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REGULAR PAPER

Molecular evidence for the polyphyletic origin of low pH adaptation in the genus *Klebsormidium* (Klebsormidiophyceae, Streptophyta)

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Background and aims – Algae living in low pH environments have been the subject of numerous studies, but their phylogenetic relationships with relatives found in non-acidic habitats are poorly known. In the present study we analyzed the morphology and phylogeny of acid-adapted strains of *Klebsormidium*, a genus of filamentous green algae frequently present in low pH environments.
Methods – Eighteen strains of *Klebsormidium* were collected from from low pH habitats in Europe and U.S.A., mainly from terrestrial sites affected directly or indirectly by carbon mining activities. These algae were isolated in culture and their phylogenetic relationships were studied using *rbc*L and ITS rDNA sequence data in a concatenated dataset.
Key results – In the molecular phylogeny the strains of *Klebsormidium* living in low pH habitats formed a polyphyletic assemblage. They were representative of sixteen lineages and corresponded morphologically to six species (*K. crenulatum*, *K. elegans*, *K. flaccidum*, *K. fluitans*, *K. nitens*, *K. scopulinum*), with the exception of four strains for which an unambiguous identification was not possible.
Conclusions – The genus *Klebsormidium* is a group of morphologically and physiologically dynamic algae in which the capacity of adaptation to low pH conditions has been developed multiple times independently. Extreme acidophilic populations probably originate from populations of various species growing locally when strongly acidic habitats become available. For the acid-adapted lineages of *Klebsormidium* examined here the current known distribution is geographically restricted, with the exception of a lineage containing strains from Czech Republic, New Zealand and Ohio.

Key words – Acid mine drainage, acidophilic algae, acidotolerant algae, extremophiles, Klebsormidiales, *Klebsormidium*, molecular systematics, phylogeny, Streptophyta, taxonomy.

INTRODUCTION

Sites characterized by low pH conditions occur both in terrestrial and aquatic environments throughout the world. In a few cases the high acidity is due to natural geochemical and biological features of the areas in which these sites are located and is not caused by anthropogenic influences. Some well-known cases are represented by certain sites affected by volcanic activities (Baffico et al. 2004, Pollio et al. 2005), streams with naturally low pH produced by leaching of fumic and fluvic acids from podocarp rainforests (Novis & Harding 2007) and rivers originating from areas with massive bodies of iron and copper sulfites (Aguilera et al. 2007a, 2007b). More often, however, the low pH is the result of human influence, mostly in the form of acid mine drainage released by mining activities (Lukešová 2001, Sabater et al. 2003, Novis & Harding 2007). The extent of these effects is usually localized, and consequently the distribution of highly acidic habitats is patchy and fragmented (Gross 2000, Weisse et al. 2011). However, sites with low pH exist in all continents and the overall extent of these habitats on global scale is not negligible; for example, in the recent past it was considered that approximately 15,000 km of streams in the U.S. were affected by acid mine drainage (Gross 2000).

Highly acidic environments pose challenging conditions for algae and cyanobacteria not only due to the low pH, but

also because acidity is often combined with extreme levels of other parameters. High concentrations of heavy metals such as Fe, Cu, Pb, Al and Zn are often recorded in soils and waters with low pH (Gross 2000, Aguilera et al. 2007b, Novis & Harding 2007, Spijkerman & Weithoff 2012), whereas at sites with geothermal activities acidity is accompanied by high temperatures (up to 83°C; Huss et al. 2002, Pollio et al. 2005, Spijkerman & Weithoff 2012). Additionally, most extremely acidic environments contain relatively low concentrations of dissolved organic carbon, and may therefore be considered oligotrophic (Johnson 1998), with further limitation for the growth of autotrophic organisms.

Due to such hostile conditions, the diversity of algae living in acidic environments is generally low (Huss et al. 2002, Sabater et al. 2003, Nancucheo & Johnson 2012, Spijkerman & Weithoff 2012), although some studies based on extended seasonal sampling and incorporating molecular data have revealed an unsuspected microbial eukaryotic diversity (Aguilera et al. 2006, 2007b). Organisms living in these environments can be separated into acidophiles (acid-loving organisms, adapted to pH values as low as 0.05 and unable to grow at neutral pH) and acidotolerant (acid-tolerating organisms, with growth optima at higher pH but able to tolerate low values) (Johnson 1998, Gross 2000). Some algal taxa are particularly able to adapt to low pH and are a recurrent presence in acidic habitats; these are mainly unicellular organisms such as species of the green algal genus *Chlamydomonas*, the euglenophyte *Euglena*, the chrysophyte *Ochromonas*, the diatom *Pinnularia* and red algae of the class Cyanidiophyceae (Johnson 1998, Huss et al. 2002, Ciniglia et al. 2004, Pollio et al. 2005, Novis & Harding 2007, Spijkerman & Weithoff 2012). However, some multicellular algae with filamentous habit may also occur in these habitats; of these, species of *Klebsormidium* P.C.Silva, Mattox & Blackwell are the most frequently recorded. Members of this genus belong to the streptophyte lineage of the Viridiplantae (Leliaert et al. 2012) and consist of uniseriate filaments formed by cells having a parietal chloroplast with a single pyrenoid and reproducing asexually by biflagellate spores (Lokhorst 1996, John 2002). *Klebsormidium* is one of the most widespread genera of green algae in the world, spanning in distribution from polar to tropical regions and occurring in a wide range of terrestrial and freshwater habitats (Lokhorst 1996, Rindi et al. 2008, Škaloud & Rindi 2013). Records of *Klebsormidium* in low pH habitats are available from many, widely separated locations all over the world, mainly in waters (Douglas et al. 1998, Stevens et al. 2001, Verb & Vis 2001, Brown & Wolfe 2006, Novis 2006, Valente & Gomes 2007, Bray et al. 2008, Lear et al. 2009, Urrea-Clos & Sabater 2009, Aguilera et al. 2010, Baffico 2010, Adlassnig et al. 2013) but also in soils (Lukešová & Hoffman 1996, Lukešová 2001, Lukešová & Hrčková 2011). Strains collected in low pH habitats are usually identified as *Klebsormidium rivulare* (Kütz.) M.O.Morison & Sheath (Morison & Sheath 1985, Stevens et al. 2001, Verb & Vis 2001), *Klebsormidium flaccidum* (Kütz.) P.C.Silva, Mattox & Blackwell (Lukešová & Hoffman 1996, Lukešová 2001, Sabater et al. 2003) and *Klebsormidium nitens* (Meneghini) Lokhorst (Lukešová 2001). Novis (2006) described *Klebsormidium acidophilum* Novis based on collections made in low pH streams in New Zealand. However, a taxonomic assessment of *Klebsormidium* at species level is hampered by several unresolved issues and the precise identity of several species, including the type species *K. flaccidum*, remains uncertain (Novis 2006, Škaloud 2006, Rindi et al. 2008, 2011, Škaloud & Rindi 2013). Currently, the molecular data available for strains of *Klebsormidium* from low pH habitats are limited, which is a major impediment for an assessment of their phylogenetic position and taxonomic identity.

Few studies have investigated the phylogenetic relationships between algae adapted to acidic conditions and their pH-neutral congeners. Besides the study of Novis (2006) for *Klebsormidium acidophilum*, results available for coccoid trebouxiophytes (Huss et al. 2002, Juárez et al. 2011) suggest that adaptation to low pH has taken place independently in different lineages and that in green microalgae acidophilic forms coexist with closely related neutrophilic forms. Here we investigate the phylogenetic relationships of low pH *Klebsormidium* using *rbc*L and ITS sequences of several strains isolated from acidic soils and rivers in Europe and U.S.A. Using the phylogenetic framework built by previous molecular studies (Novis 2006, Mikhailyuk et al. 2008, Rindi et al. 2008, 2011, Škaloud & Rindi 2013), our goal is to clarify whether adaptation to low pH in *Klebsormidium* is monophyletic or not and in which known lineages of this genus this trait occurs. The results have important implications both in terms of speciation patterns and from a biogeographic point of view.

MATERIALS AND METHODS

Origin and isolation of *Klebsormidium* strains used in the study

Eighteen strains of *Klebsormidium* were obtained from low pH terrestrial and aquatic habitats as detailed in table 1. The strains were identified based both on morphological features (Printz 1964, Ettl & Gärtner 1995, Lokhorst 1996) and molecular data from recent studies (Novis 2006, Mikhailyuk et al. 2008, Novis & Visnovsky 2011, Rindi et al. 2011, Škaloud & Rindi 2013). With the only exception of the strain from Ohio, all strains were isolated in unialgal cultures and DNA extractions were performed on cultured material. The two strains from Sardinia, Italy (SCCA009 and SCCA011) were isolated using WARIS-H culture medium without soil extract (McFadden & Melkonian 1986). Stock cultures were established and maintained axenically at 25°C, 12:12 h L:D, under cool white luminescent light (80-100 µmol photons m^{-2} s^{-1}) in the Sardinian Culture Collection of Algae (SCCA), Interdepartmental Center of Environmental Science and Engineering (CINSA), University of Cagliari. The strains from Czech Republic and Germany were isolated in unialgal cultures using BBM culture medium (Andersen et al. 2005) and grown at 20–22°C, 18:6 h L:D, under cool white luminescent light (40–60 µmol photons m^{-2} s^{-1}). Stock cultures are maintained at 15°C under continuous light below 10 µmol photons m^{-2} s^{-1} in the Culture Collection of Soil Algae and Cyanobacteria at the Institute of Soil Biology, Academy of Sciences of the Czech Republic, České Budějovice.

Table 1 – Details of the strains of *Klebsormidium* used in the study.

Strain	Identification	Collection data	GenBank accession number (*rbc*L)	GenBank accession number (ITS)
LUK S01	*Klebsormidium flaccidum*	Sokolov coal mining area, Czech Republic. Coal clay, 30-years old forest, spontaneous succession (pH 3.05). Alena Lukešová, April 2008.	KM197105	KM197123
LUK S02	*Klebsormidium elegans*	Sokolov coal mining area, Stará Chodovská, Czech Republic. Coal clay, 40-years old forest, spontaneous succession (pH 3.4). Alena Lukešová, April 2008.	KM197106	KM197124
LUK S03	*Klebsormidium fluitans*	Sokolov coal mining area, Stará Chodovská, Czech Republic. Coal clay, 40-years old forest, spontaneous succession (pH 3.4). Alena Lukešová, April 2008.	KM197107	KM197125
LUK S06	*Klebsormidium crenulatum*	Giant Mountains, Vysoké Kolo Mount (1510 m a.s.l), Czech Republic. Polygonal soil on granite, biological crust (pH 4.0). Alena Lukešová, June 2006.	KM197108	KM197126
LUK S12	*Klebsormidium* sp.	Slavkovský les, peat bog Krásno, Czech Republic. Peat, meliorated area with dense cover dominated by *Calluna vulgaris*, thick litter layer (pH 4.0). Alena Lukešová, June 2007.	KM197109	KM197127
LUK S19	*Klebsormidium flaccidum*	Sokolov coal mining area, Czech Republic. Coal clay, 30-years old forest, spontaneous succession (pH 3.0). Alena Lukešová, April 2008.	KM197110	KM197128
LUK S29	*Klebsormidium crenulatum*	Vintířov, Sokolov coal mining area, Czech Republic. Volcanic ash mixed with coal clay, 10 years after mining, spontaneous succession (pH 3.45). Alena Lukešová, April 2008.	KM197111	KM197129
LUK S46	*Klebsormidium flaccidum*	Weissacker Berg, near Cottbus, Lusatian lignite mining area, Germany. Tertiary carboniferous and pyritic sand, ameliorated with fly ash, 2 years after mining (pH 2.9). Alena Lukešová, May 1997.	KM197112	-
LUK S48	*Klebsormidium nitens*	Meuro, Lusatian lignite mining area, Germany. Sandy soil, 20-years old pine plantation (pH 5.0). Alena Lukešová, May 1997.	KM197113	KM197130
LUK S50	*Klebsormidium* sp.	Weissacker Berg, near Cottbus, Lusatian lignite mining area, Germany. Tertiary carboniferous and pyritic sand, ameliorated with fly ash and sewage sludge, 2 years after mining (pH 2.9). Alena Lukešová, May 1997.	KM197114	-
LUK S63	*Klebsormidium* sp.	Weissacker Berg, near Cottbus, Lusatian lignite mining area, Germany. Tertiary carboniferous and pyritic sand, 1 year after mining (pH 2.7). Alena Lukešová, October 1999.	KM197115	-
LUK S64	*Klebsormidium flaccidum*	Weissacker Berg, near Cottbus, Lusatian lignite mining area, Germany. Tertiary carboniferous and pyritic sand, ameliorated with fly ash and compost, 4 yrs after mining (pH 2.9). Alena Lukešová, October 1997.	KM197116	KM197131
LUK S66	*Klebsormidium* sp.	Weissacker Berg, near Cottbus, Lusatian lignite mining area, Germany. Tertiary carboniferous and pyritic sand, ameliorated with fly ash and mineral fertilizers, 2 years after mining (pH 2.9). Alena Lukešová, May 1997.	KM197117	KM197132
LUK S67	*Klebsormidium nitens*	Domsdorf, Lusatian lignite mining area, Germany. Sandy soil, 34 years old pine plantation (pH 4.1). Alena Lukešová, October 1999.	KM197118	KM197133
LUK S68	*Klebsormidium elegans*	Domsdorf, Lusatian lignite mining area, Germany. Sandy soil, 34 years old pine plantation (pH 4.0). Alena Lukešová, May 1997.	KM197119	KM197134
PRC 2378	*Klebsormidium scopulinum*	Carbondale, Ohio, U.S.A. Acidic seep of an abandoned coal mine, heavily armored with iron oxide precipitation (pH < 4.0). Sam Drerup, 3 Jan. 2014.	KM197120	-
SCCA009	*Klebsormidium flaccidum*	Arquerì, forest of Montarbu, Seui, Ogliastra, central-eastern Sardinia, Italy. About 1000 m a.s.l. Temporary pool on side of untarred road (pH 7.2). Veronica Malavasi, 18 Jan. 2009.	KM197121	-
SCCA011	*Klebsormidium flaccidum*	Rio Irvi-Piscinas, area Montevecchio-Ingurtosu, southwestern Sardinia, Italy. 370 m a.s.l. Water of Rio Irvi (pH 3.22). Veronica Malavasi, 14 Sep. 2012.	KM197122	-

DNA extraction, PCR and DNA sequencing

Total genomic DNA was extracted from the fresh cultures or silica-dried material 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-ITS2 rDNA region was amplified using the primers Klebs-ITS-F (5'-GGA AGG AGA AGT CGT AAC AAG G-3'; Škaloud & Rindi 2013) and Klebs-ITS-R (5'-TCC TCC GCT TAG TAA TAT GC-3'; Škaloud & Rindi 2013). The *rbc*L gene was amplified using the primers *rbc*L-KF2 (5'-ACT TAC TAC ACT CCT GAT TAT GA-3'; Škaloud & Rindi 2013) and *rbc*L-KR2 (5'-GGT TGC CTT CGC GAG CTA-3'; Škaloud & Rindi 2013) or the primers *rbc*L-KF376 (5'-TCA AAA CTT TCC AAG GTC CTC-3'; Rindi et al. 2008) and *rbc*L-KR2. All PCR reactions were performed in 20 µl reaction volumes, using the conditions described in Škaloud & Rindi (2013). The purified amplification products were sequenced with the PCR primers using an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730xl) at Macrogen Corp. in Seoul, Korea. Sequencing reads were assembled and edited using the SeqAssem programme (Hepperle 2004).

Sequence alignment and model selection

Multiple alignments of the newly determined ITS1 rDNA, ITS2 rDNA and *rbc*L sequences and other sequences selected from the DDBJ/EMBL/GenBank databases (electronic appendix 1) were manually built using MEGA 4 (Kumar et al. 2008), and then optimized using MAFFT, version 6, applying the Q-INS-i strategy (Katoh et al. 2002). The concatenated data matrix of unique sequences was 1,672 bp long and was 100% filled by the *rbc*L data (1,101 bp) and 79% filled by the ITS rDNA data (571 bp). The concatenated alignment used for the phylogenetic analyses consisted of 84 sequences. Sequences retrieved from GenBank were selected in order to represent all lineages currently known in the phylogeny of the Klebsormidiales (based primarily on Rindi et al. 2011); whenever possible, we used strains for which both *rbc*L and ITS sequence data were available. The appropriate substitution models for the ITS1 rDNA and ITS2 rDNA datasets and individual *rbc*L codon positions were selected using jModelTest 2.1.4 (Darriba et al. 2012). This BIC-based model selection procedure selected the following models: (1) TIM2ef + Γ for internal transcribed spacer ITS1, (2) K80 + Γ for internal transcribed spacer ITS2, (3), TIM1 + I for the first codon position of the *rbc*L gene, (4) JC + I for the second codon position of the *rbc*L gene, and (5) TRN + I + Γ for the third codon position of the *rbc*L gene.

Phylogenetic analyses

The phylogenetic tree was inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist et al. 2012). The analysis was carried out on a partitioned dataset using the different substitution models selected by jModelTest 2.1.4. The general structure of each substitution model was determined by the 'lset' command, and the model parameters were set using the priors defining the frequencies of nucleotides (statefreqpr) and nucleotide substitution rates (revmatpr) using the Dirichlet distribution. All parameters were unlinked among partitions. Two parallel MCMC runs were carried out for five 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 assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value between simultaneous runs was 0.00332. Finally, the burn-in was determined using the 'sump' command. Variance around the parameter estimates were verified in order to ensure that they were effectively modelled. Bootstrap analyses were performed by maximum likelihood (ML) and weighted parsimony (wMP) criteria using GARLI, version 0.951 (Zwickl 2006) and PAUP*, version 4.0b10 (Swofford 2002), respectively. ML analyses consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100,000). The wMP bootstrapping (1,000 pseudo-replicates) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences, and gap characters treated as missing data.

RESULTS

The strains examined in this study were collected mostly from terrestrial habitats, primarily soils in areas affected directly or indirectly by carbon mining activities. Although at several of these sites carbon mining had ceased a long time before our surveys and the sites were subsequently subjected to a natural succession, the pH measured at the time of collection was still fairly acidic, ranging between 2.0 and 5.0 (table 1). The only algae obtained from aquatic habitats were the two strains from Sardinia (SCCA009 and SCCA011, Malavasi 2012), which were growing in water bodies with very different characteristics (the Rio Irvi, where SCCA011 was collected, is a river affected by mining drainage with extremely high levels of heavy metals, where the pH of the water was 3.22; the strain SCCA009 was isolated from a large temporary pool with pH close to neutral).

Our analyses revealed a great morphological and phylogenetic diversity of the strains of *Klebsormidium* examined (figs 1, 2 & 3). Combining morphological observations and molecular data, they were referred to six species (*K. crenulatum* (Kütz.) Lokhorst (fig. 2A), *K. elegans* Lokhorst (fig. 2B), *K. flaccidum* (fig. 2C–E), *K. fluitans* (F.Gay) Lokhorst (fig. 2F), *K. nitens* (fig. 3A–B), *K. scopulinum* (Hazen) H.Ettl & G.Gärtner), with the exception of four strains for which an unambiguous identification was not possible (fig. 3C–D). Details of the morphology of the strains are reported in table 2. In the concatenated ITS1-ITS2-*rbc*L phylogeny, the low pH strains of *Klebsormidium* formed a polyphyletic assemblage (fig. 1). Overall, they were representative of sixteen lineages, which were separated in seven different clades belonging to four superclades delineated in recent studies (superclades D, E, F and G and clades E1, E2, E3, E4 as in Rindi et al. 2011 and Škaloud & Rindi 2013) (fig. 1). Some of these evolutionary units corresponded to well-circumscribed morphological species and the strains from low pH habitats belonging to these units were in good morphological agreement with them: this was the case for the superclade D (corresponding

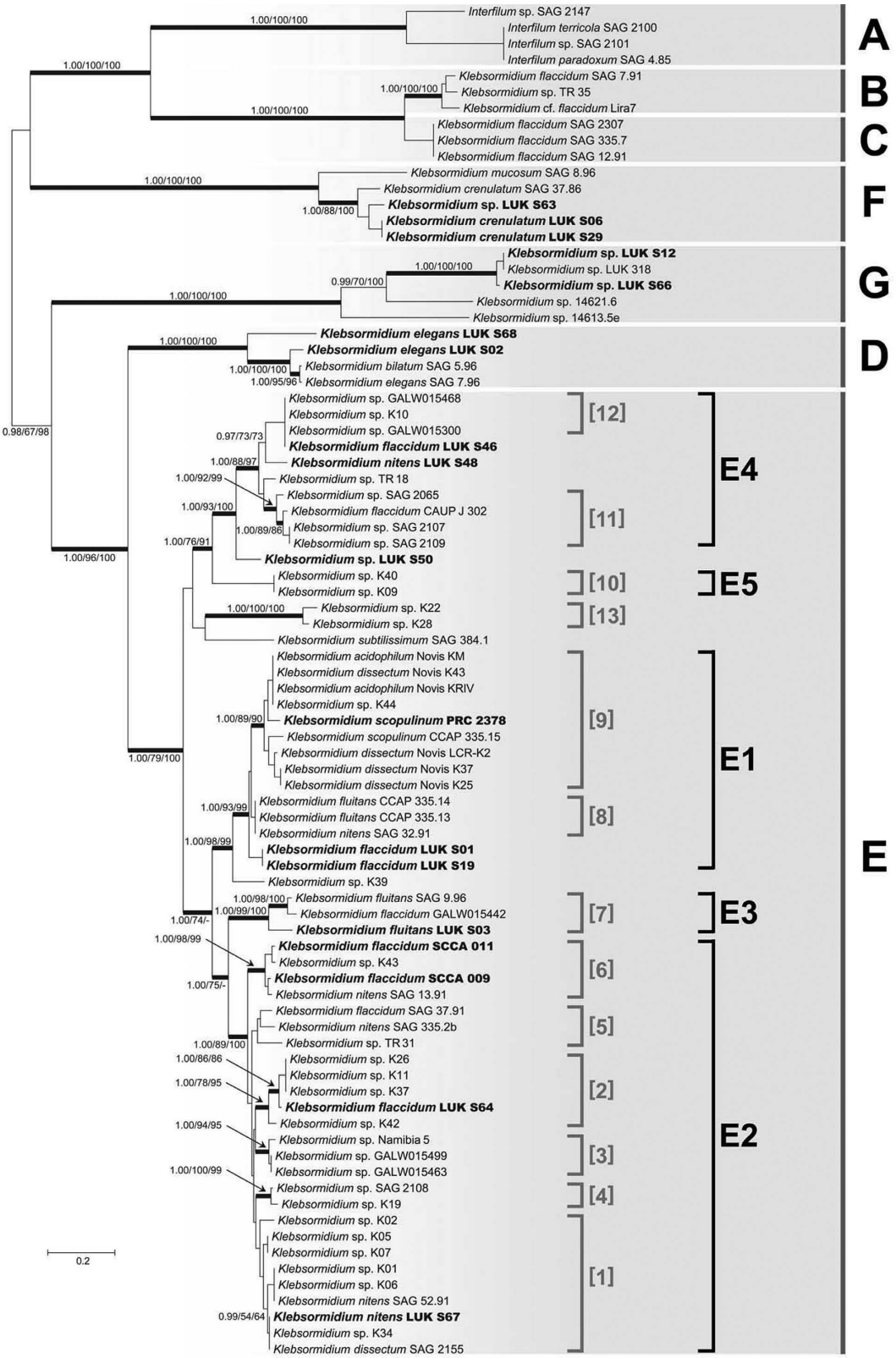

Figure 1 – Phylogram obtained from Bayesian analysis based on the combined *rbc*L and ITS rDNA dataset, showing the position of investigated *Klebsormidium* strains and their relatives. Values at the nodes indicate statistical support estimated by MrBayes posterior node probability (left), maximum likelihood bootstrap (middle) and maximum parsimony bootstrap (right). Thick branches represent nodes receiving the highest PP support (1.00). Species affiliation to seven superclades (A–G) and five clades (E1-E5) sensu Rindi et al. (2011) is indicated, as well as the affiliation to 13 lineages sensu Škaloud & Rindi (2013). Scale bar indicates number of substitutions per site.

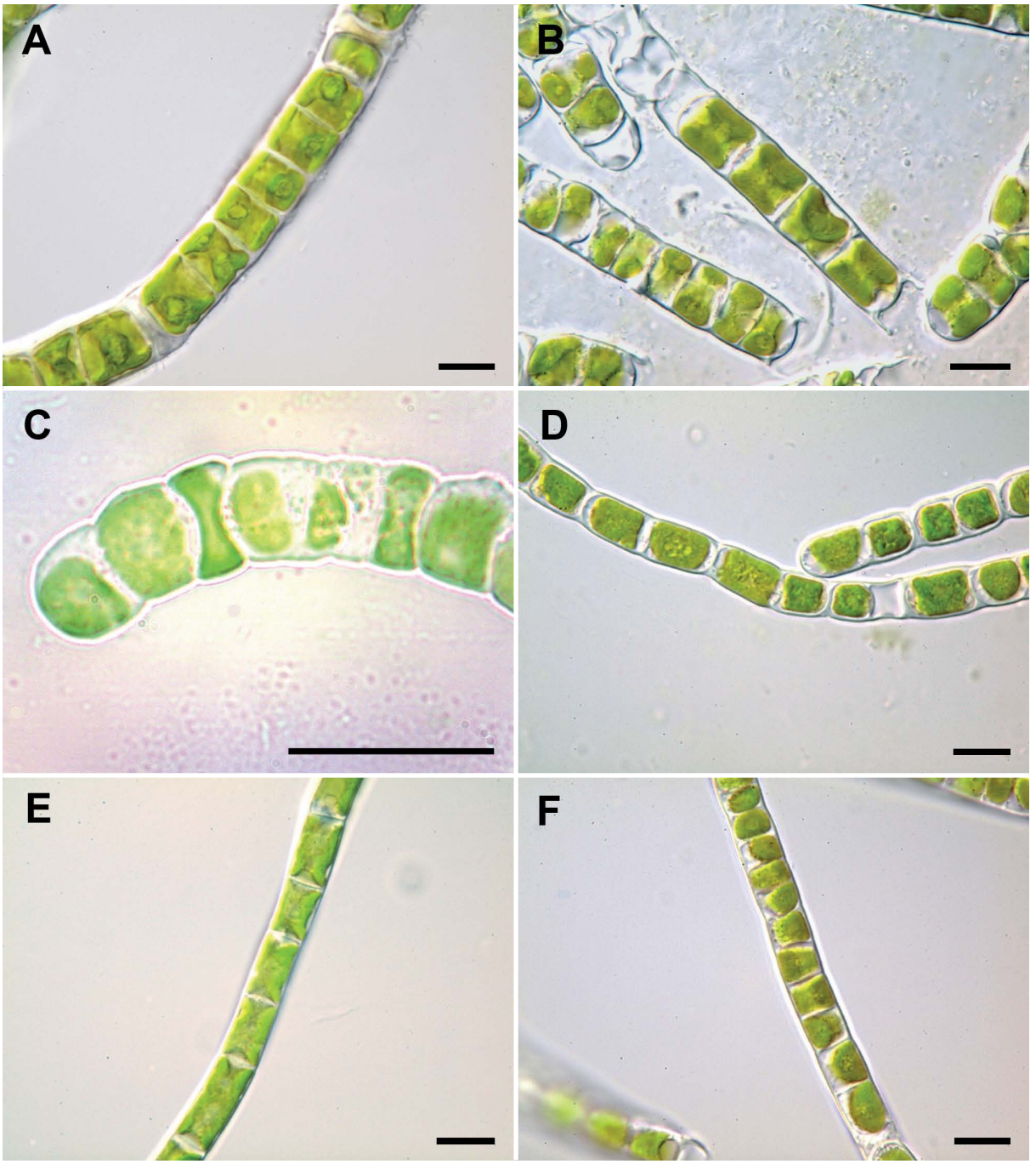

Figure 2 – Morphology of strains of *Klebsormidium* from low pH habitats: A, *Klebsormidium crenulatum* S06; B, *Klebsormidium elegans* S02; C, *Klebsormidium flaccidum* SCCA011; D, *Klebsormidium flaccidum* S19; E, *Klebsormidium flaccidum* S64; F, *Klebsormidium fluitans* S03. Scale bars represent 10 µm.

to *K. elegans*, fig. 2B), the superclade F (corresponding to *K. crenulatum*, fig. 2A) and the clade E3 (corresponding to *K. fluitans*, fig. 2F). Two unidentified strains (*Klebsormidium* sp. LUK S12 and LUK S66) belonged to the superclade G, a group discovered in recent molecular investigations (Rindi et al. 2011) and not yet resolved taxonomically; these strains displayed a key character typical of this superclade, the lobed chloroplast with a median incision (fig. 3D). Low pH strains identified morphologically as *K. flaccidum* belonged to the superclade E but did not form a monophyletic group and were scattered in three separate clades (E1, E2, E4). The clade E2 included the two aquatic strains from Sardinia (SCCA009 and SCC011); although collected from habitats with different pH conditions, they were closely related and their *rbc*L sequences differed by only a single nucleotide substitution. The two strains identified as *K. nitens* were not closely related and were recovered in separate clades (E2 and E4). The strain isolated from Ohio (PRC 2378) was identified as *K. scopulinum* and differed strikingly from all other strains for its markedly thin and long cells (4.5–5.5 µm wide, up to 10

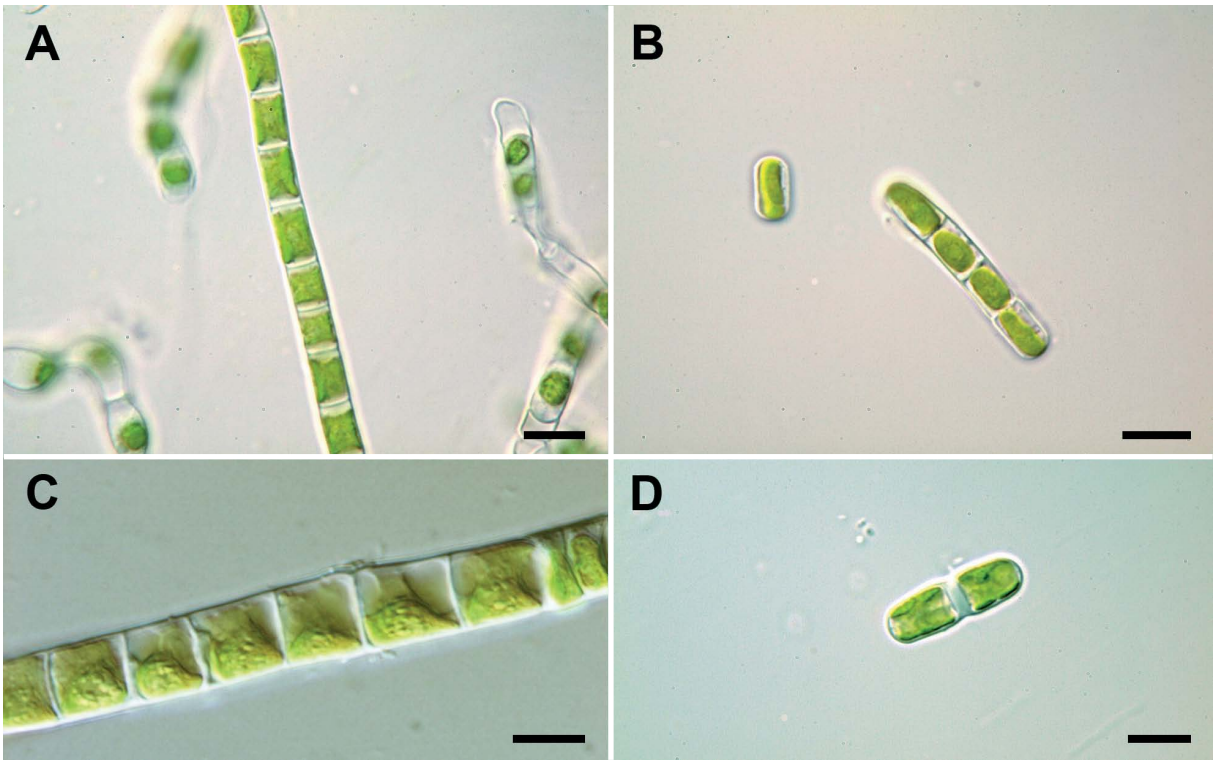

Figure 3 – Morphology of strains of *Klebsormidium* from low pH habitats: A, *Klebsormidium nitens* S48; B, *Klebsormidium nitens* S67; C, *Klebsormidium* sp. S50; D, *Klebsormidium* sp. S12. Scale bars represent 10 μm.

times as long as wide). Such morphological difference was accompanied by a sharp phylogenetic separation: in the molecular phylogeny PRC 2378 was clearly separated from all other strains from low pH habitats sequenced in this study. However, it was recovered with high support in a group including other acid-tolerant *Klebsormidium* (*K. acidophilum* from New Zealand sequenced by Novis 2006); it was also in close relationship with the only strain of *K. scopulinum* for which *rbc*L and ITS sequence data were available (culture CCAP 335.15 isolated from the river Gannel, England).

Among our strains, only three had sequences identical to records already available in GenBank: *Klebsormidium* sp. LUK S12, *K. flaccidum* LUK S46 and *K. nitens* LUK S67. Therefore, most of the strains sequenced in this study appeared to be new lineages not detected in previous molecular studies, and some of them were remarkably distinct. *Klebsormidium* sp. LUK S50, in particular, was robustly placed in the superclade E but did not belong to any of the six clades delineated in this group in previous studies; it was recovered with high support as sister taxon to the clade E4. Two strains identified as *K. flaccidum* (LUK S01 and LUK S19) had identical *rbc*L and ITS sequences; they belonged to the clade E1 but were distinct within it, representing the sister taxon to a clade formed by all other strains in this group.

Finally, it is worthy to note that none of the low pH *Klebsormidium* strains examined in this study and by Novis (2006) belonged to the superclades A, B and C. These groups include the genus *Interfilum* (superclade A), unidentified strains of *Klebsormidium* from natural habitats in eastern Europe (superclade B) and several strains of *K. flaccidum* deposited in culture collections (superclade C).

DISCUSSION

Our results clearly demonstrate that the capacity to adapt to low pH in *Klebsormidium* is phylogenetically widespread and does not represent a synapomorphy characterizing one or a few lineages. In our phylogeny, acid-adapted strains are widely interespersed among congeners living in habitats with neutral conditions. This situation is in agreement with the results of other phylogenetic studies focusing on microalgae from low pH environments, such as unicellular trebouxiophytes (Huss et al. 2002, Juarez et al. 2011), chlamydomonads (Gerloff-Elias et al. 2005, Pollio et al. 2005) and diatoms (Ciniglia et al. 2007). The fact that widely unrelated taxa possess the physiological and biochemical attributes that allow adaptation to low pH indicates that the genetic makeup on which these attributes are based is phylogenetically widespread among green algae. In the case of *Klebsormidium* this is not surprising, considering that members of this genus are equipped to withstand a wide range of extreme conditions and can grow in very hostile environments; records of *Klebsormidium* are available from biotic crusts of hot deserts (Lewis & Lewis 2005), alpine soil crusts (Karsten et al. 2010), hydrothermal springs (Brown & Wolfe 2006), Antarctic rocks and sand (Elster et al. 2008) and bases of concrete walls in trafficked urban streets (Rindi & Guiry 2004). In this genus colonization and adaptation to low pH habitats seem to be frequent events, probably more frequent than in other green algal taxa. Based on the present study

Table 2 – Morphology of the strains of *Klebsormidium* used in the study.

Strain	Identification	Morphology
LUK S01	*Klebsormidium flaccidum*	Long filaments, without tendency to break, cells cylindrical, 6–8 µm wide, 7–11 µm long, 1–1.8 times as long as wide. Chloroplast covering about 70–80% of the cell, with smooth margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S02	*Klebsormidium elegans*	Short filaments, with tendency to break into short fragments. Occasionally, filaments were spirally coiled. Cells cylindrical, 9–13 µm wide, 6–12 µm long, 0.5–1.2 times as long as wide. Chloroplast covering 70% of the cell wall, with smooth margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S03	*Klebsormidium fluitans*	Long filaments, without tendency to break, cells cylindrical (subglobular), 6–8 µm wide, 5–9 µm long, 0.6–1.2 times as long as wide. Chloroplast covering about 50% of the cell, with smooth margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S06	*Klebsormidium crenulatum*	Long filaments, without tendency to break, cells cylindrical, 11–13 µm wide, 8–12 µm long, 0.6–1 times as long as wide. Chloroplast covering about 75% of the cell, with evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S12	*Klebsormidium* sp.	Short filaments, with tendency to break into very short fragments (in many cases 1–2 cells). Cell cylindrical, 5–6 µm wide, 9–12 µm long, 1.5–2.4 times as long as wide. Chloroplast covering about 35–50% of the cell, with unclear small pyrenoid. Fully developed chloroplasts often deeply incised midway. H-shaped pieces observed. Release of zoospore not observed.
LUK S19	*Klebsormidium flaccidum*	Long filaments, without tendency to break, cells cylindrical, 5.5–8 µm wide, 6–11 µm long, 0.75–1.8 times as long as wide. Chloroplast covering about 50–60% of the cell, with smooth margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S29	*Klebsormidium crenulatum*	Long filaments, without tendency to break, cells cylindrical, 10–15 µm wide, 8–15 µm long, 0.5–1.5 times as long as wide. Chloroplast covering about 50% of the cell, with smooth or grooved margin and evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S46	*Klebsormidium flaccidum*	Short filaments, with tendency to break, cells cylindrical, 7–8 µm wide, 7–16 µm long, 0.9–2.2 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. H-shaped pieces not observed. Release of zoospore not observed.
LUK S48	*Klebsormidium nitens*	Short filaments, with tendency to break, cells cylindrical, 4–5 µm wide, 8–15 µm long, 1.6–3.8 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. H-shaped pieces not observed. Release of zoospore not observed.
LUK S50	*Klebsormidium* sp.	Long filaments, without tendency to break, cells cylindrical to subglobular, 9–11 µm wide, 8–11 µm long, 0.9–1.2 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S63	*Klebsormidium* sp.	Long filaments, without tendency to break, cells cylindrical, 8–9 µm wide, 8–18 µm long, 0.9–2.3 times as long as wide. Chloroplast covering about 50–70% of the cell, with evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.
LUK S64	*Klebsormidium flaccidum*	Short filaments, with tendency to break, cells cylindrical, 6–7 µm wide, 10–17 µm long, 1.4–2.8 times as long as wide. Chloroplast covering about 75% of the cell, without evident pyrenoid. H-shaped pieces not observed. Release of zoospore not observed.
LUK S66	*Klebsormidium* sp.	Short filaments, but without tendency to break, cells cylindrical, 6–7 µm wide, 8–13 µm long, 1.1–2.2 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. Fully developed chloroplasts occasionally incised midway. H-shaped pieces observed. Release of zoospore not observed.
LUK S67	*Klebsormidium nitens*	Short filaments, with tendency to break, cells cylindrical, 5–6 µm wide, 8–14 µm long, 1.3–2.8 times as long as wide. Chloroplast covering about 50% of the cell, without evident pyrenoid. H-shaped pieces not observed. Release of zoospore not observed.
LUK S68	*Klebsormidium elegans*	Long filaments, with tendency to break, cells cylindrical, but becoming doliiform with increasing age, 8–9 µm wide, 6–10 µm long, 0.7–1.3 times as long as wide. Chloroplast covering about 50% of the cell, with evident pyrenoid. H-shaped pieces observed. Release of zoospore not observed.

Table 2 – Morphology of the strains of *Klebsormidium* used in the study.

Strain	Identification	Morphology
PRC 2378	*Klebsormidium scopulinum*	Alga forming dense mats consisting of strong, dense filaments long up to 40 cm, without tendency to break. Cells cylindrical, 4.5–5.5 µm wide, 2–10 times as long as wide. Chloroplast extending for the whole length of the cell, covering about 50% of the cell wall, without evident pyrenoid. H-shaped pieces and constrictions between adjacent cells absent. Release of zoospores not observed.
SCCA009	*Klebsormidium flaccidum*	Alga with filamentous structure, forming long filaments with limited tendency to break. Filaments easily adhering to the substratum. Cells cylindrical, 6–7 µm wide, 0.5–3 times as long as wide (mainly about 1.5). A few intercalary cells are subglobular and distinctly larger. H-shaped pieces not observed. Constrictions between adjacent cells occasionally present, mainly in fragmenting portions of the alga. Chloroplast parietal, extending for the whole length of the cell and covering about 50% of the side wall, sometimes with irregular margins. Pyrenoid small, without a clear starch envelope. Release of zoospores not observed. In some parts the filaments are distinctly bent with elbow-like habit, suggesting that the germination pattern of the zoospores is bipolar.
SCCA011	*Klebsormidium flaccidum*	Filamentous alga, with limited tendency to break into short fragments. Cells cylindrical, 6–7.5 µm wide, 1–3 times as long as wide (mainly about 1.5). Several intercalary cells are subglobular and distinctly larger. H-shaped pieces not observed. Constrictions between adjacent cells occasionally present, mainly in fragmenting portions of the alga. Chloroplast parietal, extending for the whole length of the cell and covering about 50% of the side wall, sometimes with irregular margins. Pyrenoid small, without a clear starch envelope. Release of zoospores not observed. In some parts the filaments are distinctly bent with elbow-like habit, suggesting that the germination pattern of the zoospores is bipolar.

and the results of Novis (2006), at least sixteen lineages of *Klebsormidium* have adapted to life in acidic habitats; this is a higher number than for the other genera of green algae that have been investigated to date in this regard. The nature and extent of adaptation to low pH, however, differ in different lineages. Some acid-adapted *Klebsormidium* strains are closely related to strains from non-acidic habitats; some have in fact identical or very similar *rbc*L and ITS sequences to strains isolated from different environments, which is a strong indication of conspecificity. In these cases we are probably dealing with generalist species with large dispersal and wide pH tolerance, able to survive equally well in acidic and non-acidic environments. A situation of this type was demonstrated in other algae commonly found in low pH environments. For example, *Parachlorella kessleri* (Fott & Nováková) Krienitz et al. isolated from a mesothermal acidic pond in Argentina (pH 2.5–2.8) corresponded morphologically and had almost identical rDNA sequences to strains of the same species isolated from other environments (Juarez et al. 2011). The diatom *Pinnularia obscura* Krasske is considered a textbook example of an extremely acidotolerant species with global dispersal; strains from thermoacidic springs in Italy examined using morphological, ecophysiological and molecular data showed complete identity with strains from freshwater environments (Ciniglia et al. 2007). Conversely, other strains of *Klebsormidium* occurring in low pH environments are likely to represent evolutionary lineages that have genuinely evolved and specialized for life in acidic conditions. This type of scenario has been reported for other green algae considered real acidophilic organisms specialized for life at low pH, such as strains of *Chlamydomonas* from thermal springs in Italy (Pollio et al. 2005) and acidic mining lakes in Germany (Gerloff-Elias et al. 2005) that grow optimally at pH values < 3. In our phylogeny, good candidates are strains that form distinct lineages without a clear sister species/taxon, such as *Klebsormidium* sp. LUK S50, *K. flaccidum* LUK S01/LUK S19 and *K. nitens* LUK S48. We believe that these strains originated based on the hypothesis that extreme acidophilic populations establish from populations of various species growing locally when strongly acidic habitats become available. Other studies conducted in different environments have concluded that extreme conditions apparently unfavourable for survival and growth of green microalgae drive the evolution of these organisms (e.g. extreme aridity of North American deserts, Lewis & Lewis 2005); it can be therefore expected that the same applies to strong acidity. This would also be consistent with the possibility suggested by Škaloud & Rindi (2013) that selective sweep combined with the selection of new mutants differing in ecological niche may have played a major role in the differentiation of *Klebsormidium*. Physiological studies determining the optimal pH and the limits of the pH range in which different strains can grow would be very useful to understand better the nature and extent of adaptation to low pH conditions. Unfortunately, however, physiological data on acid adaptation in *Klebsormidium* are restricted to the experiments of Novis (2006) on *K. acidophilum* and *K. dissectum* from New Zealand. This is somewhat surprising, considering that *Klebsormidium* has been reported frequently in low pH habitats and that other aspects of the physiology

of this genus have been studied with great detail in recent years (e.g. Elster et al. 2008, Nagao et al. 2008, Karsten et al. 2010, Karsten & Rindi 2010, Kaplan et al. 2012, Karsten & Holzinger 2012, Karsten et al. 2013). Novis (2006) found that both *K. acidophilum* and *K. dissectum* were able to grow in a similar range of pH (approximately 2.0 to 9.0), but the healthiest filaments of *K. acidophilum* were observed at pH 2.4, whereas the healthiest filaments of *K. dissectum* were found at pH 4.8–6.2; combined with other morphological data, this supports the separation of *K. acidophilum* as a low pH species. Interestingly, other strains closely related to *K. acidophilum* examined in subsequent studies were collected from different geographical regions but also isolated from acidic environments: *Klebsormidium* sp. K44 from an acidic peat bog in the Czech Republic (Škaloud & Rindi 2013) and *K. scopulinum* PRC 2378 (sequenced in this study) from the acidic seep of an abandoned coal mine in Ohio.

The idea that acid-adapted strains of *Klebsormidium* originate independently from generalist populations when acidic habitats become available is also partially supported by their high biogeographical diversity. It is remarkable that almost each acid-adapted lineage discovered in this study was restricted to a single site or a few sites in the same geographical area. At present none of these lineages appear to have a wide geographical distribution, with the only major exception of the lineage containing *K. acidophilum* and *K. scopulinum* (which, as mentioned above, includes strains from Czech Republic, New Zealand and Ohio). Biogeography and dispersal of acidophilic organisms are fascinating but poorly understood topics, and have been investigated in detail only for few taxa. Some authors concluded that acidophilic species have worldwide distribution (Gimmler 2001, Gerloff-Elias et al. 2005), conforming essentially to the neutral model of ubiquitous dispersal of microorganisms (everything is everywhere, but the environment selects; Baas Becking 1934). However, molecular studies that have focused on some acid-adapted morphospecies have unraveled genetic heterogeneity correlated to geographical distribution; cyanidialean red algae belonging to the genus *Galdieria* are the best-know example (Pinto et al. 2003, Ciniglia et al. 2004). Not surprisingly, this pattern is found in algae that are obligate acidophiles restricted to thermal acidic sites. An acidic environment is essential for the survival of these organisms, that cannot establish subpopulations in non-acidic habitats (Gross et al. 2001); their inability to reach easily other sites with suitable characteristics determines geographic isolation, with consequent high genetic differentiation (Ciniglia et al. 2004). The patterns observed for *Klebsormidium* conform to some extent to this situation; this may be an indication that many low pH *Klebsormidium* strains have reached a high level of specialization to acidic habitats, and do not disperse easily. This possibility, however, requires confirmation based on physiological data and further molecular sampling from other geographic regions. At the moment the data available for low pH strains are geographically biased (most of our strains are from central Europe) and sequence data of strains from other continents could reveal a different scenario.

The morphological identification of our strains based on characters traditionally used for species circumscription in *Klebsormidium* (Printz 1964, Ettl & Gärtner 1995, Lokhorst 1996, John 2002) was straightforward for most of our strains. Our results, however, confirm the discrepancies between molecular phylogeny and morphology-based taxonomy evidenced by previous molecular studies (Mikhailyuk et al. 2008, Rindi et al. 2008, Novis & Visnovsky 2011, Rindi et al. 2011). Some species, such as *Klebsormidium crenulatum* and *K. elegans*, can be linked unambiguously with molecular phylogenetic groups, and the low pH strains exhibitng the morphology of these species belong to the expected clades. Conversely, the morphology of the type species *K. flaccidum* is homoplasious and largely widespread in the phylogeny of the genus. This situation was already highlighted by previous studies (Rindi et al. 2011, Škaloud & Rindi 2013) and is confirmed here: low pH strains morphologically referable to *K. flaccidum* belong to three different clades. The taxonomic circumscription of this species remains an open problem which cannot be solved by sequencing the type specimen, due to its poor quality (a small sample formed by a few filaments embedded in a drop of mud). Since the morphology of this species is so widespead in different clades, selection of a specimen from the type locality (Strasbourg, France) would also not guarantee to obtain the alga actually used for the description of the species (Kützing 1849). Therefore, the designation of an epitype specimen based on a subjective choice appears to be the only feasible solution of the reassessment of this species (Rindi et al. 2011). To a lesser extent, the same considerations apply to *K. nitens*, which is also polyphyletic; in this case, however, there is a phylogenetic group which appears a good candidate to be linked with this morphospecies (the clade E2, in which most strains referred to this species are recovered; Rindi et al. 2011, Škaloud & Rindi 2013).

An interesting discovery of this study is the phylogenetic positioning of the strains LUK S12 and LUK S66 in the superclade G. The superclade G represents a lineage recently discovered, formed mainly by strains isolated from biotic crusts of subdesertic areas in Namibia and South Africa (Rindi et al. 2011). Within this superclade, the strains LUK S12 and LUK S66 form a well-supported lineage with *Klebsormidium* sp. LUK 318, another strain isolated from eastern Europe (discarded material on soil previously subjected to coal mining in the Czech Republic). The two strains sequenced here exhibited a four-lobed chloroplast with a median incision, a character that appears to be diagnostic for this group. It will be very interesting to verify if this group has a wider geographical distribution and if it also occurs in different types of habitats; it can be expected, in particular, that strains of *Klebsormidium* from desertic areas in North America (Lewis & Fletchner 2002, Lewis & Lewis 2005) will turn out to belong to this superclade.

An additional point that requires some discussion is the high morphological plasticity related to environmental factors observed in *Klebsormidium*, an aspect that has complicated many morphological studies. It has been shown that the culture conditions and the age of cultures can significantly affect some morphological characters which were considered useful for species identification for a long time (Škaloud 2006, Rindi et al. 2008). The limited data available indicate that pH conditions may play a major role in this regard: Novis (2006) highlighted a strong effect of pH on the morphologies of *K. acidophilum* and *K. dissectum*, showing that

characters such as cell shape, chloroplast shape and amount of granules deposited in the cytoplasm varied considerably in different pH conditions. Variations in pH are also known to affect cell shape in other green microalgae, mainly determining an overall reduction of the cell surface relative to the cell volume (Coesel 1982, Černá & Neustupa 2010). This phenomenon is considered a functional response aimed at reducing osmotic stress. Algae living in low pH habitats are able to maintain a fairly constant, neutral, cytosolic pH over a wide range of external pH values (Gerloff-Elias et al. 2005, Bethmann & Schönknecht 2009); the maintenance of a neutral cytosolic pH under low pH conditions is an energy-demanding process that involves considerable metabolic costs, and a reduced cell surface contributes to reducing these costs (Černá & Neustupa 2010). Due to lack of experimental data, we are not able to demonstrate similar effects in our *Klebsormidium* strains and to assess their morphological variation in different pH conditions; we expect, however, that variations similar to those reported by Novis (2006) are probably general phenomenon.

In conclusion, the genus *Klebsormidium* is a morphologically and physiologically dynamic algal group in which the capacity of adaptation to low pH conditions has been developed multiple times independently. Further studies aimed at clarifying the extent of this adaptation and the molecular features that determine it have the potential to shed light into many fascinating aspects of the biology of acidophilic and acidotolerant organisms that are still poorly understood.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo.supp-data), and consist of additional sequences of Klebsormidiales used for the phylogenetic analyses.

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