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ORIGINAL ARTICLE

Ecological Differentiation of Cryptic Species within an Asexual Protist Morphospecies: A Case Study of Filamentous Green Alga *Klebsormidium* (Streptophyta)

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ABSTRACT

Taxa of microbial eukaryotes defined on morphological basis display a large degree of genetic diversity, implying the existence of numerous cryptic species. However, it has been postulated that genetic diversity merely mirrors accumulation of neutral mutations. As a case taxon to study cryptic diversity in protists, we used a widely distributed filamentous genus, *Klebsormidium*, specifically the lineage E (*K. flaccidum*/*K. nitens* complex) containing a number of morphologically similar strains. Fourteen clades were recognized in the phylogenetic analysis based on a concatenated ITS rDNA + *rbc*L data set of more than 70 strains. The results of inferred character evolution indicated the existence of phylogenetic signal in at least two phenotypic characters (production of hydro-repellent filaments and morphology of zoosporangia). Moreover, the lineages recovered exhibited strong ecological preferences to one of the three habitat types: natural subaerial substrata, artificial subaerial substrata, and aquatic habitats. We interpret these results as evidence of existence of a high number of cryptic species within the single morphospecies. We consider that the permanent existence of genetically and ecologically well-defined cryptic species is enabled by the mechanism of selective sweep.

SYSTEMATICS and species delimitation of protists has become a very controversial topic in the last 15 yrs, primarily in relation to the neutral model of ubiquitous dispersal of microorganisms raised by Finlay and Clarke (1999). Specifically, it has been postulated that global protist species diversity is much lower than for macroscopic animals and plants because most small-sized protist species have global dispersal (Fenchel 2005; Fenchel and Finlay 2004). According to this theory, the small size, extremely large populations, and high dispersal potential of protist species, should facilitate migration, making allopatric speciation almost impossible (Finlay 2002; Finlay and Fenchel 2004). Conversely, extraordinary high global protist diversity has been implied by Foissner (1999), based mainly on the observed restricted distribution of "flagship" species, i.e. species with easily recognizable morphologies whose presence/absence can be easily demonstrated (Foissner 2006, 2008). Moreover, Casteleyn et al. (2010) recently provided new evidence that allopatric speciation is possible, even in microscopic organisms with large population sizes and high dispersal potential. Consequently, the estimated total species richness differs considerably according to the viewpoint considered. Whereas Finlay and Fenchel (1999) estimated that there are approximately 20,000 protist species, Foissner (1999) estimated that there might be 30,000 species of ciliates alone. This controversy could be mainly caused by the use of different species concepts to describe protist species, by our poor knowledge of real protist diversity in most natural ecosystems (Caron 2009), or by the existence of rather smooth continuum of differences among protist populations (Boenigk et al. 2012).

Recently, many studies based on molecular data revealed a large amount of cryptic diversity within morphologically defined taxa (e.g. Fučíková et al. 2011; Kooistra et al. 2008; Leliaert et al. 2009; Škaloud and Peksa 2010; Škaloud et al. 2012; Šlapeta et al. 2006), which is in obvious contrast with the low presumed protist diversity hypothesis. Accordingly, Fenchel and Finlay (2006) postulated that the use of genetic data brought confusion into the estimations of real diversity in protists. They proposed that the variation in molecular markers reflects the accumulation of neutral mutations over historical times rather than the existence of morphologically indiscernible, cryptic species. Finally, they emphasized the usefulness of the

phenotype as the only proper feature to define real species of protists (Fenchel and Finlay 2006).

In this study, we use a cosmopolitan genus of microchlorophytes as a case taxon to test if the genetic diversity within an asexual morphospecies must be considered as the result of mere accumulation of neutral mutations, without any relation with evolutionary processes. This issue is very important, as it could contribute to our understanding of what a species is. This question is of more than academic interest. For example, better understanding of protist diversity and species richness will contribute to our knowledge about their ecosystem functions, and could indicate how we should act to preserve the earth's natural diversity.

We focused on the genus *Klebsormidium*, a filamentous green alga broadly distributed in terrestrial and freshwater habitats worldwide. This genus is an ideal model for investigating the origin and nature of genetic diversity within the protist morphospecies: it has cosmopolitan distribution, it probably lacks sexual reproduction, and its morphology is, in general, very uniform. Although *Klebsormidium* is one of the most widespread microchlorophytes in the world, the delineation of species boundaries is very problematic. Several morphological characters were used in the past to delimit particular species, including cell width, filament fragmentation, presence of a hydro-repellent layer in liquid media, and features of zoosporangia and pattern of zoospore germination (Ettl and Gärtner 1995; Lokhorst 1996; Printz 1964). However, there is often a large overlap in morphology between *Klebsormidium* species, and some features considered taxonomically important are known to show some variation depending on the age and the physiological conditions of the specimens examined (Lokhorst 1996; Škaloud 2006). These problems are due in part to the fact that the original species descriptions were brief and provided insufficient information to characterize accurately the species (e.g. Kützing 1849); this has lead to different interpretations about the significance of taxonomic characters in the subsequent literature.

Recently, some studies based on molecular data provided insights into the genetic diversity of the genus *Klebsormidium*. First, Novis (2006) performed combined morphological and molecular (*rbc*L sequences) analyses of several strains isolated from streams in New Zealand. On the basis of his results, he described the new species *Klebsormidium acidophilum* Novis. Soon after, Sluiman et al. (2008) sequenced ITS rDNA in 10 strains of *Klebsormidium* obtained mostly from culture collections. Finally, Rindi et al. (2008, 2011) published so far the most extensive molecular investigations of *Klebsormidium* species, producing data sets of *rbc*L and ITS rRNA sequences for many strains isolated from natural and urban habitats in Europe or acquired from culture collections. In general, these molecular investigations showed a high level of genetic diversity within morphologically defined species. Moreover, the data presented in these studies demonstrated a strong incongruence between morphological species circumscriptions and both the ITS rDNA and *rbc*L phylogenies. For example, one of the most commonly identified species, *Klebsormidium flaccidum* (Kützing) P.C. Silva, Mattox & Blackwell, was recovered as highly polyphyletic, occurring in five different clades. In addition, Rindi et al. (2008, 2011) failed to detect any morphological traits that could be used for an unambiguous delimitation of this species. It is clear that an unambiguous circumscription is presently not possible and will require the examination of additional samples, as well as a critical reconsideration of the characters that have been used so far to delimit species in *Klebsormidium*.

As the goal of this study was to investigate the evolutionary processes and meaningfulness of cryptic species in asexual protist morphospecies, the genetic diversity within a monophyletic lineage should be explored. Therefore, we focus on the lineage E sensu Rindi et al. (2011), which contains a number of morphologically similar strains. If the phenotype really represents the best tool for understanding the microbial diversity in the natural world (as stated by Fenchel and Finlay 2006), the substantial molecular diversity revealed within the *Klebsormidium* lineage E should simply reflect the accumulation of neutral mutations within a single, asexual protist species (Finlay et al. 2006). Generalizing this view to all asexual protists, their total species richness should be low, as estimated by Finlay and Fenchel (1999). On the other hand, the detection of even diminutive phenotypic or any functional properties specific to particular genetic lineages could point out the existence of a high number of cryptic species. If this is the case, the real species diversity of asexual protists could be in fact much higher than estimated (e.g. by Adl et al. 2007).

To resolve which of these two possibilities better interprets the detected genetic diversity, we mapped the morphological and ecological features of 71 strains isolated from various habitats in Europe (Rindi et al. 2008; Škaloud 2006) on the phylogeny of the *Klebsormidium* lineage E. To infer the phylogenetic relationships among the strains, we performed a combined analysis of nuclear ITS rDNA and plastid *rbc*L gene sequences. Although the different inheritance of these loci could potentially lead to incongruent topologies in the inferred phylogenetic trees, the analyses presented by Rindi et al. (2011) indicate that in *Klebsormidium*, the phylogenetic signals of ITS rDNA and *rbc*L are congruent, supporting the use of a combined approach.

MATERIALS AND METHODS

Origin and cultivation of investigated strains

Fifty-nine strains were obtained in a recent study that investigated the diversity of *Klebsormidium* in a variety of aeroterrestrial and (semi-)aquatic freshwater habitats in Europe (Škaloud 2006). In addition, 18 strains were obtained from three public culture collections: Culture Collection of Algae, Georg-August University Göttingen (SAG); Culture Collection of Algae and Protozoa (CCAP), Oban, Scotland, UK; and Culture Collection of Algae at The University of Texas at Austin (UTEX). The strains were cultivated on agarized BBM medium (Bischoff and

Bold 1963) at 15 °C under a constant illumination of 50–200 μmol photons/m^2/s provided by 18W cool fluorescent tubes (Philips TLD 18W/33, Royal Philips Electronics, Amsterdam, the Netherlands). Collection data and other characteristics of each strain are listed in Table S1.

Morphological analyses

To obtain morphological data comparable to the phenotypic characteristics of strains isolated from urban habitats by Rindi et al. (2008), we cultivated the strains in the following conditions: A 50–100 cells long fragment of each strain was transferred to glass dishes filled by liquid BBM medium (without neither vitamins nor soil extract added) and cultivated at 15 °C, 16:8 light:dark, 20–30 μmol photons/m^2/s (laboratory thermostat ST1; Pol-Eko-Aparatura, Wodzisław Śląski, Poland). The morphology of *Klebsormidium* strains was examined after 5 wk since the beginning of the cultures, during their exponential growth phase (to avoid depletion of nutrients). The following characters were observed: (1) width of filaments (calculated from at least 40 replicates); (2) growth habit (long filaments; fragmented filaments, with the habit of a green soup; or a mixture of these two growth forms); (3) presence/absence of a superficial layer of hydro-repellent filaments; (4) shape of release aperture in lateral wall of zoosporangial cell (distinct or indistinct); (5) germination pattern of zoospores (unipolar and bipolar or unipolar only); and (6) formation of H-shaped pieces or cap-like remnants of the mother cell wall. Formation of zoospores was stimulated according to the procedure described by Škaloud (2006).

DNA extraction, polymerase chain reaction, and DNA sequencing

Total genomic DNA was extracted from the fresh cultures using the Invisorb® Spin Plant Mini Kit (Invitek, Berlin, Germany). Algal DNA was resuspended in sterile dH_2O and amplified by polymerase chain reaction (PCR). The ITS1-5.8S rDNA-ITS2 region was amplified using the newly designed primers Klebs-ITS-F (5′-GGA AGG AGA AGT CGT AAC AAG G-3′), Klebs-ITS-R (5′-TCC TCC GCT TAG TAA TAT GC-3′), Klebs-ITS1-F (5′-GAA GCT GTG AGA AGT TCA TTA AAC C-3′), and Klebs-LSU-R (5′-CTC TCA CCC TCT CTG ACG TCC CAT T-3′). The *rbc*L gene was amplified using the primers *rbc*L-B (5′-ATG TCA CCA CAA ACA GAA ACT AAA GCA-3′; Zechman 2003) and *rbc*L-Q (5′-GAT CTC CTT CCA TAC TTC ACA AGC-3′; Zechman 2003) or the newly designed primers *rbc*L-KF2 (5′-ACT TAC TAC ACT CCT GAT TAT GA-3′) and *rbc*L-KR2 (5′-GGT TGC CTT CGC GAG CTA-3′). All new primers were designed to specifically amplify streptophyte organisms, especially *Klebsormidium* species. Furthermore, the ITS rDNA primers were designed to avoid the amplification of fungal contaminants. All PCR reactions were performed in 20 μl reaction volumes (15.1 μl sterile Milli-Q Water (Millipore Inc., Bedford, MA), 2 μl 10′ PCR buffer (Sigma St. Louis, MO), 0.4 μl dNTP (10 μM), 0.25 μl of primers (25 pmol/ml), 0.5 μl Red Taq DNA Polymerase (Sigma) (1 U/ml), 0.5 μl of MgCl2, 1 μl of DNA (not quantified)). Polymerase chain reaction was performed in either a XP thermal cycler (Bioer, Tokyo, Japan) or a Touchgene gradient cycler (Techne, Cambridge, UK). Polymerase chain reaction amplification of the ITS rDNA region began with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 1.5 min, with a final extension at 72 °C for 7 min. The amplification of the *rbc*L gene began with the initial denaturation at 95 °C for 2 min, and was followed by 40 cycles of denaturing at 94 °C for 1 min, annealing at 47 °C for 1 min, and elongation at 72 °C for 3 min, with a final extension at 72 °C for 8 min. The PCR products were quantified on a 1% agarose gel stained with ethidium bromide and cleaned with the JetQuick PCR Purification Kit (Genomed, Löhne, Germany) according to the manufacturer's protocol. The purified amplification products were sequenced with the PCR primers using an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730xl) at Macrogen Corp., Seoul, Korea. Sequencing reads were assembled and edited using the SeqAssem programme (Hepperle 2004).

Sequence alignment, model selection, and ITS2 secondary structure

Multiple alignments of the newly determined ITS rDNA and *rbc*L sequences and other sequences selected from the DDBJ/EMBL/GenBank databases were built using ClustalX 1.83 (Thompson et al. 1997) and MUSCLE (Edgar 2004). Congruence between ITS rDNA and *rbc*L data sets was tested using the incongruence length difference (ILD) test (Farris et al. 1995), as implemented by the partition homogeneity test in PAUP* (heuristic search, simple addition, TBR branching swapping, 1,000 replicates). The concatenated data matrix of unique sequences was 1,902 bp long and was 95% filled by the *rbc*L data and 76% filled by the ITS rDNA.

A suitable partitioning strategy and partition-specific substitution models were selected in a multistep process (Verbruggen et al. 2010). Initially, a guide tree was obtained by carrying out a second-level maximum likelihood (ML) search on the unpartitioned data set with a HKY + Γ_8 model using TREEFINDER (Jobb et al. 2004). Then, the data set was divided by 11 different partitioning strategies (combining different levels of loci segmentation). Subsequently, Bayesian Information Criterion (BIC) calculations were performed for all 11 potential partitioning strategies, assuming the guide tree and HKY + Γ_8 model for each partition. The three best-scoring partitioning strategies (lowest BIC scores) were retained for further analysis. These were: (1) ITS region, first codon position of *rbc*L, second codon position of *rbc*L, third codon position of *rbc*L, (2) ITS region, first and second codon position of *rbc*L, third codon position of *rbc*L, (3) ITS1 + ITS2, 5.8S rDNA, 1st codon position of *rbc*L, second codon position of *rbc*L, third codon position of *rbc*L. In the next step, models of sequence evolution were selected for individual partitions using the BIC. For each partition present in the

three retained partitioning strategies, 12 different nucleotide substitution models were evaluated (F81, HKY, GTR, and their combinations with Γ, I, and Γ + I). Finally, the partitioning strategies were re-evaluated using the selected models for the particular partitions. This BIC-based model selection procedure selected the following model with five partitions: (1) first codon position of the *rbc*L gene – GTR + G, (2) second codon position of the *rbc*L gene – F81, (3) third codon position of the *rbc*L gene – HKY + G, (4) internal transcribed spacers ITS1 and ITS2 – HKY + G, and (5) 5.8S ribosomal locus – F81.

The ITS2 secondary structure of the selected *Klebsormidium* strain (CAUP J302) was constructed using the mfold computer program v. 2.3 (Zuker 2003), with folding temperature set to 18 °C. To obtain secondary structures for all *Klebsormidium* strains, we used homology modelling based on the CAUP J302 ITS2 secondary structure selected as a template, using the ITS2 Database v. 3.0.5. (Koetschan et al. 2010). Consensus secondary structure (Fig. S1) was drawn using VARNA (Darty et al. 2009). Compensatory base change (CBC) matrices were derived by 4SALE (Seibel et al. 2006), according to the sequence-structure alignments refined from the ITS2 Database.

Phylogenetic analyses

New ITS rDNA and *rbc*L sequences were produced for 29 *Klebsormidium* strains. After including additional sequences deposited in GenBank, the final data matrix consisted of 77 sequences. Selection of the additional sequences was based on information published in recent studies (Mikhailyuk et al. 2008; Rindi et al. 2008, 2011). The phylogenetic tree was inferred by Bayesian inference using MrBayes version 3.1 (Ronquist and Huelsenbeck 2003). The analysis was carried out on partitioned data set using the strategy selected in a multistep process described above. All parameters were unlinked among partitions. Two parallel MCMC runs were carried out for 5 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was checked and burn-in was determined using the "sump" command. Bootstrap analyses were performed by ML and weighted parsimony (wMP) criteria using GARLI, version 0.951 (Zwickl 2006) and PAUP*, version 4.0b10 (Swofford 2002) respectively. Maximum likelihood analyses consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100,000). The wMP bootstrapping (1,000 replications) was performed using heuristic searches with 100 random sequence addition replicates, Tree bisection reconnection swapping, random addition of sequences (the number limited to 10,000 for each replicate), and gap characters treated as a fifth character state.

Character evolution

For each of the measured morphological characters, we tested the existence of phylogenetic signal by conducting two different tests. First, we fitted Pagel's lambda using ML (Pagel 1999). This test uses a tree transformation parameter that has the effect of gradually eliminating phylogenetic structure. The ML optimization of lambda value was performed using the "fitDiscrete" or "fitContinuous" functions of the Geiger package (Harmon et al. 2008). To test for the existence of phylogenetic signal in the data set, we compared the negative log-likelihoods obtained from a tree without phylogenetic signal ($\lambda = 0$) and the original topology, using likelihood ratio test. Second, the phylogenetic signal was tested using *K* statistic (Blomberg et al. 2003). This statistics quantify the phylogenetic signal by estimating the accuracy of the original phylogeny to describe the variance–covariance pattern observed in the data set. The *K*-value and randomization test were calculated by "Kcalc" and "phylosignal" functions of the Picante package (Kembel et al. 2010). All calculations were done in the program R, ver. 2.9.2 (The R Foundation for Statistical Computing 2009, http://www.r-project.org/).

The evolution of morphological characters was traced along the tree using maximum parsimony in the Mesquite software package (Maddison and Maddison 2006). Along with the morphological data obtained in this study, published morphological observations were used to characterize following strains: Lokhorst KL1, Lokhorst KL37, SAG 5.96, SAG 6.96, SAG 7.96, SAG 8.96 (Lokhorst 1996); Novis K25, Novis K37, Novis K48, Novis KM, Novis KRIV (Novis 2006); and Novis LCR-K2 (Novis et al. 2008). The evolution of habitat preferences was reconstructed in the program BayesTraits (Pagel and Meade 2006). The ancestral state probabilities for the three habitat preferences (natural substrate, artificial substrate, freshwater) were calculated for each node using BayesMultiState in an ML framework (using the "addNnode" command). We adjusted the "MItries" parameter to 100 to increase the number of optimalization attempts. The BayesTraits output was mapped onto the reference tree with TreeGradients v1.03 (Verbruggen 2009). This program plots ancestral state probabilities on a phylogenetic tree as colours along a colour gradient. Comparisons among the means of cell widths accounted for the three habitat types were examined using Sheffé Test in Statistica ver. 8 (StatSoft, Inc., Tulsa, OK).

RESULTS

Morphological data

The morphological characteristics of 65 *Klebsormidium* strains, investigated both in this study and by Rindi et al. (2008, 2011), are summarized in Table S1. The filaments were uniseriate and unbranched, with a smooth cell wall (Fig. 1). The strains consisted of either tufts of long filaments (typically more than 150 cells long) or a cocktail of short fragments (typically 10–50 celled). However, in many strains, a mixture of the two habits was observed. In the majority of the strains, a superficial layer of hydro-repellent filaments with parallel arrangement was produced (Fig. 2). In all strains, the cells were cylindrical, without any

Figure 10 Phylogenetic relationships among the *Klebsormidium* lineage E strains. The phylogenetic tree was inferred from the combined *rbc*L + ITS rDNA data set using Bayesian analysis under a partitioned, unrooted model. Values at the nodes indicate statistical support estimated by three methods – MrBayes posterior node probability (left), maximum likelihood bootstrap (in the middle), and maximum parsimony bootstrap (right). Thick branches represent nodes receiving high posterior probability support ($\geq$ 0.99). Sequences determined in this study are given in bold. Strain affiliation to 14 lineages (1–14) and six clades sensu Rindi et al. (2011) (E1–E6) is indicated. Scale bar – expected number of substitutions per site.

pronounced constrictions at the transverse cell walls. In very young, germinating filaments, mucilaginous sheets covering the cell walls were occasionally observed. The average cell width was strain-specific, ranging from 4.4 to 8.0 μm. Although the strains with thin filaments (e.g. K07, K13) were morphologically well discernible from those having broad cells (e.g. GALW015499, TR 18), we observed a continuum within the range of cell width. Thus, no evident groups of strains with markedly different widths could be discerned on the basis of cell width measurements.

Each cell contained one parietal chloroplast with smooth or slightly lobed margin, which encircled a half to 2/3 of the cell wall and was extended for the whole length of the cell. Incisions described for *Klebsormidium elegans* and *Klebsormidium bilatum* (Lokhorst 1996) were not observed. The chloroplast contained usually one pyrenoid surrounded by a distinct layer of starch grains. In several strains, the vegetative cell division gave rise to the formation of specific cell wall structures. During the process of growth of the daughter cells, the nonelastic mother cell wall was ruptured, leading to the formation of either H-pieces (Fig. 3) or mother cell wall caps (Fig. 4, 5). Interestingly, similar structures (mother cell wall "halves") have been recently reported for the closely related genus *Interfilum* (Mikhailyuk et al. 2008).

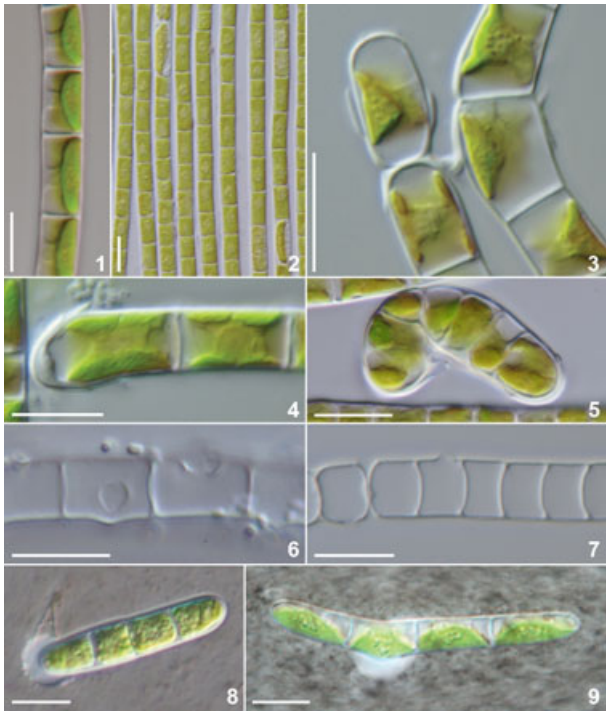


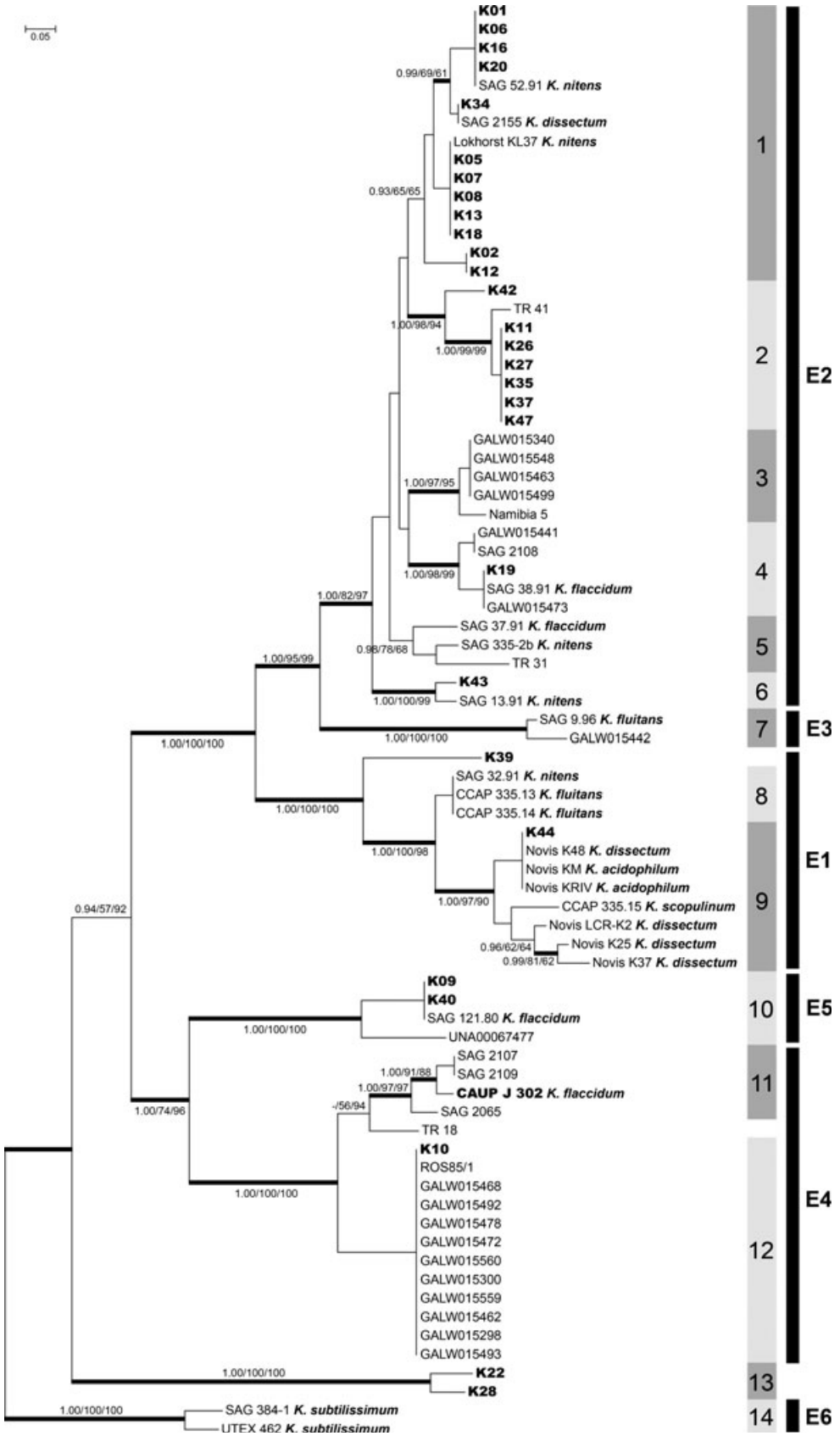
Figure 1–9 Morphological features of investigated *Klebsormidium* strains. **1.** Typical morphology of vegetative filament composed of cylindrical cells containing one chloroplast (K10). **2.** Superficial layer of hydro-repellent filaments with parallel arrangement (SAG 2155). **3.** H-shaped wall remnants (K 27). **4.** Cap-shaped wall remnant (SAG 9.96). **5.** Short filament wrapped by mother cap-shaped cell wall remnants (K 44). **6.** Distinct apertures in zoosporangial cell wall (K19). **7.** Indistinct apertures in zoosporangial cell wall (SAG 121.80). **8.** Unipolar germination of zoospores (K 19). **9.** Bipolar germination of zoospores (K 22). Scale bars = 10 μm.

The release of zoospores was successfully induced in 28 strains. In all these strains, the structure of release apertures in empty zoosporangial cell wall and the zoospore germination were observed. In 22 strains, the apertures were distinct and could be easily recognized already at lower magnification. The apertures were spherical, showing a distinctive margin (Fig. 6). In the rest of the strains, the margin of the aperture was inconspicuous and discernible only at high magnification (1,000X) (Fig. 7). Furthermore, two types of zoospore germination were observed. In 23 strains, the zoospores germinated exclusively with unipolar pattern (Fig. 8). In other strains, the prevailing unipolar germination was accompanied by the bipolar germination of some zoospores in the same cultures (Fig. 9). When bipolar germination occurred, the young, germinating filaments were often bent, even including those with unipolar pattern.

Molecular data and phylogenetic analysis

The final data matrix used for the analyses consisted of 77 sequences. The *rbc*L data provided most characters (1,181 bp) from the total of 1,902 base pairs analysed. The ITS rDNA and *rbc*L loci differed considerably in the amount of phylogenetic signal. The *rbc*L data set contained significantly higher amount of parsimony informative sites (236, corresponding to 20% of the characters analysed) than the ITS region (55, corresponding to 7.6%). Visual inspection of single-locus trees revealed that all moderately to well-supported groups (receiving above 70% bootstrap) were monophyletic in both the ITS rDNA and *rbc*L trees. The data sets did not show strong support for the conflicting rival nodes. Moreover, according to the partition homogeneity test (ILD test), the ITS rDNA and *rbc*L data sets were not significantly heterogeneous ($p = 0.86$), justifying a combined data approach.

Fourteen lineages (indicated as 1–14) were recognized in the concatenated ITS rDNA + *rbc*L phylogeny of the *Klebsormidium* lineage E (Fig. 10). With the exception of a newly identified lineage 13, each resolved lineage could be identified as a portion of one of the six highly sup-



ported clades E1–E6 sensu Rindi et al. (2011). The phylogeny revealed considerable variability within the genus *Klebsormidium*, which did not reflect the traditional assessment of its diversity based on morphological data. For example, four strains deposited in culture collections under the name *K. flaccidum* (CAUP J 302, SAG 37.91, SAG 38.91, SAG 121.80) occurred within four separate lineages in the phylogram (Fig. 10). Similarly, strains identified morphologically as *Klebsormidium nitens* and *Klebsormidium dissectum* were also polyphyletic, occurring in four or two different lineages respectively.

Evolution of morphological characters

To determine whether particular *Klebsormidium* lineages could be characterized by specific phenotypic properties, we tested for the existence of phylogenetic signal in all measured morphological characters. Pagel's lambda and *K*-statistics calculations revealed significant phylogenetic signal for four of the six characters (Table 1). Variance of neither zoospore germination nor growth habit was significantly correlated by inferred phylogeny. However, both statistic calculations indicated strong signal for the production of hydro-repellent superficial layer (Fig. 11). A more or less developed superficial layer was observed in a majority of strains. However, this characteristic growth form was completely or mostly absent in the strains of the lineages 4, 9, and 12.

A strong phylogenetic pattern was detected for the structure of release apertures in zoosporangial cell wall, as well (Fig. 12). The majority of lineages exhibited exclusively the production of distinct apertures in empty zoosporangial cell walls. On the other hand, the lineages 7, 10, and 13 could be characterized by the indistinct apertures. Furthermore, the existence of phylogenetic signal was revealed in the formation of H-shaped pieces and cell wall remnants. However, this feature seemed to have low potential to define particular *Klebsormidium* lineages (Fig. 13). Although some lineages could be well defined by their ability to produce H-shaped pieces on cell wall constrictions (8, 14), most lineages contained strains showing both ability and incapability to form these cell wall structures.

Despite the existence of significant phylogenetic signal in cell width variance, the parsimony reconstruction of its evolution showed only partial usefulness of this character to characterize particular genetic lineages (Fig. 14). The *Klebsormidium* strains with higher average cell width were spread through several lineages (2, 3, 7, 9, 12). Moreover, the cell width exhibited rather large variability among genetically identical strains. For example, the average cell width of genetically uniform strains K19, SAG 38.91, and GALW015473 (clade 4) was 5.9, 5.6, and 7.2 μm respectively. On the other hand, some lineages included strains morphologically very similar, and could be easily distinguished from the closely related lineages on the basis of their average cell width. For example, the strains belonging to the lineage 1 were clearly thinner than the strains forming the related lineage 2. Similarly, the lineage 7 could be clearly distinguished on the basis of the high average cell width (7.4 μm).

Table 1. Statistics for randomization tests showing the significance of phylogenetic signal for the variables investigated. For each trait, Pagel's lambda and *K*-statistics were calculated to show influence of inferred phylogeny (Fig. 1) on trait variance across *Klebsormidium* strains

	Pagel's lambda			*K*-statistics	
Trait	λ	Likelihood ratio	*p*-value	*K*-value	*p*-value
Superficial layer	0.985	1.69	**< 0.0001**	0.00461	**0.001**
Apertures	1.000	2.57	**< 0.0001**	3.82795	**0.001**
Germination	0.769	1.14	0.0675	0.00090	0.251
H-pieces	0.998	1.21	**0.0002**	0.00178	**0.001**
Habit	0.956	1.11	**0.0001**	0.00069	0.168
Cell width	0.901	1.30	**< 0.0001**	0.00228	**0.001**
Habitat	1.000	2.51	**< 0.0001**	0.66164	**0.001**

Lambda values could vary from 0 (no influence of phylogeny) to 1 (strong phylogenetic influence). Likelihood ratio indicates comparison of the log-likelihoods of a model with the maximum likelihood estimate of lambda for a given trait to the log-likelihood of a model where lambda was set to zero. The *K*-values indicate how perfectly is the species trait correlated to the phylogeny as expected under Brownian motion (higher *K*-values mean better correlation). Significant traits are given in bold.

Mapping the habitat preferences

In addition to several above-mentioned phenotypic properties, Pagel's lambda and *K*-statistics calculations detected significant habitat preferences for particular *Klebsormidium* lineages (Table 1), which allowed mapping the evolution of the habitat preferences along the phylogenetic tree (Fig. 15). Almost all lineages exhibited strong ecological preferences for one of the three selected habitat types: natural subaerial substrata (soil, wood, rock), artificial subaerial substrata (concrete), and aquatic habitats (submerged in freshwater). A preference for natural substrata was detected for the lineages 1, 2, and 10, whereas the organisms of the lineages 3, 4, 7, and 12 significantly inhabited the artificial substrata. Preference for aquatic habitats was associated with the lineages 5, 8, 9, 13, and 14. Moreover, further habitat associations of particular lineages appeared to be present within these three habitat types. For example, whereas the lineage 10 comprised strains isolated from either moss vegetation or bark surface, almost all strains from the lineages 1 and 2 grew on the rock surface or in the soil. Similarly, the lineages 9 and 13 were differentiated for their distribution in streams or peat bogs respectively.

DISCUSSION

The results presented here demonstrated the existence of phenotypic and ecological traits specific to particular genetic lineages within the *Klebsormidium* lineage E

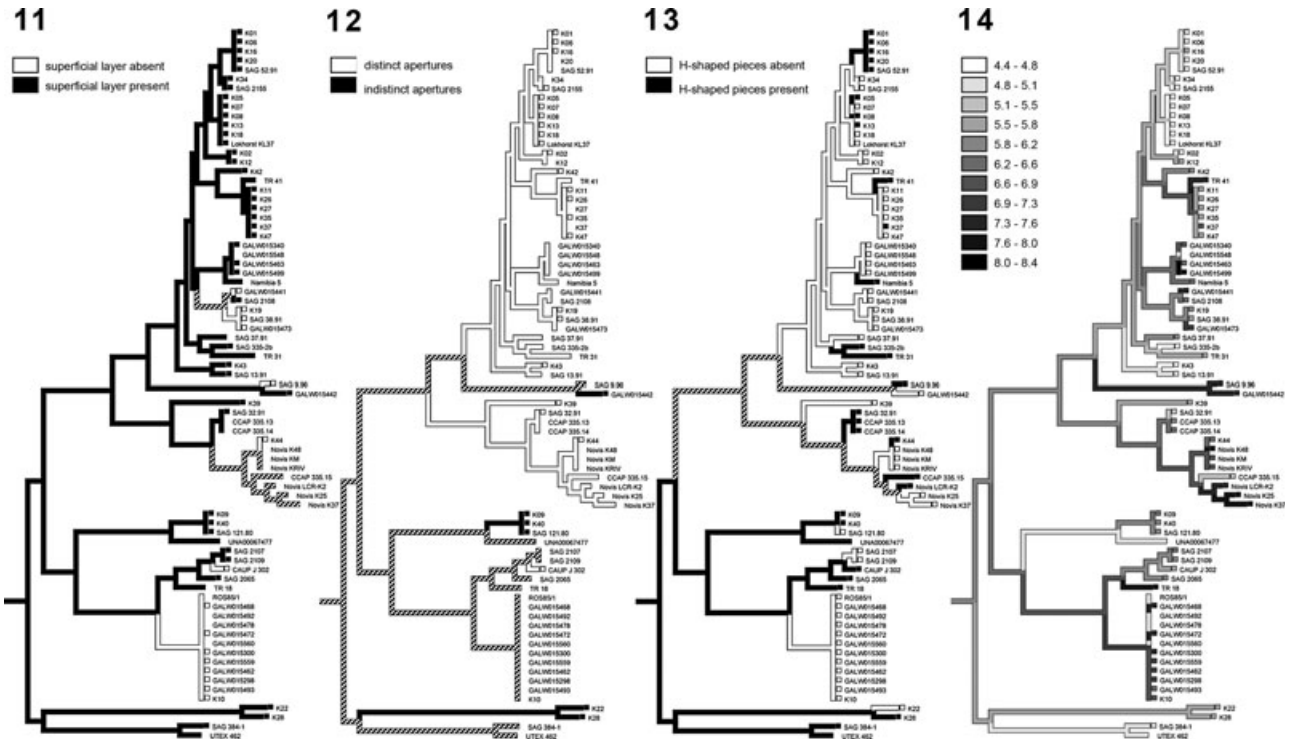


Figure 11–14 Evolution of morphological characters mapped onto the phylogenetic tree. Ancestral traits were reconstructed using maximum parsimony. **11.** Superficial layer of filaments. **12.** Release apertures. **13.** Formation of H-shaped pieces. **14.** Average cell width (μm).

sensu Rindi et al. (2011). In general, we detected a strong phylogenetic pattern of habitat preferences. In strains growing aerophytically, we detected a differentiation between genotypes growing on natural and artificial substrata. We believe that this separation reflects different physiological attributes. In the case of *Klebsormidium*, several detailed studies have been published recently (Holzinger et al. 2011; Kaplan et al. 2012; Karsten and Holzinger 2012; Karsten and Rindi 2010; Karsten et al. 2010). These, however, have mostly focused on individual strains and at this stage their results, although very interesting, cannot be generalized to whole lineages. The study of Kaplan et al. (2012) provides the most useful information. These authors examined the osmotic potential in a strain of *Klebsormidium crenulatum* isolated from alpine soil crusts and a strain of *K. nitens* isolated from a concrete panel in the city of Innsbruck. Their molecular analyses show that the strain of *K. nitens* belongs to the lineage 3 of our phylogenetic tree (which indeed includes strains isolated from artificial surfaces). Kaplan et al. (2012) demonstrated that these algae have extraordinarily negative osmotic potentials and *K. nitens* had a broader amplitude to tolerate osmotic water stress than *K. crenulatum*, and concluded that this could explain its survival on the concrete panel where it was isolated (whereas *K. crenulatum* was found in a soil crust, which presumably has better water-retaining capacities). Other attributes, such as preference for substrata with different pH (Hoffmann 1989), soil chemical properties (Starks and Schubert 1982), or textures might also play an important role, but conclusions in this regard require experimental data, which are presently not available.

The results of habitat mapping suggest that certain lineages are able to switch easily between different habitats, whereas this is not the case for other lineages. Moreover, the capacity to switch between freshwater and natural aerophytic habitats seems to be more frequent. Whereas the habitat switch towards the aerophytic habit was detected in three aquatic lineages (5, 6, 9), the switch between natural and artificial aerophytic habits was detected only once within the lineage 11.

Among the morphological characters, statistical tests and character mapping revealed a strong phylogenetic pattern in the ability to produce a superficial layer of hydrorepellent filaments and the structure of release apertures in zoosporangial cell wall. By contrast, the formation of H-shaped pieces and cell width could be used for the characterization of only a few lineages. Our data indicate that cell width may be influenced by the environmental conditions, mainly the pH. Within the lineage 9, the cell width of four strains with identical *rbc*L sequences (Novis K48, Novis KM, Novis KRIV, K44) was correlated with the pH of the environment. According to the field and culture investigations of Novis (2006), the cell width of a strain isolated from the neutral stream (Novis K48, cell width 8–9 μm) was consistently larger than that of two strains isolated from the acidic streams (Novis KM, Novis KRIV; cell width 6–8 μm), as well as of the strain K44, isolated

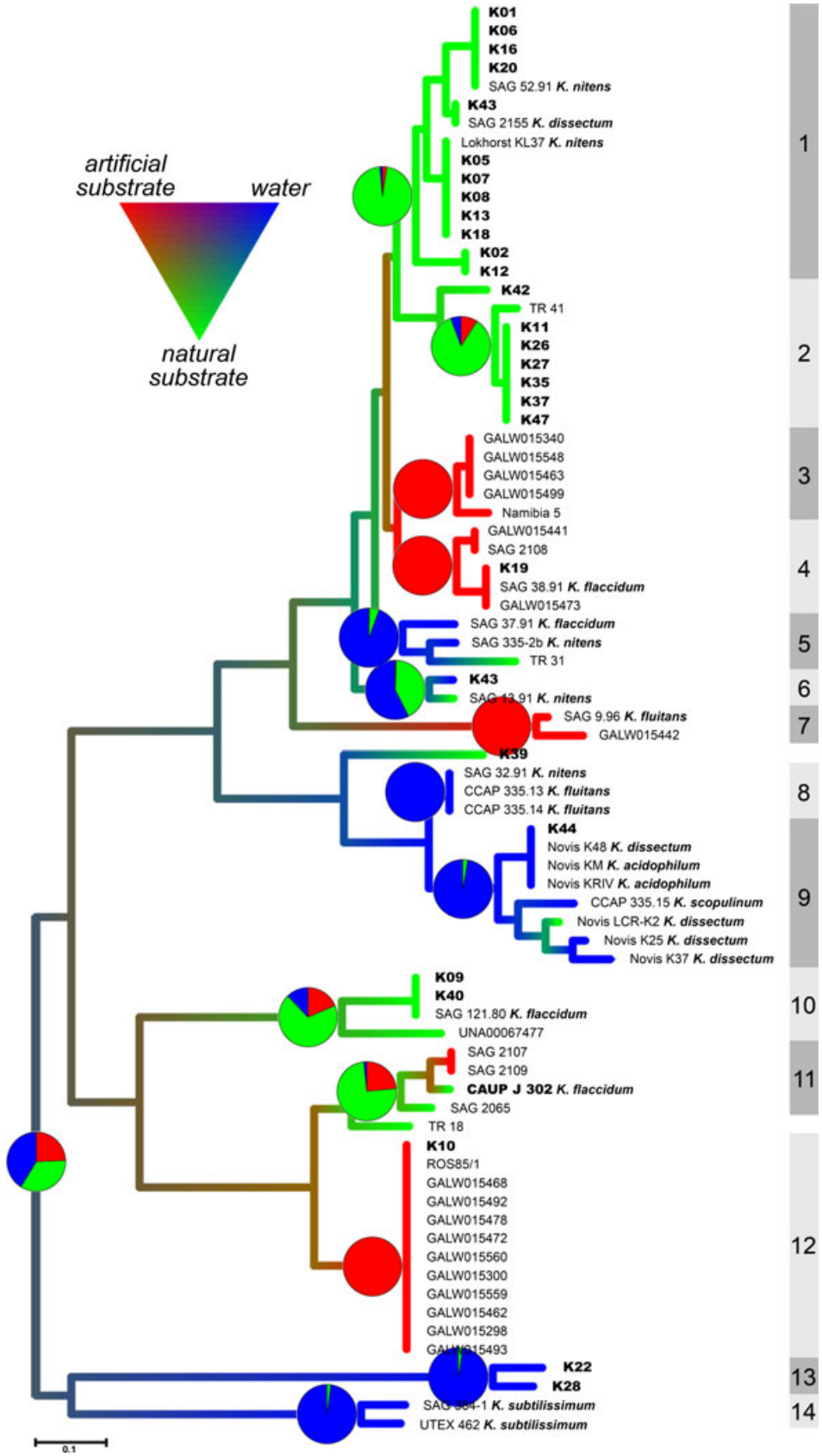
Figure 15 Estimated evolution of habitat preferences, showing strong ecological preferences to one of the three selected habitat types: natural substrates (soil, wood, rock), artificial substrates (concrete), and aquatic habitat (submerged in freshwater). Pie charts indicate the ancestral maximum likelihood probabilities of three habitat types, reconstructed for selected nodes receiving high statistical support.

from an acidic peat bog (average cell width 6.3 μm). Moreover, according to the Sheffé Test comparing the means of cell widths accounted for the three habitat types, the average cell width of strains growing on artificial substrata (6.93 μm) was significantly larger than the cell width of strains growing on both natural aerophytic (5.58 μm) and aquatic (6.07 μm) habitats ($p < 0.001$). Accordingly, although the cell width traditionally represents one of the most used morphological features to distinguish species in green filamentous algae, at least in *Klebsormidium* it could also be affected by environmental conditions. Interestingly, since the average cell width was measured on cultured strains, our data indicate that these phenotypic morphological differences could be at least for some time retained in culture, despite identical culture conditions.

Recently, several studies revealed the significant ecological differentiation of particular genotypes within morphologically defined protist species. First, de Vargas et al. (1999) uncovered the existence of three distinct genotypes within the foraminiferan species *Orbulina universa*, whose distributions were primarily correlated to the productivity of the surface waters. Ecological differentiation of distinct genotypes within nominal protist morphospecies has been subsequently reported for another foraminiferan species (de Vargas et al. 2002; Kucera and Darling 2002), for ciliates (Finlay et al. 2006), flagellates (Koch and Ekelund 2005; Lowe et al. 2005; Pfandl et al. 2009), and diatoms (Casteleyn et al. 2010; Poulíčková et al. 2008; Vanelslander et al. 2009). Habitat preferences were also noted for some *Klebsormidium* lineages (Rindi et al. 2011). In all the above-mentioned studies, the ecological differentiation, as well as slight morphological differences of particular genotypes, led authors to consider these genotypes as different cryptic species.

In the case of eukaryotic green microalgae, ecological and physiological traits have been largely neglected in comparison with other types of characters. Some studies published in recent years, however, have strongly indicated that ecological features may bear phylogenetic significance and in some cases may be used to define taxa. Fawley et al. (2005), describing the new genus *Meyerella*, remarked that this alga has a strikingly different seasonal distribution in comparison to other morphologically similar planktonic microchlorophytes. For *Prasiola* (a widespread genus of leafy and filamentous Trebouxiophyceae), Rindi et al. (2007) showed a striking separation of three clades formed by species associated with different environments (marine, freshwater, and terrestrial). Still for this genus, Moniz et al. (2012) described the new species *Prasiola glacialis* using the habitat occupied as primary character distinguishing it from species with similar morphology. Peksa and Škaloud (2011) demonstrated that the particular lineages within the lichen photobiont genus *Asterochloris* exhibit clear environmental preferences, such as for the rain and sun exposure, substrate, and climatic preferences. For *Klebsormidium*, the recent studies have shown ecophysiological differences among different strains, which were correlated with the habitats in which these algae occurred (Holzinger et al. 2011; Kaplan et al. 2012; Karsten and Holzinger 2012; Karsten and Rindi 2010; Karsten et al. 2010). Karsten and Holzinger (2012) regarded *K. crenulatum* (a species with thick filaments and slower growth) as a K-strategist and *K. dissectum* (a thinner species) as an r-strategist (unfortunately these authors did not present molecular data that give the possibility to refer their strain of *K. dissectum* to any lineages of our phylogeny).

Despite two and a half centuries of study, definition and circumscription of species in green microalgae remains one of the most problematic areas in eukaryotic microbiology. The importance of molecular data is now universally accepted, but the interpretation of phenotypic data is subject to a margin of subjectivity (especially in the case of subtle morphological differences, as observed for several characters in *Klebsormidium*). Overall, our results and the information available from previous studies (Novis 2006; Rindi et al. 2008, 2011) strongly indicate that ecological traits are reliable and phylogenetically significant in *Klebsormidium*. For this reason, we consider them suitable for the circumscription of taxa within this genus. A complete taxonomic reassessment is better left to a subsequent stage in which more complete ecophysiological data will be available for all lineages. Once these data become available, we feel that to recognize the 14 lineages of our phylogenetic tree as separate species, distinguished on molecular and ecological basis, will be the appropriate solution. Some striking biogeographical associations can be noted in our lineages. For example, the lineage 9 is formed entirely by strains from New Zealand, and the lineage 5 consists also of extra-European samples (strains from Australia, Peru and U.S.). Other associations of this type might become evident with additional taxon sampling; generalizations in this regard, however, look premature, as the vast majority of the molecular data currently available for *Klebsormidium* has been obtained from European strains. Biochemical information concerning secondary metabolites in species of *Klebsormidium* would also be valuable. Secondary metabolites have an ecological significance, having evolved as a response of the organism in its interactions with the environment. Some recent studies have shown that in aeroterrestrial green algae, some of these compounds may be used as chemotaxonomic markers (Darienko et al. 2010; Görs et al. 2010; Gustavs et al. 2011). It would be interesting to verify if similar patterns may be detected in *Klebsormidium*.



In conclusion, we interpret the ecological variation detected in morphologically barely distinguishable *Klebsormidium* strains as evidence for the existence of cryptic species. Since this genus lacks sexual reproduction, the separation of species cannot be based on the biological species concept. It has been proposed that even a single CBC in the helices 2 and 3 of the ITS2 secondary structure indicates sexual incompatibility and thus separates biological species (Coleman 2000, 2009). However, none of recognized *Klebsormidium* clades was differentiated by single CBCs (Fig. S1).

On the basis of the results of this study and data for other microalgae, we conclude that the predominantly cosmopolitan distribution of protists does not imply the low species diversity suggested by Fenchel and Finlay (2004). The ecological differentiation facilitates both allopatric (our data; de Vargas et al. 1999) and sympatric (e.g. Amato et al. 2007; Vanelslander et al. 2009; Weisse 2008) speciation of protistan cryptic species. Congruently with the recent studies of Fontaneto et al. (2007) and Birky et al. (2010), our data point to the existence of distinct species units and sympatric speciation in asexual protists. We consider that the permanent existence of genetically and ecologically well-defined cryptic species is enabled by the mechanism referred to as "periodic selection" or "selective sweep" (Atwood et al. 1951; Cohan 2001; Finlay 2004; Smith and Haigh 1974). Given selective sweep, a single ecologically defined cryptic species is not expected to expand its diversity indefinitely. This process should yield a cycling of diversity levels, whereby diversity is accumulated and then a wave of selective sweep crashes the accumulated diversity back to near zero (Cohan 2001). However, if a new mutant differs in its ecological niche, it could escape the diversity-purging effects of selective sweep, giving rise to the new, ecologically defined species.

It is possible that such speciation events took place several times during the evolution of protist genera such as *Klebsormidium*. Particular lineages could be either fully or partially driven out by the process of selective sweep, and subsequently substituted by newly emerged species. Such dynamic and evolutionary fast speciation processes might have generated a large amount of evolutionarily young cryptic diversity, as recently reported for other protist taxa (e.g. Evans et al. 2008; Kooistra et al. 2008; Škaloud and Peksa 2010). It is well possible that rare and little diverse protist lineages represent relicts of originally abundant and diversified lineages, driven nearly to extinction by the rapid evolutionary expansion of closely related lineages. In the Klebsormidiophyceae, this scenario could be well used to interpret the strong differences in the diversities of the closely related genera *Klebsormidium*, *Hormidiella*, and *Entransia* (Sluiman et al. 2008).

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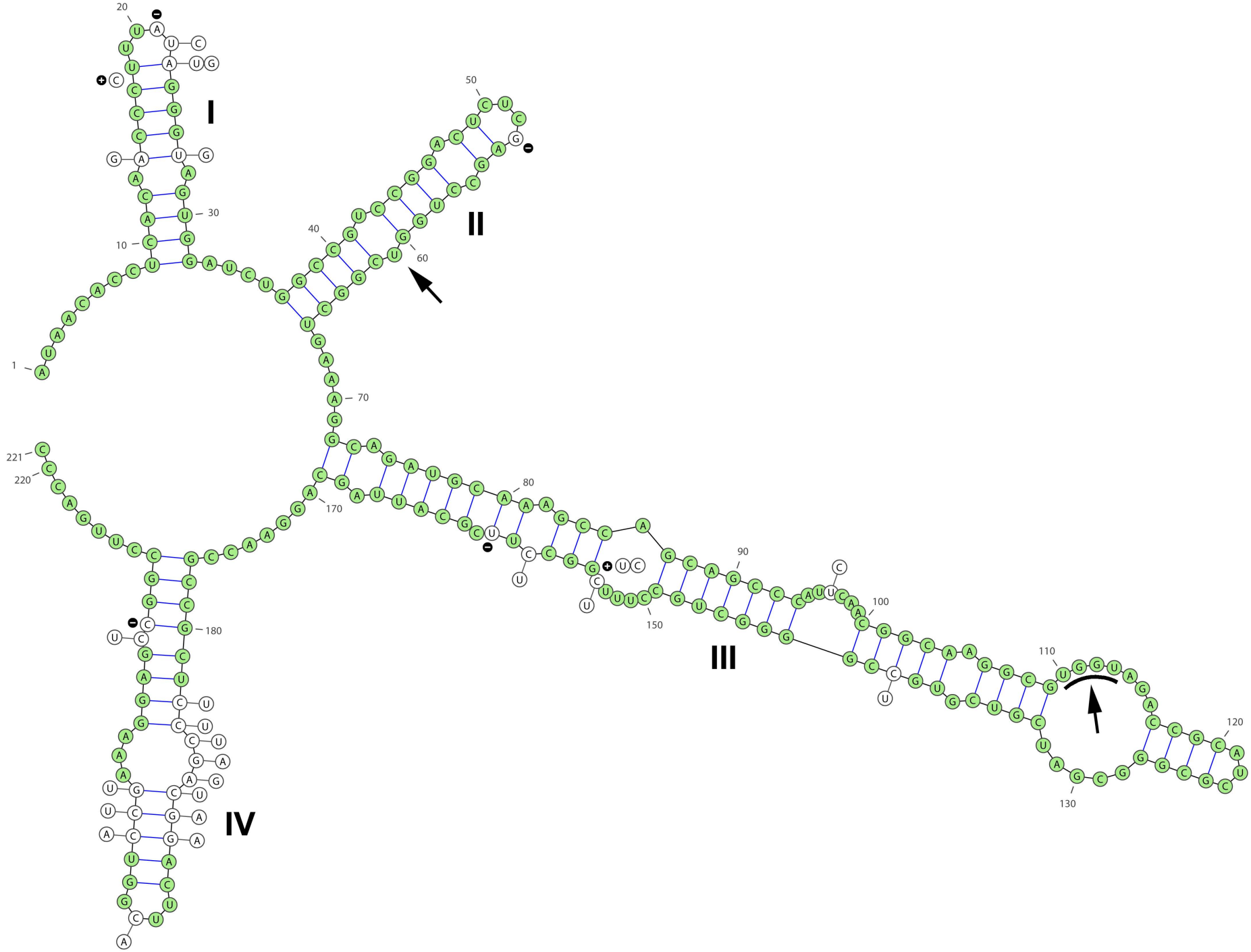
SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Predicted ITS2 secondary structure model for *Klebsormidium* lineage E (strain CAUP J302). Base numbering is indicated every 10 bases, and the four helices are numbered with Roman numerals. Bases conserved among all sequences are given in green. Single base changes among the different *Klebsormidium* genotypes are marked in circles. Plus and minus symbols indicate insertions and deletions respectively. The highly conserved U–U mismatch and UGGU motif are highlighted by arrows.

Table S1. List of the *Klebsormidium* strains used in this study, including collection data, accession numbers of ITS and rbcL sequences, and investigated morphological features.


I
II
III
IV
1
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120
130
150
170
180
220
221

Table S1. List of the *Klebsormidium* strains used in this study, including collection data, accession numbers of ITS and rbcL sequences, and investigated morphological features.

Herbarium/culture	Clade affiliation	Locality	Habitat	Accession No.		Species assignation	Cell width	Growth habit	Superficial layer	Reproductive features		H-pieces, cell wall remnants
				ITS	rbcL					Apertures	Germination	
K01	1	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649310**	**HE649339**	sp.	4.9 ± 0.26	Frag	yes	distinct	unipolar	yes
K06	1	České Středohoří Mts., Czech Republic	moss vegetation	**HE649311**	**HE649340**	sp.	5.0 ± 0.29	Frag	yes	distinct	unipolar, bipolar	yes
K16	1	Šumava Mts., Czech Republic	neuston (cutoff of a river)	**HE649312**	**HE649341**	sp.	5.6 ± 0.28	Frag	yes	distinct	unipolar	yes
K20	1	Milská stráň, Czech Republic	soil	**HE649313**	**HE649342**	sp.	4.8 ± 0.35	Frag	yes	-	-	yes
SAG 52.91	1	Mors Island, Denmark	soil	-	EU477446	*nitens*	4.8 ± 0.22	Frag	yes	-	-	yes
K34	1	Velké Přílepy, Czech Republic	rock (lydite)	**HE649314**	**HE649343**	sp.	4.9 ± 0.32	Frag	yes	-	-	no
SAG 2155	1	Col du Bussang, France	soil	EF372518	EU477429	*dissectum*	4.6 ± 0.20	Frag	yes	distinct	unipolar	no
K05	1	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649315**	**HE649344**	sp.	4.6 ± 0.31	Frag	yes	distinct	unipolar	yes
K07	1	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649316**	**HE649345**	sp.	4.4 ± 0.19	Frag	yes	distinct	unipolar	no
K08	1	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649317**	**HE649346**	sp.	4.6 ± 0.29	Frag	yes	distinct	unipolar	yes
K13	1	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649318**	**HE649347**	sp.	4.4 ± 0.19	Frag	yes	distinct	unipolar	yes
K18	1	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649319**	**HE649348**	sp.	4.5 ± 0.19	Frag	yes	distinct	unipolar	no
Lokhorst KL 37	1	Middelbeers, Belgium	soil (bank of a river)	AM490843	-	*nitens*	4.4 ± 0.13[a]	Frag[a]	yes[a]	distinct[a]	unipolar[a]	no[a]
K02	1	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649320**	**HE649349**	sp.	5.0 ± 0.21	Mix	yes	distinct	unipolar	no
K12	1	České Středohoří Mts., Czech Republic	soil (forest)	**HE649321**	**HE649350**	sp.	5.5 ± 0.31	Fil	yes	-	-	no
K42	2	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649322**	**HE649351**	sp.	6.1 ± 0.58	Mix	yes	distinct	unipolar	no
TR 41	2	Khokhitva, Ukraine	rock (granite)	HQ654172,HQ654238	HQ613261		7.3 ± 0.50	Mix	-	-	-	yes
K11	2	České Středohoří Mts., Czech Republic	soil	**HE649323**	**HE649352**	sp.	5.6 ± 0.18	Frag	yes	distinct	unipolar	no
K26	2	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649324**	**HE649353**	sp.	5.8 ± 0.25	Frag	yes	distinct	unipolar	no
K27	2	České Středohoří Mts., Czech Republic	rock (phonolite)	**HE649325**	**HE649354**	sp.	5.7 ± 0.34	Mix	yes	-	-	no
K35	2	Drahanské údolí, Czech Republic	rock (schist)	**HE649326**	**HE649355**	sp.	5.5 ± 0.27	Frag	yes	distinct	unipolar	no
K37	2	Drahanské údolí, Czech Republic	rock (schist)	**HE649327**	**HE649356**	sp.	6.9 ± 0.60	Frag	yes	-	-	yes
K47	2	Drahanské údolí, Czech Republic	rock (schist)	**HE649328**	**HE649357**	sp.	5.8 ± 0.34	Mix	yes	distinct	unipolar	no
GALW015340	3	Bergen, Norway	concrete (wall)	-	EU477424	sp.	6.7 ± 0.62	Mix	yes	-	-	no
GALW015548	3	Graskop, South Africa	concrete (wall)	-	EU477439	sp.	-	-	-	-	-	no
GALW015463	3	Hamburg, Germany	concrete (wall)	-	EU477440	sp.	6.5 ± 0.62	Mix	yes	-	-	no
GALW015499	3	Konstans, Germany	concrete (wall)	-	EU477441	sp.	8.0 ± 0.83	Fil	yes	-	-	no
Namibia5	3	Namibia	artificial stone substrate	HQ654162,HQ654228	HQ613252	sp.	6.4 ± 0.10	-	-	-	-	yes
GALW015441	4	Porto, Portugal	concrete (pavement)	-	EU477451	sp.	7.3 ± 0.71	Fil	no	-	-	no
SAG 2108	4	Zingst, Germany	rooftile	HQ654157,HQ654223	HQ613255	sp.	6.2 ± 0.15	Frag	yes	-	-	no
K19	4	Koleč, Czech Republic	concrete (moss vegetation at wall)	**HE649329**	**HE649358**	sp.	5.9 ± 0.39	Frag	no	distinct	unipolar, bipolar	no
GALW015473	4	Bordeaux, France	concrete (wall)	-	EU477427	sp.	7.2 ± 0.40	Fil	no	-	-	no
SAG 38.91	4	Witzenhausen, Germany	probably concrete (sewage plant)	-	EU477433	*flaccidum*	5.6 ± 0.24	Mix	no	distinct	unipolar	no
SAG 37.91	5	Lake Titicaca, Peru	freshwater	HQ654131,HQ654197	HQ613244	*flaccidum*	6.0 ± 0.15	Mix	-	-	-	no
SAG 335-2b	5	Barlow, USA	freshwater	AM490844	AF408254	*nitens*	4.7 ± 0.21	Frag	yes	-	-	yes
TR 31	5	Australia	soil	HQ654168,HQ654234	HQ613259	sp.	5.7 ± 0.35	Mix	-	-	-	yes
K43	6	Šumava Mts., Czech Republic	freshwater (sandy sediment in a small brook)	**HE649330**	**HE649359**	sp.	4.7 ± 0.27	Fil	yes	distinct	unipolar	no
SAG 13.91	6	Tehoa, New Zealand	soil	HQ654146,HQ654212	HQ613248	*nitens*	5.1 ± 0.30	Frag	yes	-	-	no
SAG 9.96	7	Rijsenhout, The Netherlands	concrete (piling in a lake)	AM490839	EU477438	*fluitans*	7.4 ± 0.46	Fil	no	-	-	yes
GALW015442	7	Galway, Ireland	concrete (pavement)	-	EU477432	*flaccidum*	7.4 ± 0.35	Fil	yes	indistinct	unipolar, bipolar	no
K39	-	Budapest, Hungary	rock (calcite?)	**HE649331**	**HE649360**	sp.	5.5 ± 0.25	Frag	yes	distinct	unipolar	no
SAG 32.91	8	River Gannel, U.K.	freshwater	-	EU477447	*nitens*	5.2 ± 0.50	Mix	yes	distinct	unipolar	yes
CCAP 335.13	8	River Hayle, U.K.	freshwater	HQ654143,HQ654209	HQ613246	*fluitans*	6.0 ± 0.25	Mix	-	-	-	yes
CCAP 335.14	8	River Hayle, U.K.	freshwater	HQ654144,HQ654210	HQ613247	*fluitans*	6.3 ± 0.15	Mix	-	-	-	yes
K44	9	Krkonoše Mts., Czech Republic	freshwater (peat bog)	**HE649332**	**HE649361**	sp.	6.3 ± 0.69	Fil	no	distinct	unipolar	yes
Novis K48	9	Agility Creek, New Zealand	freshwater (neutral stream)	-	DQ028576	*dissectum*	8.2 ± 0.43[b]	-	-	-	-	no[b]
Novis KM	9	Millerton, New Zealand	freshwater (acidic stream)	-	DQ028577	*acidophilum*	6.8 ± 0.91[b]	-	-	-	-	no[b]

Novis KRIV	9	Sullivan's Mine, New Zealand	freshwater (acidic stream)	-	DQ028578	*acidophilum*	6.8 ± 0.91[b]	-	-	-	-	no[b]
CCAP 335.15	9	Wheal Godolphin adit, U.K.	freshwater	HQ654152,HQ654218	HQ613263	*scopulinum*	5.3 ± 0.25	Mix	-	-	-	yes
Novis LCR-K2	9	Mt. Philistinem New Zealand	soil	-	EF589144	*dissectum*	7.6 ± 0.20[c]	-	-	-	-	yes[c]
Novis K25	9	Acheron River, New Zealand	freshwater (neutral stream)	-	DQ028574	*dissectum*	7.8 ± 0.37[b]	-	-	-	-	no[b]
Novis K37	9	Ryton River, New Zealand	freshwater (slightly alkaline stream)	-	DQ028575	*dissectum*	7.8 ± 0.37[b]	-	-	-	-	no[b]
K09	10	České Středohoří Mts., Czech Republic	moss vegetation	**HE649333**	**HE649362**	sp.	6.4 ± 0.52	Frag	yes	indistinct	unipolar	yes
K40	10	České Středohoří Mts., Czech Republic	moss vegetation	**HE649334**	**HE649363**	sp.	6.0 ± 0.30	Mix	yes	indistinct	unipolar	yes
SAG 121.80	10	Solling, Germany	wood	-	EU477431	*flaccidum*	5.9 ± 0.32	Frag	yes	indistinct	unipolar	no
UNA00067477	10	Lake June, Florida, USA	wood	-	EU477437	sp.	-	-	-	-	-	-
SAG 2107	11	Zingst, Germany	rooftile	HQ654156,HQ654222	HQ613254	sp.	6.2 ± 0.35	Frag	yes	-	-	no
SAG 2109	11	Zingst, Germany	rooftile	HQ654158,HQ654224	HQ613256	sp.	5.9 ± 0.30	Frag	yes	-	-	no
CAUP J302	11	Adršpach, Czech Republic	rock (sandstone)	**HE649335**	**HE649364**	*flaccidum*	5.8 ± 0.34	Fil	no	distinct	unipolar	yes
SAG 2065	11	Roskilde, Denmark	probably soil (Christmas tree plantage)	EU434032	HQ613253	sp.	6.0 ± 0.50	Frag	yes	-	-	yes
TR 18	-	Australia	soil	HQ654165,HQ654231	HQ613257	sp.	7.8 ± 0.65	Mix	-	-	-	yes
K10	12	Prague, Czech Republic	concrete (fountain wall)	**HE649336**	**HE649365**	sp.	6.7 ± 0.25	Fil	no	-	-	no
ROS85/1	12	Borchen, Germany	concrete	-	EU477426	sp.	-	-	-	-	-	no
GALW015468	12	Copenhagen, Denmark	concrete (pavement)	-	EU477428	sp.	7.5 ± 0.37	Fil	no	-	-	no
GALW015492	12	Koper, Slovenia	concrete (wall)	-	EU477442	sp.	-	Fil	-	-	-	no
GALW015478	12	London, U.K.	concrete (wall)	-	EU477443	sp.	-	-	-	-	-	no
GALW015472	12	Marseilles, France	concrete (wall)	-	EU477444	sp.	7.3 ± 0.42	Mix	no	-	-	no
GALW015560	12	Pavia, Italy	concrete (pavement)	-	EU477448	sp.	-	-	-	-	-	no
GALW015300	12	Pisa, Italy	concrete (wall)	-	EU477449	sp.	7.5 ± 0.53	Mix	no	-	-	no
GALW015559	12	Plymouth, U.K.	concrete (wall)	-	EU477450	sp.	7.0 ± 0.34	Fil	no	-	-	no
GALW015462	12	Prague, Czech Republic	concrete (wall)	-	EU477452	sp.	7.1 ± 0.40	Fil	no	-	-	no
GALW015298	12	Siena, Italy	concrete (pavement)	-	EU477453	sp.	7.0 ± 0.45	Fil	no	-	-	no
GALW015493	12	La Valletta, Malta	concrete (pavement)	-	EU477455	sp.	6.3 ± 0.56	Frag	no	-	-	no
K22	13	České Švýcarsko, Czech Republic	freshwater (acidic peat bog)	**HE649337**	**HE649366**	sp.	5.6 ± 0.22	Mix	yes	indistinct	unipolar, bipolar	no
K28	13	Krkonoše Mts., Czech Republic	freshwater (acidic peat bog)	**HE649338**	**HE649367**	sp.	5.5 ± 0.37	Mix	yes	indistinct	unipolar, bipolar	yes
SAG 384-1	14	Port Barrow, Alaska, USA	freshwater, snow	EF372517	EU477454	*subtilissimum*	4.9 ± 0.31	Frag	yes	-	-	yes
UTEX 462	14	Port Barrow, Alaska, USA	freshwater, snow	**-**	AF408253	*subtilissimum*	5.8 ± 0.40	Mix	-	-	-	yes

a) according to Lokhorst (1996); **b)** according to Novis (2006); **c)** according to Novis et al. (2008)