155), the type of *K. elegans* (SAG7.96), and *K. flaccidum* from Galway. With regard to this character, the position of the last two strains is noteworthy. *K. flaccidum* from Galway forms a well-supported clade with the type culture of *K. fluitans* (SAG9.96); these strains had an almost identical *rbc*L sequence (2 bp difference). While the Galway strain produced the superficial layer in certain culture conditions, in *K. fluitans* this feature is typically absent (Lokhorst 1996). The genetic similarity of *K. elegans* and *K. bilatum* has been discussed above; remarkably, the superficial layer (present in *K. elegans*, absent in *K. bilatum*) is one of the main characters that Lokhorst (1996) used to separate these species.

The tendency to break into short fragments in liquid cultures is a well-known phenomenon, reported in many studies of *Klebsormidium* (Gay 1891, Klebs 1896, Lokhorst 1996, Dřímalová and Poulíčková 2003). Its taxonomic value, however, is unclear. Our results suggest that its taxonomic and phylogenetic relevance is probably very limited. Our clade 1 includes 11 strains with almost identical *rbc*L sequence, some of which showed rapid and intense fragmentation (Marseilles, Pisa, and La Valletta), whereas others retained for years a morphology of long filaments (e.g., Copenhagen, Prague, and Siena). Furthermore, in fragmented strains, the extent of fragmentation was affected by the culture conditions. In general, the fragmentation was more pronounced at higher temperatures and irradiances, and in JM than in BBM. Effects of the culture conditions were also noted by Dřímalová and Poulíčková (2003), who reported that the extent of fragmentation in *K. flaccidum* increases with decreasing N/P ratio in the culture medium.

Reproductive characters observed in culture might possibly bear more phylogenetic significance. Our strain from Galway (the only one in which zoosporulation was easily induced) was in this regard very similar to *K. fluitans* SAG9.96, with which it formed a well-supported clade; the presence of sporelings with bipolar germination in Galway was the only difference between these strains. However, other recent studies reported that some of these features, in particular, the size of the release aperture, may also be influenced by environmental conditions (Škaloud 2006). We feel that the importance of these characters merits further investigation, but robust conclusions will be possible only after additional data sets of molecular data are available and all the common species of *Klebsormidium* are well characterized from a molecular point of view. This problem is particularly obvious for *K. flaccidum*. In our phylogeny, strains of this species occur in several separate clades. This finding suggests that there is great confusion in the circumscription of this species, and, at present, it would be speculative to decide which of these clades represents the real *K. flaccidum*. The logical solution to clarify such confusion would be to obtain sequences from the type specimen of *Ulothrix flaccida* (L 93967905). If this is not possible, a realistic alternative would be to make extensive collections of *Klebsormidium* from the streets of Strasbourg, isolate them in culture, and sequence them. This is a particularly important aspect, as *K. flaccidum* is the type species of *Klebsormidium,* and its characterization may have substantial taxonomic and nomenclatural implications for the whole genus. Evidence from ITS sequences indicates that species of *Interfilum* are nested in *Klebsormidium* and render it paraphyletic (Thomas Friedl, personal communication), separating the large clade from the small clade containing the strains of K. *flaccidum* 335-7, 12.91, and 7.91 from SAG. If strains of the small clade represent the real *K. flaccidum*, the species of the large clade would have to be transferred to a new separate genus. In fact, a genus-level separation might be an appropriate solution regardless of the position of *Interfilum*. The pair-wise distances between the strains of the small clade and those of the large clade are in a range (11%–13%) that may justify such a separation.

A similar molecular characterization will be necessary for other species with no available neotype cultures. More generally, all species of *Klebsormidium* for which no molecular data are currently available will need to be sequenced. We also believe that a definitive clarification of the phylogeny of *Klebsormidium* will require a considerable amount of fieldwork and collections from a wide range of natural and artificial habitats. Since our analyses have shown a higher genetic diversity than suggested by the morphology, there is the realistic possibility that some lineages with wide geographic distribution, which have not yet been discovered, may exist in nature. Finally, the availability of further molecular data sets will play a fundamental role. The *rbc*L phylogeny presented here is supported by a strong phylogenetic signal, and its conclusions are in basic agreement with analyses of ITS sequences (Sluiman et al. 2008, Thomas Friedl, unpublished data). Analyses of multiple data sets, however, can be expected to clarify relationships in the internal parts of the trees that are unresolved in single-locus phylogenies. The use of more variable molecular markers (e.g., chloroplast spacers that are currently being tested for DNA barcoding in flowering plants, Kress et al. 2005) can also be expected to be of great help to define species-level boundaries in *Klebsormidium*.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Details of the strains of *Klebsormidium* used in the study.

Table S2. Primers used for amplification of the *rbc*L gene.

This material is available as part of the online article.

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