

Influence of substrate and pH on the diversity of the aeroterrestrial alga *Klebsormidium* (Klebsormidiales, Streptophyta): a potentially important factor for sympatric speciation

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ABSTRACT: Our knowledge of the processes involved in speciation of microalgae remains highly limited. In the present study, we investigated a potential role of ecological speciation processes in diversification of the filamentous green alga *Klebsormidium*. We examined 12 strains representing four different genotypes. The strains were collected from sandstone and limestone rocks and were cultivated at five different pH levels ranging from pH 4 to pH 8. We determined the responses of the 12 strains to the experimental pH conditions by (1) measuring the effective quantum yield of photosystem II, and (2) determining the growth rates after cultivation at different pH levels. Strong differences were found between the results obtained by these two methods. Direct counting of cells revealed a strong ecological differentiation of strains of *Klebsormidium* isolated from different substrate types. Strains isolated from limestone showed the highest growth rates at higher pH levels; whereas, the strains isolated from sandstone exhibited two distinct growth responses with optima at pH 5 and 6, respectively. In contrast, the effective quantum yield of photosystem II was always down-regulated at lower pH values, probably due to dissolved inorganic carbon limitation. In general, we determined distinct ecophysiological differentiation among distantly and closely related lineages, thereby corroborating our hypothesis that the sympatric speciation of terrestrial algae is driven by ecological divergence. We clearly showed that pH is a critical ecological factor that influences the diversity of autotrophic protists in terrestrial habitats.

KEY WORDS: Diversity, *Klebsormidium*, pH, Protist, Speciation

INTRODUCTION

Protist species diversity has been the subject of considerable debate (Finlay 2002; Foissner 2006; Caron 2009). On one hand, some researchers think that diversity is very low because protists show a cosmopolitan distribution without endemic species. This ubiquity model predicts a very low probability of local extinction within protist populations (Fenchel & Finlay 2003). The consequence of this very low local extinction, coupled with extremely large population size, is high local protist diversity and low global diversity (Fenchel & Finlay 2003, 2004). Supporters of the ubiquity model estimate that approximately 20,000 protist species exist (Finlay & Fenchel 1999). Fenchel & Finlay (2003) argued that the distribution of the smallest organisms is dependent solely on habitat properties and not on contingencies of the evolutionary history, as is the case for multicellular organisms. On the other hand, other researchers think that diversity is very high because microorganisms also have a significant portion of endemic species. The existence of endemic species has been demonstrated through the example of flagship species, i.e. species with easily recognizable morphologies for which presence/absence can readily be demonstrated (Foissner 2006; Foissner *et al.* 2008). The considerable protist diversity is puzzling because the current paradigm holds that dispersal in microbes is ubiquitous, and therefore allopatric speciation must be

strongly impeded by global gene flow. However, virtually nothing is known about alternative speciation mechanisms. Allopatric processes may play an important role in diatom speciation (Evans *et al.* 2009; Casteleyn *et al.* 2010). Moreover, some studies have demonstrated sympatric speciation (reviewed in Benton & Pearson 2001) based on fossil evidence. Nevertheless, it is clear that the distribution of some protists on the Earth's surface is restricted by their habitat requirements. In addition, convergent morphological evolution has frequently led to the existence of simple morphotypes, which show extremely high phylogenetic diversity (Huss *et al.* 1999). Recently, many molecular studies have demonstrated the existence of very high cryptic diversity in different protistan taxa (de Vargas *et al.* 1999; von der Heyden *et al.* 2004; Škaloud & Peksa 2010; Škaloud *et al.* 2012).

Ecological adaptation is also known to influence diversity in algae (Huss *et al.* 2002; Logares *et al.* 2007; Rindi *et al.* 2008; Škaloud & Rindi 2013; Škaloud *et al.* 2014). For example, Rindi and Guiry (2002) reported that some species of *Trentepohlia* and *Printzina* formed perennial populations on different substrates in urban habitats in western Ireland. Similar ecological differentiation was reported for some species of *Prasiola* (Trebouxiophyceae) (Moniz *et al.* 2012). The influence of environmental factors was further demonstrated by Peksa & Škaloud (2011), who showed that specific lineages within the lichen photobiont genus *Asterochloris* exhibited clear environmental preferences, in relation to factors such as exposure to rain or sunlight, substrate type, and climate.

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In comparison with the factors that determine the diversity of marine and freshwater algal communities (Machová-Černá & Neustupa 2009; Desrosiers *et al.* 2013; Svoboda *et al.* 2014), the critical factors that influence the distribution of aeroterrestrial algae remain unclear. The key factors that influence the community structure of terrestrial algae include light, humidity, temperature, nutrients, and pH (Hoffmann 1989). Pietrasiak *et al.* (2011) showed that in the absence of disturbance, several abiotic factors, particularly soil texture, pH, and electrical conductivity, seemed to be important for the development of biotic crusts. The pH seems to influence the dominance of the major groups of soil photoautotrophic organisms; cyanobacteria are known to prefer neutral and alkaline soils (Shields & Durell 1964; Brock 1973); whereas, green algae predominantly prefer acidic soils (Starks *et al.* 1981; Lukešová & Hoffmann 1995; Lukešová 2001). Bates *et al.* (2012) think that soil protistan richness and diversity were primarily influenced by climatic conditions that regulate annual moisture availability in soils (Bates *et al.* 2012). Similarly, climatic factors probably control the species composition of aerophytic algal communities growing on urban walls, e.g. Prasiolales-dominated assemblages in Atlantic parts of Europe vs *Klebsormidium*-dominated growths in continental and Mediterranean cities (Rindi & Guiry 2004; Rindi *et al.* 2007). Algal communities isolated from sandstone that was used as building blocks for a German castle differed markedly according to their exposure to direct sunlight (Hallmann *et al.* 2013).

In the present study, we investigated the genus *Klebsormidium* (Silva *et al.* 1972; Sluiman *et al.* 2008; Rindi *et al.* 2011) as a model to obtain a better insight into the ecologically driven speciation of microalgae. This genus comprises cosmopolitan filamentous green algae broadly distributed in various terrestrial and freshwater habitats (Rindi *et al.* 2008, 2011; Škaloud & Rindi 2013; Mikhailiuk *et al.* 2014, 2015; Ryšánek *et al.* 2015). The local distribution of *Klebsormidium* is generally influenced by different substrate preferences (Novis 2006; Rindi *et al.* 2008, 2011; Škaloud & Rindi 2013; Škaloud *et al.* 2014), which repeatedly originated during the evolution of the genus (Škaloud *et al.* 2014). Strains of *Klebsormidium* show a wide range of adaptation to unfavourable conditions, such as prolonged desiccation (Karsten & Holzinger 2012; Karsten *et al.* 2013; Holzinger *et al.* 2014; Herburger & Holzinger 2015; Herburger *et al.* 2016; Karsten *et al.* 2016), low temperature (Elster *et al.* 2008; Nagao *et al.* 2008), heavy metals (Gaysina *et al.* 2009), and ultraviolet (UV) radiation (Nagao *et al.* 2008; Kitzing *et al.* 2014).

The main objective of the present study was to understand whether ecological preferences may be drivers of sympatric speciation in *Klebsormidium*. We focused on pH as one of the major factors influencing the diversity of terrestrial photoautotrophs (see above) and investigated its influence on the effective quantum yield and growth rate of several strains of *Klebsormidium* isolated from two different substrates, sandstone and limestone. We posed the following questions: (1) Do strains isolated from different substrates show differences in their response to pH? (2) Do different genotypes that inhabit the same substrate show differences

in their physiological and growth responses? (3) Do closely related strains isolated from different substrates show differences in their response to pH?

MATERIAL AND METHODS

Sampling sites and cultivation methods

We collected and isolated algal samples from rocks in the Czech Republic during the autumn seasons of 2012 and 2013. The strains isolated from sandstone (P05, P08, P09, AD31, AD32, and AD36) were collected in Labské pískovce (P05, P08, P09) (50°48'N, 14°14'E) and Adršpach (AD31, AD32, AD36) (50°36'N, 16°7'E). The strains isolated from limestone (J06, J07, J11, MA12, MA16, and MA24) were collected near the village of Vápenná (J06, J07, J11) (50°16'N, 17°5'E) and in Moravský kras (MA12, MA16, MA24) (49°22'N, 16°43'E). The pH levels of the sandstone substrata from Labské pískovce and Adršpach were 5.05 and 5.17, respectively. The pH levels of the limestone substrata from Vápenná and Moravský kras were 6.97 and 7.06, respectively. The pH was measured by WTW pH-330 set with a flathead electrode (WTW Sentix Sur, Weilheim, Germany). The pH was measured on 10 different sides of rock, at each locality. All strains of *Klebsormidium* were isolated by cultivating samples from rock on 1.5% agar supplemented with Bold's basal medium (BBM) (Starr & Zeikus 1993). The selected algal filaments were transferred repeatedly to fresh Petri dishes. After three changes of each isolate to fresh Petri dishes, the obtained cultures were observed to be unialgal by examination under an Olympus CX 31 light microscope (Olympus Corp., Tokyo, Japan). Unialgal stock cultures of *Klebsormidium* were maintained in BBM at 20°C under white fluorescent illumination of 30–50 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by 18 W cool tubes (Philips TLD 18W/33, Amsterdam, the Netherlands), with a light:dark (L:D) cycle of 14:10 hours. We examined the morphology of 5-week-old cultures during the exponential growth phase.

Molecular analyses

The DNA was isolated according to the protocol of Ryšánek *et al.* (2015) and stored at –20°C. We used 12 microcolonies of *Klebsormidium* for subsequent molecular analyses. For molecular screening of the isolated strains we used partial sequences of the plastid-encoded *rbcL* gene (the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase). The *rbcL* sequences were obtained by polymerase chain reaction (PCR) with a Touchgene Gradient cycler (Techne, Cambridge, United Kingdom) using the primers KF590 (5'-GAT GAA AAC GTA AAC TCT CAG C-3') and *rbcL*-KR2 (5'-GGT TGC CTT CGC GAG CTA-3'; Škaloud & Rindi 2013). Both primers were designed specifically to amplify species of *Klebsormidium*. Each 20- μl reaction for PCR was composed as described by Ryšánek *et al.* (2015). The PCR protocol followed that of Škaloud & Rindi (2013). Sequencing reads were assembled and edited by using the SeqAssem software (Hepperle 2004).

For phylogenetic analyses, we used the newly obtained *rbcL* sequences of *Klebsormidium* and a selection of *rbcL* sequences of Klebsormidiales available in GenBank to produce an alignment. The final alignment of 632 base pairs (bp) was constructed by using ClustalW (Thompson *et al.* 1994) with MEGA 5.05 (Tamura *et al.* 2011). The aligned data set was analysed by using maximum parsimony with Phylogenetic Analysis Using Parsimony (PAUP 4.0b10; Swofford 2002), maximum likelihood with the Genetic Algorithm for Rapid Likelihood Inference (Zwickl 2006, unpublished Ph.D. dissertation), and Bayesian inference (BI) analysis with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). The evolutionary model was determined by using PAUP/MrModeltest 2.3 (Nylander 2004). The model selected under the Akaike information criterion was GTR + I + G. The BI analysis was performed by using the priors set as default in MrBayes. The robustness of the tree topologies was assessed by bootstrapping the data set as described by Škaloud & Rindi (2013).

Effective quantum yield in liquid medium

Six strains of *Klebsormidium* (limestone strains J06, J07, J11 and sandstone strains P05, P08, P09) were selected to evaluate their physiological performances in liquid medium. The exponentially growing strains were inoculated into 50-ml Erlenmeyer flasks containing fresh BBM medium. The strains were grown at five different pH levels (pH 4, pH 5, pH 6, pH 7, and pH 8). Liquid BBM medium was buffered to pH 4, pH 5, or pH 6 with 1 mM 2-(*N*-morpholino) ethanesulphonic acid and to pH 7 or pH 8 with 1 mM *N*-(2-hydroxyethyl) piperazine-*N'*-2-ethanesulfonic acid hemisodium salt. The pH was checked and adjusted at 3-day intervals by using 0.1 M NaOH or 0.1 M HCl (InoLab pH/conductometer 720, WTW). The strains were cultivated at an optimum growth temperature of 20°C under continuous white fluorescent illumination of 20 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{second}^{-1}$ for 8 days. Cultures of *Klebsormidium* (each with an approximate volume of 100 μl four times) were harvested daily and were concentrated on Whatman GF/F glass fibre filters (Whatman, Seattle, Washington USA). The filters were saturated with BBM at a similar pH as in harvested bottles and were maintained for 2 hours in Petri dishes at ambient room temperature ($\sim 22^\circ\text{C}$) under continuous white fluorescent illumination of 20–25 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The effective quantum yield ($\Delta F/F_m'$) of photochemistry was determined at regular intervals by using a pulse-amplitude modulated fluorimeter (PAM 2500; Heinz Walz GmbH, Pfullingen, Germany). The physiological state of the photosynthetic apparatus was determined by measuring the effective quantum yield of photosystem II photochemistry in a dark-acclimated state ($\Delta F/F_m'$). The measurements were made after 2 and 3 hours, with four replicates per strain. In total, we performed eight measurements for each pH level.

Effective quantum yield on rock substrate

Eight strains of *Klebsormidium* (limestone strains MA12, MA24, J06, J07 and sandstone strains AD31, AD32, AD36, P05) were selected to investigate their performances when

growing directly on rocky substrata. Small pieces of rock (*c.* 1 cm in diameter) were transferred to Petri dishes and were stabilized by using 1.5% solidified agar (in distilled water). Each strain was cultivated on sandstone ($n = 4$) and limestone ($n = 4$) rocks (Fig. S1) at an optimum growth temperature of 20°C under continuous white fluorescent illumination of $\sim 20 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{second}^{-1}$ for 10 days. The effective quantum yield ($\Delta F/F_m'$) of photochemistry was determined daily by using a pulse-amplitude modulated fluorimeter (PAM 2500; Heinz Walz GmbH), with four replicates per strain.

Growth rate estimations

All 12 cultivated strains were used for growth rate measurements. After 2–3 weeks, approximately 1–1.5 ml of the experimental cultures growing in 50-ml Erlenmeyer flasks with fresh BBM medium were harvested into 2-ml tubes (Eppendorf, Hamburg, Germany) containing 2–3 glass balls, each with a diameter of 0.5 mm (Sigma-Aldrich, St Louis, Missouri USA). The tubes were inserted into a mill for grinding plant material (Retsch MM400, Haan, Germany) to fragment filaments into single cells; the mill was operated for 2–3 minutes at 18–24 frequencies/second. Next, approximately 80–120 μl of the solution (containing filament fragments of differing sizes) were pipetted onto Petri dishes (diameter 8 cm). To monitor the subsequent growth of single-cell fragments, we pipetted very low cell densities (*c.* 2600 cell fragments/ml). All strains were grown at different pH levels (pH 4, pH 5, pH 6, pH 7, and pH 8) in Petri dishes containing 1.5% agar supplemented with BBM. The solidified BBM medium was buffered in the same manner as described above for the experiment in liquid medium. The pH was measured at 2-day intervals by using a WTW 330/SET1 with SenTix Sur electrode. Strains were cultivated at an optimum growth temperature of 20°C under continuous white fluorescent illumination of 20 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{second}^{-1}$ for 4 days. The culture time was relatively short because the pH increased slowly, and we were unable to adjust the pH of the agar plates. At the start of the experiment, we selected approximately 30–40 single cells for cultivation at each pH level, and we subsequently monitored the growth of each single cell over four consecutive days. Each day, we recorded the length of the filaments (number of cells) grown from these single cells; we used these measurements to determine the growth rates based on 30–40 replicates. We counted the cells by direct observation using an Olympus CX 31 light microscope.

Data analysis

The effect of different pH values and rock surfaces on effective quantum yield ($\Delta F/F_m'$) was evaluated by multi-sample nonparametric Friedman two-way analysis of variance (ANOVA) tests by ranks, in connection with the Statistica software (StatSoft Inc, Tulsa, Oklahoma USA). Post hoc nonparametric multiple comparisons were computed by applying the 'Post Hoc For Friedman.svb' macro, comparing the absolute values of the differences for all analyzed pairs. Pair differences in mean ranks were displayed in R (R Core Team 2016), using the package corplot. Growth response to different pH values was tested

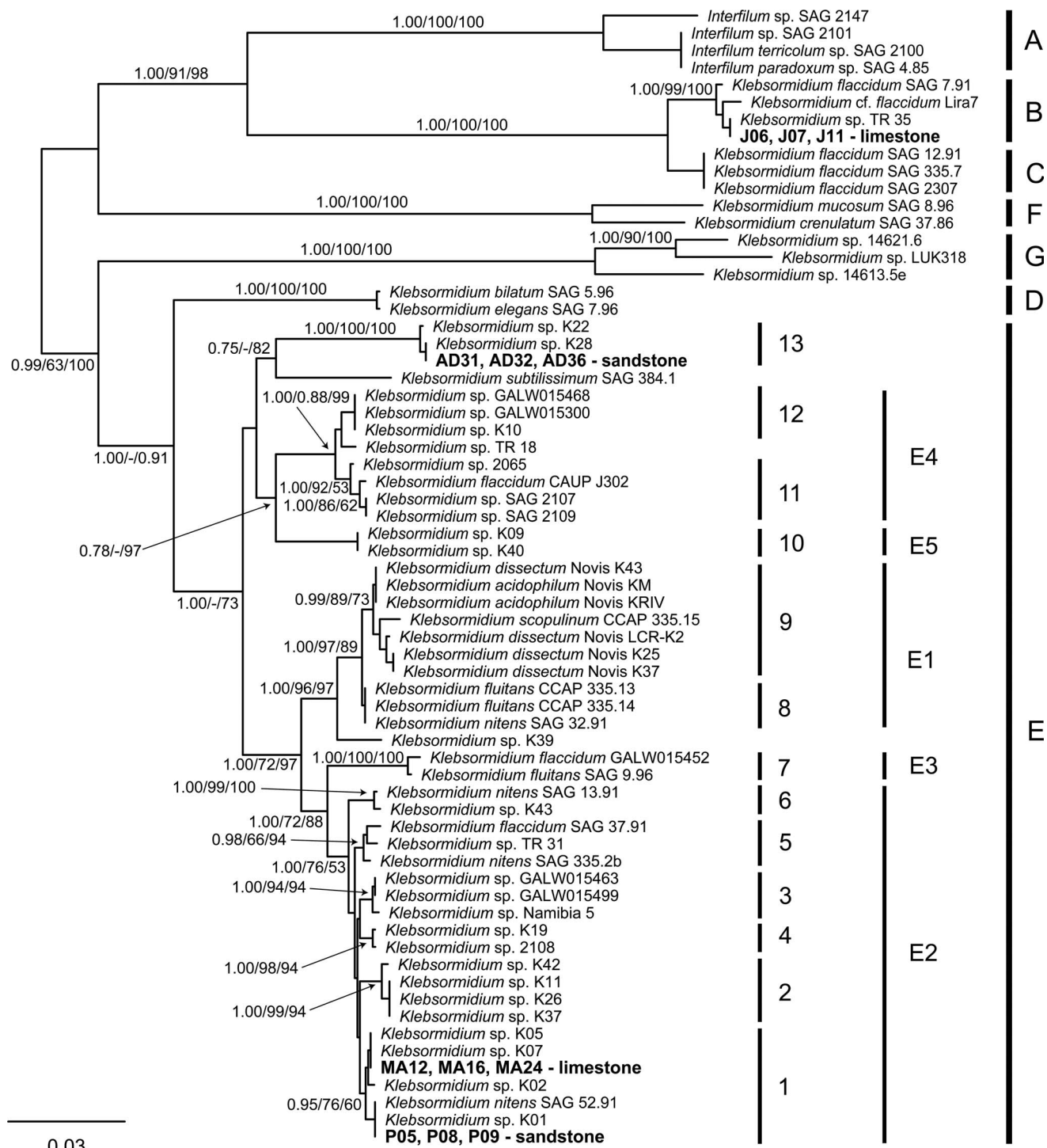


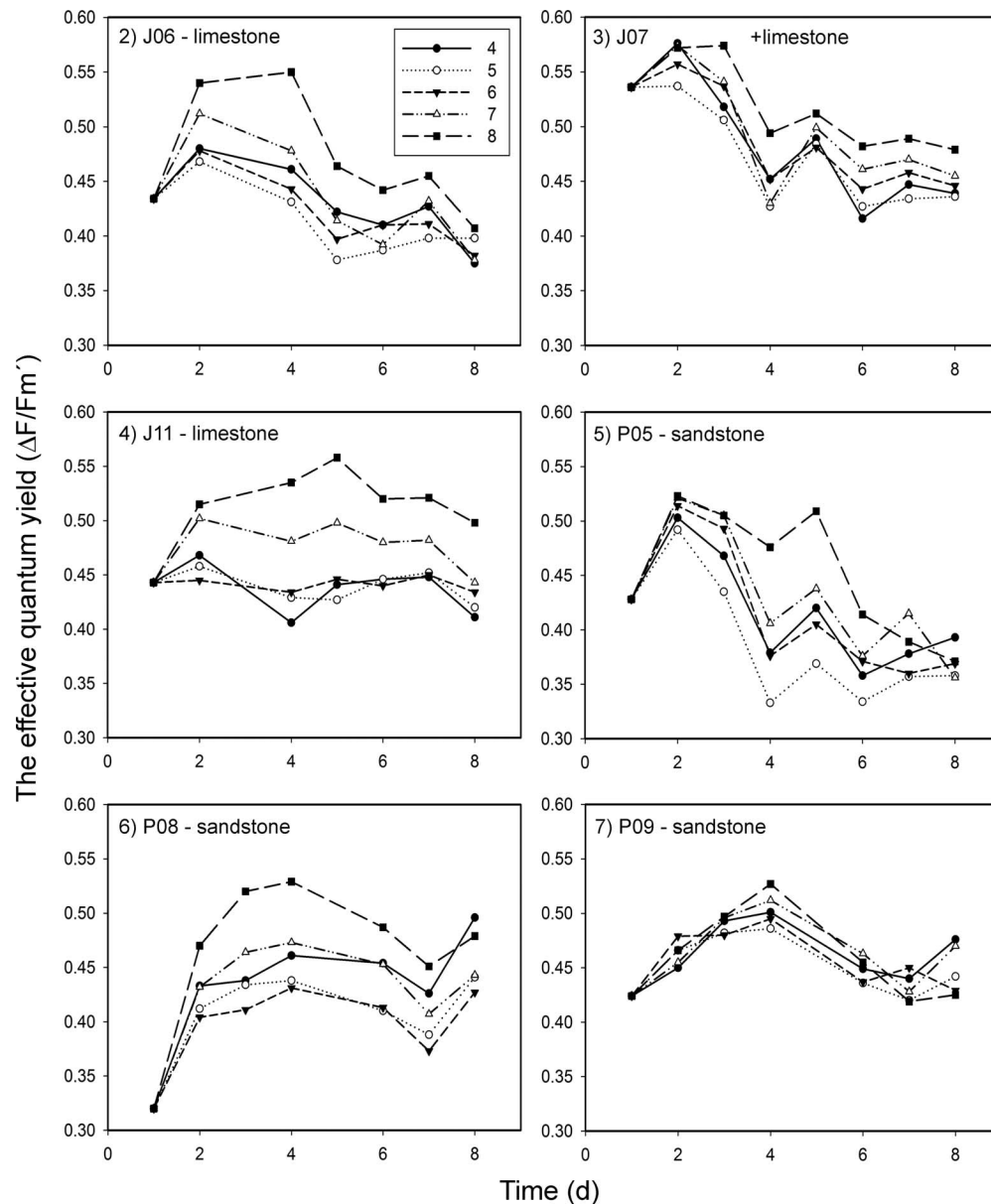
Fig. 1. Phylogenetic tree obtained from Bayesian analysis based on *rbcL* dataset, showing the position of investigated strains of *Klebsormidium* and their relatives. Values at the nodes indicate statistical support estimated by MrBayes posterior probabilities (left), maximum likelihood bootstrap (middle), and maximum parsimony bootstrap (right). The clade numbering (A–G, E1–E6) follows Rindi *et al.* (2011) and clades (1–13) are according to Škaloud and Rindi (2013).

by one-way ANOVA tests with the Tukey's pairwise comparisons in the program PAST 2.17c (Hammer *et al.* 2001). The graphs were created in SigmaPlot. The significance was tested to three levels: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

RESULTS

Strain morphology and molecular analyses

The results of our molecular analyses revealed that the isolated strains were representative of four different



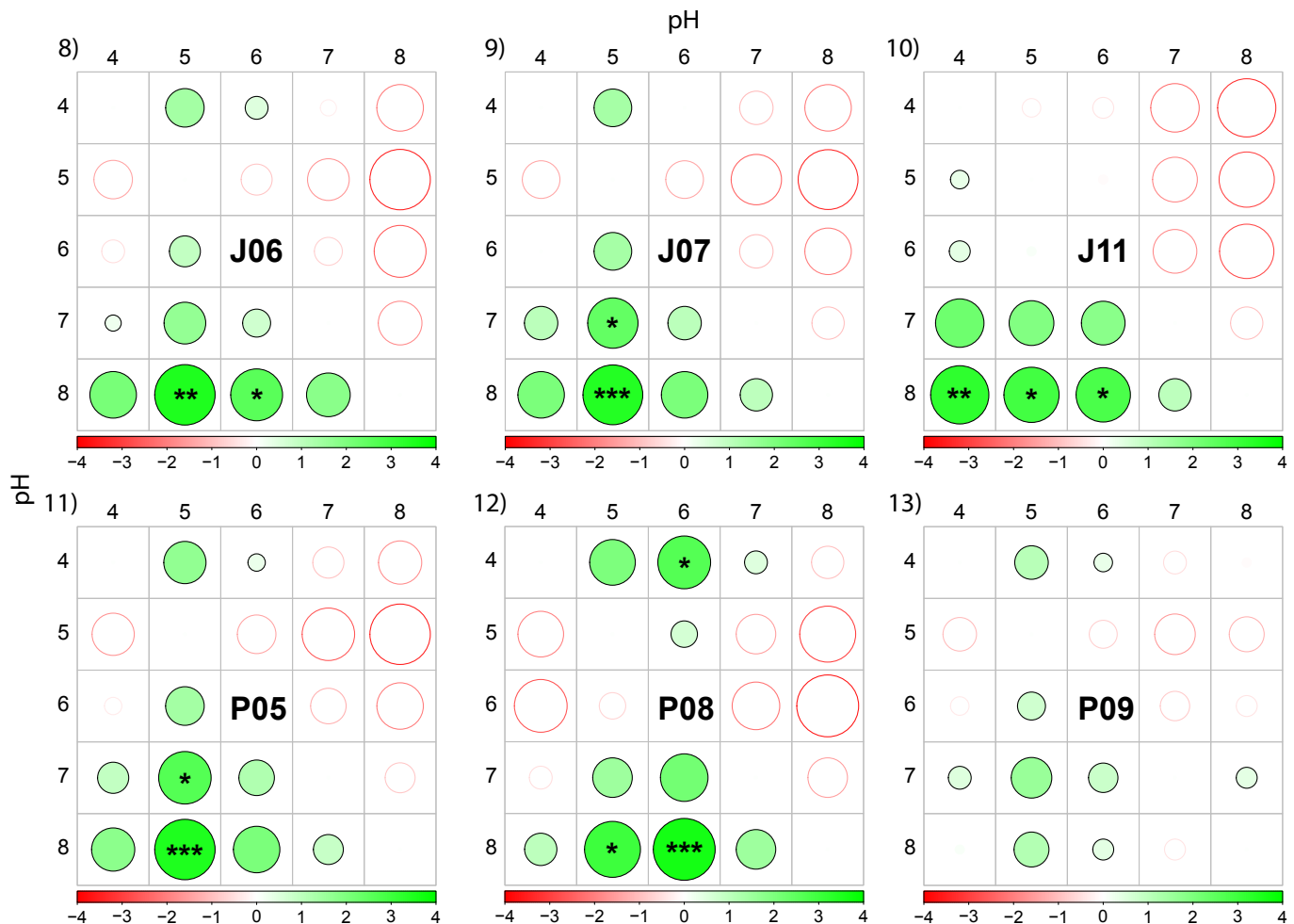
Figs 2–7. The effective quantum yield ($\Delta F/F_m'$) of PS II measured for three limestone (J06, J07, J11) and three sandstone (P05, P08, P09) strains of *Klebsormidium* at five different pH levels (4–8). The values plotted represent means of four replicated measurements. Differences were evaluated by post hoc comparisons of nonparametric Friedman two-way ANOVA tests (see Figs 8–13). Standard deviations of those measurements are given in Tables S1–S2.

genotypes, which were specific to the sampled localities (Fig. 1). These four genotypes were inferred within three distinct clades. Two genotypes (sandstone strains P05, P08, and P09 and limestone strains MA12, MA16, and MA24) were inferred within clade E1 *sensu* Škaloud & Rindi (2013); sandstone strains AD31, AD32, and AD36 were inferred within clade E13 *sensu* Škaloud & Rindi (2013); and limestone strains J06, J07, and J11 were shown to belong to superclade B *sensu* Rindi *et al.* (2011). The *rbcL* sequences of the 12 investigated strains of *Klebsormidium* were deposited in GenBank under accession numbers KU528666–KU528677. With the exception of superclade B, in which small differences between strains were observed, the strain morphology was rela-

tively uniform within and among clades. In most of the strains, the filaments were short, 4–6 μm wide, and tended to segregate into shorter filaments or individual cells. Cells were 1.4–3.2 times longer than wide; the chloroplast covered 50% of the cell wall and usually possessed a small pyrenoid. With the exception of strain J11, H-shaped pieces were not observed.

Response to pH

EFFECTIVE QUANTUM YIELD IN LIQUID MEDIUM: In our first approach to evaluate the organisms' response to different pH values, we determined the physiological performance of three limestone strains (J06, J07, and J11) and three



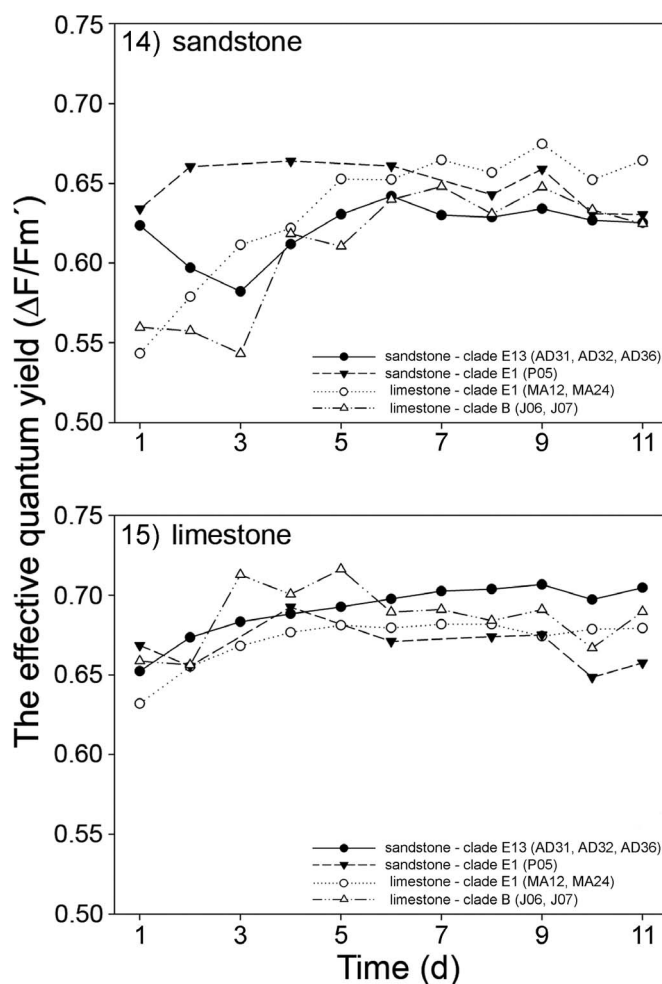
Figs 8–13. Differences in the effective quantum yield (DF/F_m^0) of PS II measured at five different pH levels (4–8) on six selected strains (see Figs 2–7 for measured data). Differences were evaluated by post hoc comparisons of nonparametric Friedman two-way ANOVA tests. First, repeated DF/F_m^0 measurements at different pH values were ordered from highest to lowest. Then, mean ranks were calculated for every pH level. Within each row (a particular pH level), positive and negative differences in DF/F_m^0 mean ranks are displayed by a symbol size and shading. Filled and empty circles display positive and negative differences in mean ranks, respectively, varying from -4 to 4 (see the colour shade legend). For example, in the strain J06 mean rank in DF/F_m^0 at pH 4 was higher relative to pH 5 and 6 but lower relative to pH 8. Significant differences as determined by Friedman two-way ANOVA tests are indicated by asterisks (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$).

sandstone strains (P05, P08, and P09) in liquid media buffered to distinct pH values. We used a pulse-amplitude modulated fluorimeter to measure the effective quantum yield of photosystem II (Figs 2–7; Tables S1–S2). In all investigated strains, the effective quantum yield (DF/F_m^0) showed different responses to pH: DF/F_m^0 was significantly higher at pH 8 than at lower pH values (Figs 8–13), as shown by the two-way Friedman ANOVA tests, with the single exception of strain P09 (Fig. 13). In the strain P08 (Fig. 12), the effective quantum yield was significantly higher at pH 4 than at pH 6, as well. Throughout the experiments, the lowest DF/F_m^0 values were measured at pH 5 (Fig. 8).

EFFECTIVE QUANTUM YIELD ON ROCK SUBSTRATE : In our second approach, we cultivated four sandstone (AD31, AD32, AD36, and P05) and four limestone (MA12, MA24, J06, and J07) strains, respectively, directly on pieces of sandstone and limestone. As with the previous experiments, the physiological performance of investigated strains was

evaluated by measuring the effective quantum yield of photosystem II (DF/F_m^0). We found no significant differences in physiological performances of limestone and sandstone strains when they were cultivated on the same type of rock (Figs 14–15; Tables S3–S4). However, Friedman two-way ANOVA tests revealed significant differences in the pairwise comparisons of the physiological performance of some strains (Fig. 16). DF/F_m^0 was significantly higher on the strains cultivated on limestone, as determined by two-way Friedman ANOVA tests. Interestingly, the DF/F_m^0 of the limestone strains were inhibited during the first 4 days of cultivation on sandstone (Fig. 14). After this acclimation period, their physiological performance was, however, comparable to the sandstone strains.

GROWTH RATE ESTIMATIONS : Finally, we evaluated the influence of pH on the specific growth rates of all 12 cultivated strains of *Klebsormidium*. We found that all strains isolated from limestone (MA12, MA16, MA24, J06, J07, and



Figs 14–15. The effective quantum yield ($\Delta F/F_m'$) of PS II measured for four limestone (MA12, MA24, J06, J07) and four sandstone (AD31, AD32, AD36, P05) strains when growing directly on sandstone (Fig. 14) and limestone (Fig. 15) rock substrate. The values plotted represent means of four replicated measurements. Genetically identical strains were displayed by a single value for better clarity. Differences were evaluated by post hoc comparisons of nonparametric Friedman two-way ANOVA tests (see Fig. 16). Standard deviations of the measurements are given in Tables S3–S4.

J11) exhibited similar responses to pH; these strains showed increases in growth rate as the pH of the medium increased (Figs 17–18). After 4 days of cultivation, we identically counted only three cells at pH 4 but >10 cells at pH 7 and pH 8. However, strains J06, J11, MA16, and MA24 showed the highest growth rates at pH 7; whereas, strains J07 and MA12 showed the highest growth rates at pH 8. Strains MA12, MA16, and MA24 grew slightly faster than did strains J06, J07, and J11. The statistical tests revealed no significant differences in growth rates between pH 7 and 8 but the growth rates at other pH values were significantly different ($P < 0.001$), as determined by one-way ANOVA tests.

We observed different responses to pH between the two sandstone genotypes (Figs 19–20). Strains belonging to clade E13 (AD31, AD32, and AD36; Fig. 19) showed significantly different growth patterns ($P < 0.001$) at the investigated pH

levels. These strains exhibited the highest growth rates at pH 6; at this pH, they produced an average of 10 cells after 4 days of cultivation. In contrast, at pH 4 and pH 8, these strains grew more slowly and produced only approximately two cells after 4 days. Strains belonging to clade E1 (Fig. 20) grew well at all investigated pH levels; however, they showed higher growth rates at pH 5 than at pH 8 (c. 9 cells vs c. 4–6 cells). For E1 sandstone strains, the statistical tests supported the significant differentiation of growth rates at pH 5 and 8, respectively ($P < 0.001$). The highest growth rate of E13 sandstone strains at pH 6 was also significantly supported ($P < 0.001$).

DISCUSSION

In this study, we applied two different methods to monitor the response of strains of *Klebsormidium* to different pH levels. First, we used a pulse-amplitude modulated fluorimeter to measure the effective quantum yield ($\Delta F/F_m'$) of photosystem II. The main advantage of this method is that it enables the maintenance of cultures in liquid medium, thereby allowing pH adjustment during cultivation. This in turn enables experiments to be continued for prolonged periods (up to 8 days in the present study). Second, we used direct counting of cells on agar plates. This method is straightforward and provides an accurate estimate of cell growth rates at different pH levels. However, it is almost impossible to adjust the pH during the experiment. After 4 days in the present study, the pH had increased by c. 0.5 pH units. This increase in pH during cultivation experiments is caused by the photosynthetic activity of phototrophic organisms (Shiraiwa *et al.* 1993). Therefore, this technique should be used only for algal strains with relatively high growth rates.

Interestingly, we found clear differences between the results obtained by these two methods. Direct counting of cells revealed a strong ecological differentiation of strains of *Klebsormidium* isolated from different substrate types. Strains isolated from limestone showed the highest growth rates at pH 7 and pH 8 and had significantly lower growth rates at pH 4 and pH 5. The strains isolated from sandstone exhibited distinct growth responses. While strains belonging to clade E1 showed generally similar growth rates at all investigated pH levels, the strains inferred within clade E13 showed the highest growth rate at pH 6.

In contrast, the effective quantum yield ($\Delta F/F_m'$) of photosystem II did not show any differentiation between the strains isolated from different rock types. Instead, all investigated strains showed the highest $\Delta F/F_m'$ at pH 8, and its down-regulation at lower pH values. Such coincident responses indicate the presence of a common mechanism inducing an increase of photosynthetic efficiency at higher pH values.

Since the intracellular pH of photoautotrophs is usually maintained fairly constant over a wide range of external pH values (Lane & Burris 1981), we hypothesize that a strong positive effect of pH to $\Delta F/F_m'$ was probably caused by dissolved inorganic carbon (DIC) limitation at lower pH values. In aqueous solutions, the dissolved CO_2 dissociates

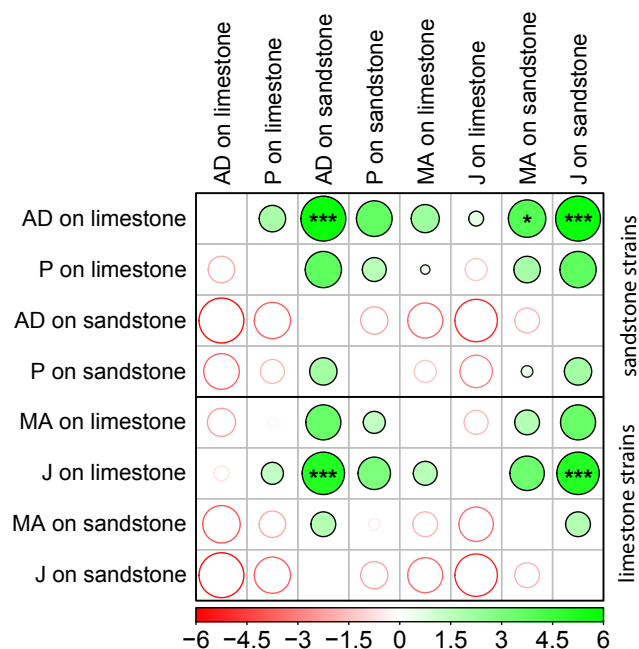


Fig. 16. Differences in the effective quantum yield (DF/F_m^0) of PS II measured at two different natural substrate (sandstone and limestone) on eight selected strains (see Figs 14–15 for measured data). Differences were evaluated by post hoc comparisons of nonparametric Friedman two-way ANOVA tests (see Figs 8–13 legend for further explanation). Filled and empty circles display positive and negative differences in mean ranks, respectively, varying from –6 to 6 (see the colour shade legend). Significant differences as determined by Friedman two-way ANOVA tests are indicated by asterisks (*** P , 0.001, ** P , 0.01, * P , 0.05).

into bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}), maintaining a certain ratio depending on pH, ion concentrations, and salinity (Falkowski & Raven 2007). At high pH values, HCO_3^- is the dominant carbon species. However, Rubisco reacts only with dCO_2 , not bicarbonate or carbonate ions (Baba & Shiraiwa 2012). The majority of microbial phototrophs use dCO_2 when it is freely available (Reynolds 1984). However, some Cyanobacteria and green algae use both dCO_2 and HCO_3^- in photosynthesis due to the extra- and intracellular activity of carbonic anhydrase (Allen & Spence 1981). Although the use of different DIC components has not been studied in *Klebsormidium*, we consider the stimulation of photosynthesis by HCO_3^- as the most likely explanation of the positive correlation of DF/F_m^0 and pH in investigated strains (Shelp & Canvin 1980). In general, our results clearly show that, at least in our experimental system, DF/F_m^0 does not reflect the overall fitness of studied organisms but rather indicates the efficiency of PSII under different DIC conditions. We therefore consider growth rate responses to different pH as the only measured data useful for assessing the ecological adaptation of the studied strains.

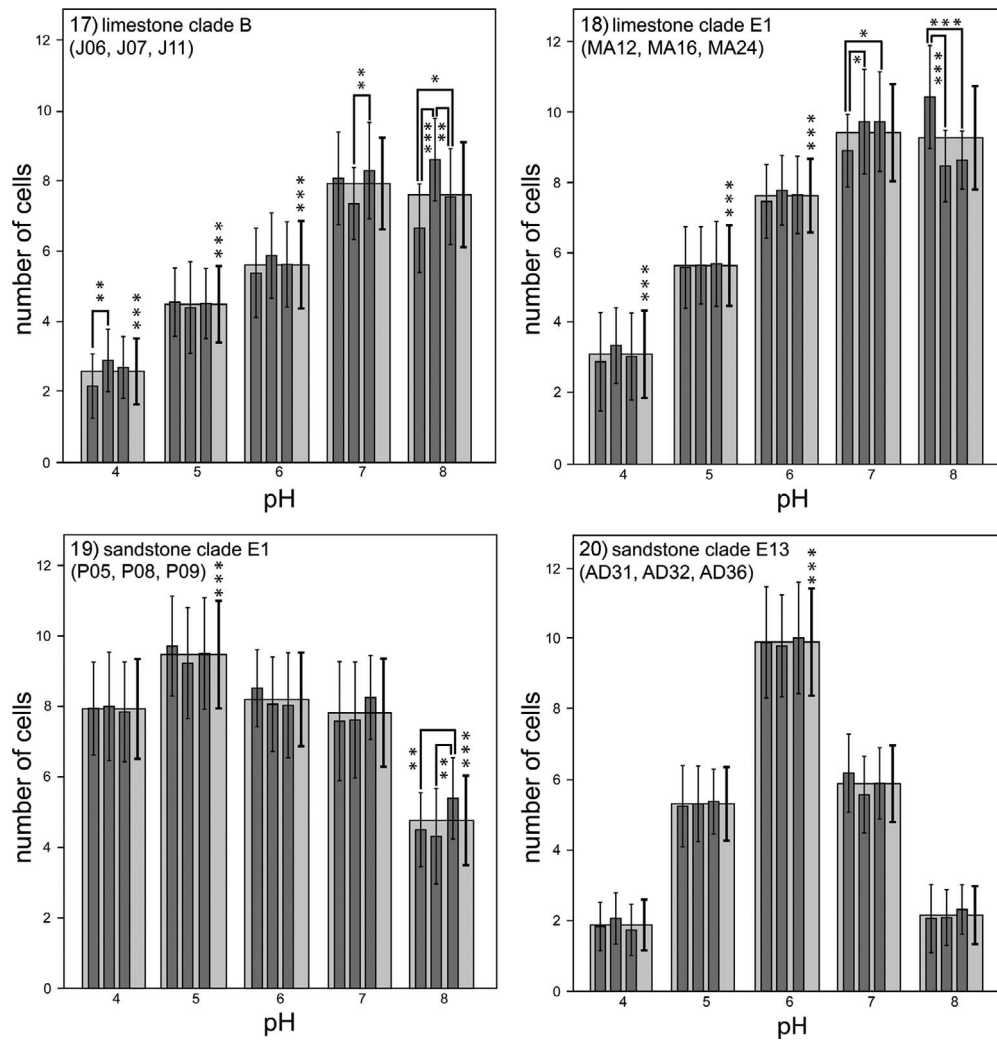
The tolerance of the investigated strains of *Klebsormidium* to a wide range of pH levels is in accordance with the results of previous investigations of the ecophysiology of this genus (Karsten et al. 2014). In general, strains of *Klebsormidium* are relatively tolerant to various physiological stresses such as UV radiation (Nagao et al. 2008; Kitzing et al. 2014;

Kitzing & Karsten 2015), desiccation (Karsten et al. 2010, 2016; Karsten & Holzinger 2014), and osmotic stress (Kaplan et al. 2012). The genomic machinery required for adaptation to terrestrial environments was detected in the genome of *Klebsormidium flaccidum* (Kitzing et al. 2014), which is a member of clade E5 sensu Škaloud & Rindi (2013). This machinery includes genes involved in the signalling pathways for the phytohormones cytokinin, ABA, and ethylene, which was recently confirmed in the desiccation transcriptome of *Klebsormidium crenulatum* (Holzinger & Becker 2015). This is interesting, as the receptors of, e.g. cytokinin signalling show a strictly pH-dependent ligand binding in vascular plants (Lomin et al. 2015).

This ability to adapt to a wide range of conditions typical of terrestrial environments may explain the cosmopolitan distribution of the genus *Klebsormidium* (Ryšánek et al. 2015). Nevertheless, the relatively high abundance of this genus in different types of localities might simply be a sampling artefact. In other words, strains that are common and geographically widespread have been discovered and studied; whereas, rarer strains (e.g. such as those belonging to clade E13) have either not yet been studied or have been studied only sporadically. In the present study, strains belonging to clade E13 grew optimally in a very narrow range of pH levels; they showed the highest growth rates at pH 6 but grew very slowly at pH 4 and pH 8. This finding might imply that clade E13 is a specialist rather than a generalist, and this in turn may reflect its ecologically restricted occurrence. Generalists have a wider range of suitable habitats and are therefore discovered more frequently; in addition, they are geographically more widespread than are specialists (Finlay et al. 2002).

In the present study, we showed clear differences in growth responses between strains isolated from sandstone (lower pH preference) and limestone (higher pH preference) substrates. Our results are similar to those of Lowe et al. (2007), who reported a relatively important influence of pH on the structure of an algal community growing on a wet wall. Indeed, acidic conditions strongly increase the chemical solubility, and thus mobility of metals, resulting in high concentrations of heavy metals such as Fe, Cu, Pb, Al, and Zn in soils and waters with low pH levels (Gross 2000; Aguilera et al. 2007; Novis & Harding 2007). High concentrations of heavy metals can therefore substantially influence the diversity of terrestrial microalgae growing on acidic substrates.

Our results suggest that different lineages of *Klebsormidium* are adapted to the substrate on which they originally occur, independently of their evolutionary distance. We found that closely related lineages differed ecophysiologically to the same extent as unrelated clades. Our findings may indicate the widespread existence of sympatric speciation in *Klebsormidium* through ecological divergence, and we hypothesize that this situation is probably common among other taxa of terrestrial algae. The mechanisms of genetic differentiation are not yet fully understood. Specializations to habitats (Gächter & Weisse 2006; Logares et al. 2007), selection pressures (Vanormelingen et al. 2009), and/or persistent founder effects (De Meester et



Figs 17–20. Growth response of four lineages of *Klebsormidium* (limestone clades B and E1; sandstone clades E1 and E13) to different pH levels (4–8). The graphs display the total number of cells in young filaments grown from single cells after 4 days of cultivation on agar plates. Each lineage is represented by three investigated strains. Dark grey bars display mean number of cells determined for each studied strain, light grey bars display the overall means for the lineage. The mean values were calculated from 30–40 replicates, and the standard deviations are displayed for each measurement. Significant differences as determined by one-way ANOVAs Tukey's pairwise comparisons are indicated by asterisks (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$).

al. 2002) have been hypothesized as important factors contributing to the structure of protist populations. Ecological differentiation facilitates allopatric (our data; de Vargas *et al.* 1999) and sympatric (e.g. Amato *et al.* 2007; Weisse 2008; Vanellander *et al.* 2009) speciation of protistan cryptic species. Congruent with the recent studies of Fontaneto *et al.* (2007) and Birky *et al.* (2010), our data suggest the existence of distinct species units and sympatric speciation in asexual protists.

In addition, we found that adaption to specific substrates has originated many times during the evolutionary history of *Klebsormidium*. Strains belonging to clade E13 grew optimally in a very narrow range of pH levels. Moreover, two closely related genotypes inferred within clade E1 showed clear ecophysiological adaptation to the substrate from which they were originally sampled. These genotypes differed in their ecology and ecophysiology but were genetically similar. An analogous situation was reported by

Logares *et al.* (2007), who investigated two dinophytes that had identical ribosomal DNAs but which differed from each other ecologically, physiologically, and even phenetically. Such clear differentiation has been attributed to rapid adaptive evolution, which has not yet been reflected in the ribosomal divergence.

In summary, in the present study, we showed that all strains isolated from sandstone and limestone were able to grow over the range of investigated pH levels but to differing extents. Strains isolated from limestone showed the highest growth rates at pH 7 and pH 8; these strains grew very slowly at pH 4 and pH 5. Strains isolated from sandstone exhibited two different growth responses. Strains from one of the investigated genotypes showed the highest growth rate at pH 6; whereas, strains of the other genotype had almost identical growth rates at all of the investigated pH levels. We conclude that pH is a critical ecological factor that influences the diversity of *Klebsormidium* in terrestrial habitats.

Moreover, our data highlighted distinct ecophysiological differentiation among distantly and closely related lineages, thereby corroborating our hypothesis that the common sympatric speciation of terrestrial algae is driven by ecological divergence. However, further research will be necessary to provide support to this general conclusion.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at <http://dx.doi.org/10.2216/15-110.1.s1>

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