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Variation and taxonomic significance of some morphological features in European strains of *Klebsormidium* (Klebsormidiophyceae, Streptophyta)

by

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Abstract: Forty uni-algal strains of *Klebsormidium* Silva, Mattox & Blackwell (Klebsormidiophyceae, Streptophyta) were examined by light microscopy to test the stability of current discriminating features for the distinction of the narrowly related species *Klebsormidium flaccidum* (Kützing) Silva, Mattox & Blackwell and *K. nitens* (Meneghini in Kützing) Lokhorst. Cell dimensions, filament length, character of zoosporangia and zoospore germination were studied. The habit of cell morphology in relation to the culture age was studied in six randomly chosen strains. The effect of physico-chemical parameters (temperature, humidity, illumination and pH) on cell width was studied on two selected strains. Each strain was transferred to two extreme conditions of one physico-chemical parameter, whereas the other parameters remained the same. The study showed variability in taxonomically relevant morphological features during growing of the species in cultures. For example, there are strong indications that the type of aperture in empty zoosporangial cell walls, considered as a species-specific character, is dependent on environmental factors, in particular on habitat humidity.

In conclusion, it is possible to significantly divide the strains according to three attributes - cell width, character of zoosporangia and microbiotope of habitat. However, each character divides the strains into dissimilar groups. Therefore, it is impossible to decide which feature is the most suitable for proper differentiation of *Klebsormidium nitens* and *K. flaccidum*.

Introduction

The genus *Klebsormidium* contains unbranched filamentous green algae, common in aero-terrestrial habitats. It was originally proposed by Kützing (1843) under the

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name *Hormidium* to accommodate three filamentous green algae. However, this group of species was later found out to be heterogeneous (Lokhorst 1996). The generic name *Klebsormidium* was proposed by Silva et al. (1972) as a solution to the nomenclatural chaos emerged after Fott's discovery of the illegitimate use of the generic name *Hormidium* (Fott 1960). The European representatives of this genus were studied in detail by Lokhorst (1996), who presented a new classification of the genus. Form of growth in culture, cell diameter, shape of the chloroplast, length of filaments, presence of H-shaped pieces, the germination type of zoospores and the shape of emptied zoosporangia were used to distinguish the eight *Klebsormidium* species, reported for Europe.

Klebsormidium flaccidum and *K. nitens* are among the first described and the most investigated species of the genus. Both species were described by Kützing (1849) as representatives of genus *Ulothrix*, section *Hormidium*. These species were studied in detail by Klebs (1896), who distinguished them on the basis of a different cell width. Similar morphology of these species led several authors (Chodat 1902, Farooqui 1969) to consider *K. nitens* as a variety of *K. flaccidum*. Lokhorst (1996) regarded *K. flaccidum* and *K. nitens* as separate species, with different combination of zoosporangial types and zoospore germination.

These two taxa represent the most common species of the genus *Klebsormidium* (Lokhorst 1996, own observations), especially in aero-terrestrial habitats (Flechtner et al. 1998, Lukešová 2001, Neustupa 2001). In ecological and floristical studies, the species composition of terrestrial algal assemblages is often identified only by examination of algal colonies, grown up on agar plates (Ettl & Gärtner 1995). There are mainly two reasons for it: the need of studying the vegetative and reproductive features for correct identification and to trace secondarily in situ most of the species present. However, the correct delimitation of thin *Klebsormidium* species (such as *K. nitens* and *K. flaccidum*) in culture conditions seems to be still problematic, mainly due to similar morphologies and considerable variability in many features (for example cell dimensions and length of filaments). The main goal of the present investigation was therefore to test the usefulness of the current discriminating features (cell dimensions and reproductive characters) in culture conditions with emphasis on how environmental factors may affect the filament appearance during long-term laboratory cultivation. The implications of the results with regard to the species delimitation and taxonomy of genus *Klebsormidium* are discussed in detail.

Materials and methods

A total of 40 isolated strains of *Klebsormidium* were obtained from field algal samples collected from a variety of aero-terrestrial and (semi-)aquatic freshwater habitats, primarily from the Czech Republic. The samples were spread on the Petri dishes with agarized BBM medium (Bischoff & Bold 1963) at 25°C under a constant illumination of 50 - 200 μmol m^{-2} s^{-1} provided by 18W cool fluorescent tubes (Philips TLD 18W/33). Algal microcolonies grown up after 5-10 weeks were isolated into unialgal cultures and further cultivated under the same conditions. After several weeks, the strains were transferred to both agarized and liquid BBM culture tubes and then cultivated at 15°C under daylight conditions (the tubes were placed in a thermostat, placed beside a north facing window). One strain was obtained from the Collection of Algae at the Charles University of Prague, Czech Republic - CAUP (Škaloud & Neustupa 2005). Collection data and other characteristics of each strain are listed in Table 1.

The variation of cell morphology in relation to nutrient depletion was studied in six randomly chosen strains (K9, K13, K16, K29, K43 and K49). The individual strains were incubated on BBM agar plates containing approximately 42 ml of medium and cultivated at 25°C under the constant illumination of 200 μmol m^{-2} s^{-1}. Once a week, at the same time, a small part of the growing population was removed for determination of average cell width and length. The average values were counted from 50 randomly selected cells, measured to the nearest 0.5 μm.

The effect of several physico-chemical parameters (temperature, humidity, illumination and pH) on cell width was studied on two strains for which morphology and other characters (cell dimensions, germination of zoospores and type of zoosporangia) corresponded to the descriptions of *K. flaccidum* (CAUP J302) and *K. nitens* (K13) in Lokhorst (1996). Algal suspensions were prepared by adding a well-growing culture of each strain to distilled water (0.05 g of alga in 100 ml H_2O). In Erlenmeyer flasks sterile wet soil (5 g soil with 15 ml distilled water) was then inoculated by 2 ml of algal suspension. The flasks were well plugged to prevent soil drying.

To study the influence of temperature, two flasks of each strain were kept at 8°C and 26°C. The influence of soil humidity was studied by transferring each strain into the two flasks with soil wetted by 2.5 ml and 50 ml distilled water. To study the effect of illumination, two flasks of each strain were covered by aluminium foil to prevent the entrance of light; the remaining two flasks were kept under continuous illumination of about 200 mmol m^{-2} s^{-1}. The influence of pH was studied by transferring the strains to biphasic cultures (Pringsheim 1954). The pH in the cultures was maintained at 4.5 and 8.5 by adding drops of HCl or NaOH (approximately once per two weeks). During the experiments, only one of the physico-chemical parameters varied, while the others were kept constant (the standard parameters were as follows: temperature 25°C, humidity - 15 ml of sterile water in 5 g soil, illumination of about 200 μmol m^{-2} s^{-1}, pH 6.5). The strains were kept in the above-mentioned conditions for a period of four months; thereafter the cell width range was recorded.

Formation of zoospores was stimulated according to the following procedure: uni-algal cultures were transferred to agarized BBM medium and subsequently cultivated at 25°C under a constant illumination of about 200 μmol m^{-2} s^{-1}. After about 2 - 3 weeks, filaments were collected by sterile razor blades and transferred to tubes with both diluted BBM medium and fresh distilled water. After centrifuging, the tubes with diluted BBM medium were placed in darkness at a temperature of about 10°C. After zoospore formation (within one to two weeks), the tubes were placed in continuous lightness to induce their germination. Consequently, the germinating zoospores were carefully scraped off and observed in a solution of Indian ink.

A correlation-based protocol was used to describe the relations among the characters used for strain differentiation (Table 4). Due to irregular data distribution, a nonparametric Spearman correlation matrix was used to examine individual relationships between variables (Sokal & Rohlf 1995). Similarities between isolated strains were estimated using the Gower distance measure for mixed data (Gower 1971). This coefficient is appropriate for comparisons based on quantitative and qualitative (binary) data. An UPGMA dendrogram tree was calculated from standardized data using the SYN-TAX 2000 program, module HIERCLUS (J.Podani, L.Eötvös University, Budapest, Hungary) (Fig. 16). A principal component analysis (PCA) using Canoco for Windows 4.5 (Ter Braak & Šmilauer 1998) was performed to illustrate the relationship among the characters used in analyses and to show the position of studied strains in the ordination space (Fig. 17). Using the same program, a redundancy analysis (RDA) was performed to test the influence of humidity to strain variability (Ter Braak & Šmilauer 1998). The results of ordination were summarized using the program CanoDraw for Windows 4.0 (Ter Braak & Šmilauer 2002). The differentiation of isolated strains in relation to the selected variables was tested via a two-group multivariate permutation test and canonical discriminant analysis (Table 5), performed in program PAST, version 1.29 (Hammer et al. 2001) and program NCSS (Hintze 2004), respectively. Finally, two-sample t-test in the program NCSS was performed to test the influence of humidity on the aperture type (Brown & Rothery 1993): The means of habitat humidity were compared between two groups, defined by aperture type. Only strains, for which the habitat humidity was measured, were used in the analysis. The habitat humidity was measured by a digital hygrometer Svelin TM977H. The soil humidity was determined following the method described in Neustupa (2001). If the strain was cultivated from water biotope, the humidity was accounted as 100 %.

Table 1. List of studied strains with their origin and other basic characteristics. The range of cell dimensions was ascertained from cultures grown on agar in culture tubes during whole life cycle. Annotation to habitat: Rock - mainly dry, only randomly wetted rock surface; Soil - occasionally wetted bare soil; Moss - the thallus of terrestrial moss; Water – continuously submerged environment (mainly plankton samples).

Strain	Location	Habitat	Habitat humidity (%)	Cell width (μm)	Cell length (μm)	Aperture in empty zoosporangial cell walls	Germination of zoospores
K1	České Středohoří Mts.	Rock	68	5(-6)	5-10	Distinct	Unipolar
K2	České Středohoří Mts.	Rock	68	(4.5-)5-6	5-15(-17)	Distinct	Unipolar
K3	České Středohoří Mts.	Rock	100	6.5-8	5.5-11	Inconspicuous	Unipolar
K4	České Středohoří Mts.	Soil	34	5-6	6-12	Distinct	Unipolar, bipolar
K5	České Středohoří Mts.	Rock	66	4.5(-5)	7-14(-18)	Distinct	Unipolar
K6	České Středohoří Mts.	Moss	66	6-6.5	5-14	Distinct	Unipolar, bipolar
K7	České Středohoří Mts.	Rock	78	4.5-6	5-17(-20)	Distinct	Unipolar
K8	České Středohoří Mts.	Rock	78	4.5-5	4-14	Distinct	Unipolar
K9	České Středohoří Mts.	Moss	-	6-6.5	5-18	Inconspicuous	Unipolar, bipolar
K10	Prague	Water	-	7	7-20	-	-
K11	České Středohoří Mts.	Soil	20	6-6.5(-7)	5-14	Distinct	Unipolar
K13	České Středohoří Mts.	Rock	67	4.5-5	5-15(-20)	Distinct	Unipolar
K14	České Středohoří Mts.	Moss	68	5.5-6	5-15	Inconspicuous	Unipolar, bipolar
K16	Šumava Mts.	Water surface	100	5-5.5	5-12	Distinct	Unipolar
K18	České Středohoří Mts.	Rock	76	4.5-5	5-14	Distinct	Unipolar
K19	Koleč. Central Bohemia	Moss	-	6-7	5-20(-26)	Distinct	Unipolar, bipolar
K20	Milská stráň. Central Bohemia	Soil	-	6-7.5	5-15	-	-
K22	NP České Švýcarsko	Water	100	5-6	8-18	Inconspicuous	Unipolar, bipolar
K25	České Středohoří Mts.	Rock	-	7.5-10(-11)	(4-)7-17(-26)	-	-
K26	České Středohoří Mts.	Rock	80	6-7	7-20	Distinct	Unipolar

K28	Krkonoše Mts.	Water	100	5-6	5-18	Inconspicuous	Unipolar
K29	Krkonoše Mts.	Water	-	(5-)5.5-6	5-24	-	-
K30	Krkonoše Mts.	Water	-	5.5-6	4-11.5	-	-
K31	Šumava Mts.	Soil	-	5-5.5	5-18	Inconspicuous	Unipolar
K32	Krkonoše Mts.	Water	100	5-6	6-20	Inconspicuous	Unipolar, bipolar
K33	Krkonoše Mts.	Water	100	5-6	7-20	Inconspicuous	Unipolar
K35	Drahanské údolí. Central Bohemia	Rock	-	6.5-8	7-25	Distinct	Unipolar
K36	Drahanské údolí. Central Bohemia	Rock	-	6.5-7	(4-)5-29	Distinct	Unipolar
K38	Klecany. Central Bohemia	Rock	-	6.5-7	7-25	-	-
K39	Budapest. Hungary	Rock	100	6-7	4-12	Distinct	Unipolar
K40	České Středohoří Mts.	Moss	66	7-7.5	8-15	Inconspicuous	Unipolar
K41	Prague	Wall	-	6-7	3-18	Inconspicuous	Unipolar, bipolar
K42	České Středohoří Mts.	Rock	-	6-7(-8)	5-19	Distinct	Unipolar
K43	Šumava Mts.	Water	100	5.5-6	4.5-15	Distinct	Unipolar
K44	Krkonoše Mts.	Water	100	6.5-7	4-11	Inconspicuous	Unipolar
K46	Drahanské údolí. Central Bohemia	Soil	-	8	6-20	Distinct	Unipolar
K47	Drahanské údolí. Central Bohemia	Rock	-	6.5-7	4-13	Distinct	Unipolar
K48	Drahanské údolí. Central Bohemia	Soil	-	7-7.5	7-20	Inconspicuous	Unipolar, bipolar
K49	Šumava Mts.	Water	100	8	6-16	Inconspicuous	Unipolar, bipolar
CAUP J302	Adršpach Mts.	Rock	-	6-7	6-18	Distinct	Unipolar

Results

Overall thallus morphology and life cycle

On agar plates, all strains show similar growth forms, composed of circular colonies several millimeters in diameter. The colonies differ only in the size and the degree to which they appeared filamentous at low magnification. In liquid cultures, most strains exhibit two different growth forms: free-floating tufts of filaments and a surface layer of water-repellent filaments, often organized in a parallel pattern. In some strains, the latter type was not observed.

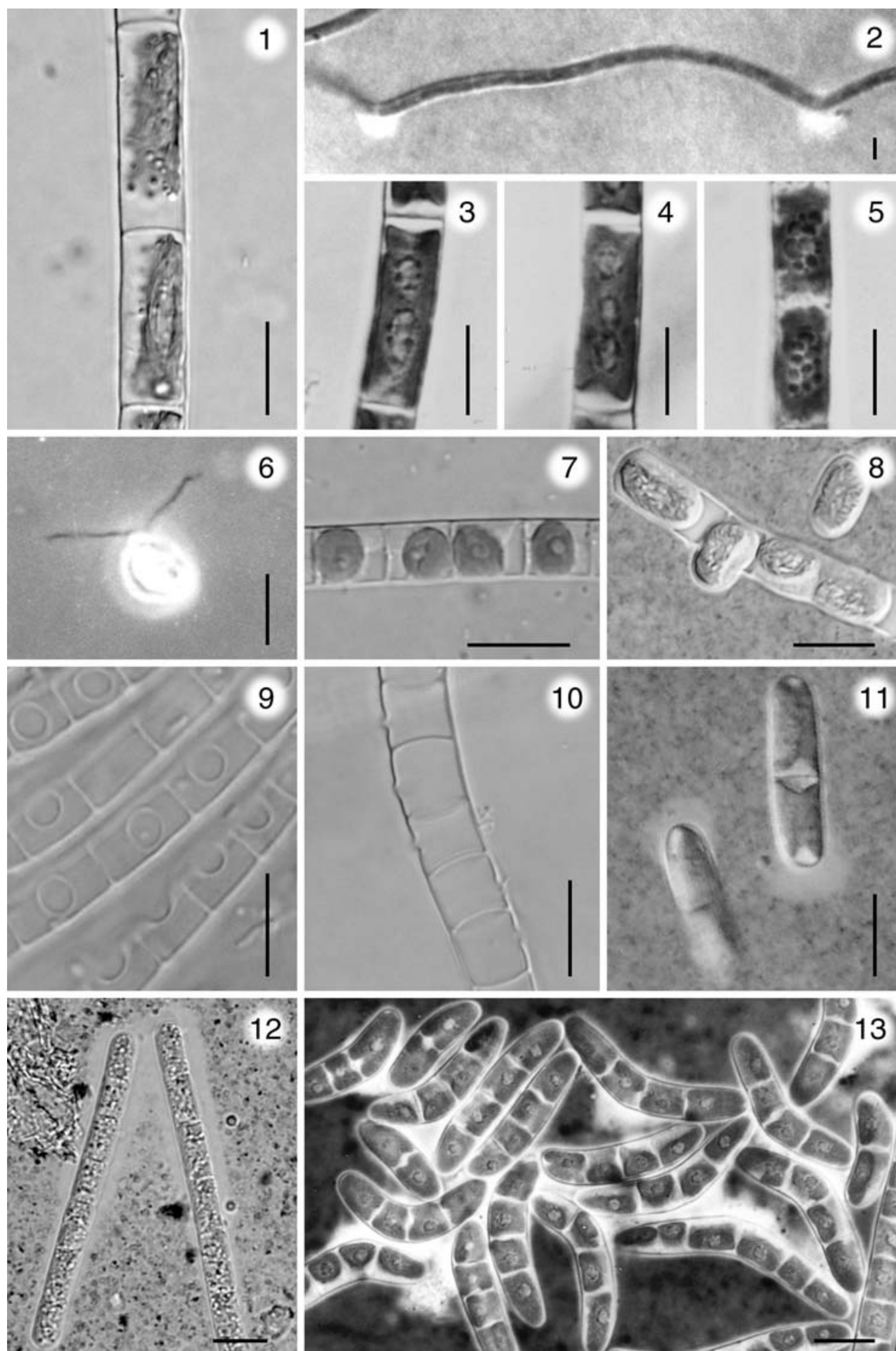
The general growth habit of filaments is identical in all strains. The filaments are uniseriate, unbranched, without morphologically differentiated cells or H-shaped pieces at the transverse cell walls (Fig. 1). The cell wall is smooth. In some strains, the production of mucilaginous discs was sometimes observed in liquid cultures. The discs are produced at regular intervals along the filament and apparently served as attachment (Fig. 2). Length of filaments varies from short, few-celled to very long (more than 1000 cells per one filament). In young cultures, the filaments are mostly short and straight, in old ones they are often bent and disintegrated easily into few-celled fragments. The cells are normally cylindrical; in older cultures they are sometimes slightly constricted at the transverse walls (doliiform). The length of the cells usually exceeds their width; the width/length ratio is quite variable through the life cycle. Cell dimensions of studied strains are given in Table 1. The cells contain one parietal chloroplast, covering about 40-70(-80)% of cell circumference. The chloroplast margin is usually straight, sometimes in older populations lobed along its longitudinal axis. Each chloroplast contains one, less frequently two (Fig. 3), rarely three (Fig. 4) pyrenoids, covered by a distinct layer of starch grains. With increasing age of culture, the starch grains become larger (Fig. 5).

The strains reproduce mostly by means of filament disintegration into few-celled fragments, produced at the ends of filaments. Less commonly, spontaneous production of biflagellate zoospores occurs in liquid cultures (Fig. 6). The zoospores are dorsiventral in structure, with flat ventral and rounded dorsal sides. They are produced in unspecialized cells of the filaments (Fig. 7). Very rarely, aplanospores are produced (Fig. 8).

Characteristics of zoosporangia and zoospore germination

From a total of 40 isolated strains, production of zoospores was induced in 34 strains. In all of these strains, the structure of empty zoosporangial cell wall including the release apertures and the zoospore germination were observed (see Table 1). Two

Fig. 1. Typical morphotype of studied strains. One chloroplast with one pyrenoid is visible in the cells. Fig. 2. Production of mucilaginous discs, serving filament attachment. Figs 3-4. Chloroplast morphology with distinct pyrenoids. Fig. 5. Ageing filament with large starch grains. Fig. 6. Zoospore. Fig. 7. Zoosporangia. Fig. 8. Aplanospore production. Fig. 9. Empty zoosporangia with distinct apertures. Fig. 10. Empty zoosporangia with inconspicuous margin of the apertures. Fig. 11. Unipolar germination of zoospores. Fig. 12. Specific unipolar germination of zoospores observed in strain K43. Fig. 13. Bipolar germination of zoospores. Scale bars = 10 μm.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types of the apertures were observed. In most strains the apertures were clearly visible by showing a distinctive margin (Fig. 9). In other strains the margin of the aperture was inconspicuous and the aperture was only distinct in side view (Fig. 10).

Two types of zoospore germination were observed. Unipolar germination was noticed in all reproductive strains (Fig. 11). A specific type of unipolar germination, characterized by mucilage production over the whole filament surface, was observed in all germinated filaments of strain K43 (Fig. 12). In 10 strains bipolar zoospore germination was observed (Fig. 13), always accompanied by unipolar germination of other zoospores in a culture.

Variability of cell morphology in relation to the nutrient depletion

A different cell width and length range was observed in the individual strains (Table 2). Whereas the average cell width fluctuated only in a range of several tenths of a µm, the average length of the cells often more strongly varied. The cell length variability of individual strains is demonstrated in Fig. 14. The average values of cell length differed both among all isolated strains and during ageing of the culture populations. Although the differences among the strains were obvious, the influence of nutrient depletion displayed identical features. During the first few weeks, a distinct reduction of cell length was observed, probably influenced by intensive cell dividing. However, at a certain stage the cells started to become longer. This phenomenon was observed in all strains, but they differed in timing at that particular stage (in strains K9 and K43 the shortest cells developed in the 3rd week, whilst in strain K49 those cells were not observed until the 5th week).

The cell length changes were notably linked with the length of filaments. The young cultures were typically quick in producing new cells per filament. Conversely, at maturity the filaments started to disintegrate into few-celled fragments. Around the sixth week after inoculation, fragments with symptoms of ageing (distinct starch grains covering the pyrenoid, disintegration of the chloroplast, production of abnormally-shaped cells) predominated the cultures. Interestingly, the start of filament disintegration corresponds with this time, when the shortest average cell length occurs (see Table 2).

The effect of physico-chemical parameters on the cell morphology variation

In the experiment, two selected strains were exposed to 8 different conditions of physico-chemical parameters. Differences in both cell length and width were observed between miscellaneous experimental populations of the same strain. The results of the experiment are listed in Table 3. In standard laboratory conditions (at 15°C under daylight conditions), the strains differed especially by cell width. The cell width of strain CAUP J302 (*K. flaccidum*) varied between 6-7 µm while the width of cells in strain K13 (*K. nitens*) fluctuated in the range of 4.5-5.0 µm (Table 1). By then, the cells of strain CAUP J302 were markedly wider. However, this distinctive cell width behavior was not hold in all experiments, as illustrated in Fig. 15.

At a lower pH, some widening of the cells of strain K13 was observed, whilst the cells of strain CAUP J302 remained unchanged. A high pH caused an increase of cell

Table 2. Variability of cell dimensions and number of cells per filament of six selected strains during population ageing. A - average, St.D. - standard deviation, St.E. - standard error of mean.

			1. week	2. week	3. week	4. week	5. week	6. week
K9	Cell length (µm)	**A**	**14**	**12.3**	**9.6**	**10.7**	**11.9**	**12.9**
		St.D.	3.63	3.23	2.26	2.12	2.65	2.64
		St.E.	0.51	0.46	0.32	0.3	0.37	0.37
	Cell width (µm)	**A**	**6.375**	**6.5**	**6.5**	**6.725**	**6.375**	**6.625**
		St.D.	0.30	0.32	0.39	0.28	0.27	0.28
		St.E.	0.04	0.04	0.06	0.04	0.04	0.04
	Cells per filament		**5-18**	**7-80**	**1-42**	**1-26**	**1-14**	**1-6**
K13	Cell length (µm)	**A**	**11**	**9.9**	**8.2**	**7.5**	**8.2**	**8.5**
		St.D.	2.76	2.02	2.14	1.62	2.14	1.63
		St.E.	0.39	0.29	0.30	0.23	0.3	0.23
	Cell width (µm)	**A**	**5.125**	**5**	**4.75**	**5.125**	**5.25**	**4.875**
		St.D.	0.13	0.19	0.20	0.26	0.22	0.13
		St.E.	0.02	0.03	0.03	0.04	0.03	0.02
	Cells per filament		**6-16(-30)**	**17-110**	**2-600**	**2-320**	**1-500**	**1-350**
K16	Cell length (µm)	**A**	**11.1**	**9.1**	**9**	**8.9**	**9.1**	**10**
		St.D.	2.46	2.34	2.44	1.67	2.08	1.83
		St.E.	0.35	0.33	0.35	0.24	0.29	0.26
	Cell width (µm)	**A**	**5.625**	**5.25**	**5.25**	**5.375**	**5.375**	**5.25**
		St.D.	0.31	0.18	0.18	0.13	0.13	0.20
		St.E.	0.04	0.03	0.03	0.02	0.02	0.03
	Cells per filament		**8-22**	**25-220**	**2-350**	**1-60**	**1-7**	**1-5**
K29	Cell length (µm)	**A**	**11.9**	**12.5**	**12.55**	**10.7**	**10.4**	**10.9**
		St.D.	2.97	3.03	3.39	2.60	2.22	3.08
		St.E.	0.42	0.43	0.48	0.37	0.31	0.44
	Cell width (µm)	**A**	**5.75**	**5.5**	**5.5**	**5.25**	**5.625**	**5.5**
		St.D.	0.21	0.20	0.19	0.20	0.29	0.18
		St.E.	0.03	0.03	0.03	0.03	0.04	0.03
	Cells per filament		**5-50**	**2-50**	**1-50**	**1-16**	**1-20**	**1-18**
K43	Cell length (µm)	**A**	**10.1**	**9.5**	**6.7**	**7.6**	**8.1**	**9**
		St.D.	2.49	2.24	1.71	1.66	1.86	1.80
		St.E.	0.35	0.32	0.24	0.24	0.26	0.25
	Cell width (µm)	**A**	**5.75**	**5.5**	**5.375**	**5.65**	**5.75**	**5.5**
		St.D.	0.21	0.20	0.13	0.13	0.19	0.00
		St.E.	0.03	0.03	0.02	0.02	0.03	0.00
	Cells per filament		**1-90**	**2-70**	**1-20**	**1-10**	**1-5**	**1-4**
K49	Cell length (µm)	**A**	**14**	**11.9**	**10.7**	**9**	**8**	**8.5**
		St.D.	5.37	2.98	2.34	1.97	2.29	2.23
		St.E.	0.76	0.42	0.33	0.28	0.32	0.32
	Cell width (µm)	**A**	**7.75**	**8**	**7.875**	**8**	**8.25**	**8**
		St.D.	0.21	0.21	0.13	0.21	0.19	0.22
		St.E.	0.03	0.03	0.02	0.02	0.02	0.03
	Cells per filament		**20-500**	**> 1000**	**> 1000**	**> 1000**	**> 1000**	**> 1000**

width range in both studied strains. Moreover, a high proportion of dead cells, disintegration of chloroplast and formation of abnormal-shaped cells (not included to the measured cell width) expressed the poor condition of both populations. No

Fig. 14. Development of average cell length of six selected strains during six weeks of cultivation. For each strain, box plot on the left presents the variability of cell length in young cultures; the right box plot illustrates the cell length variability in six weeks old cultures.

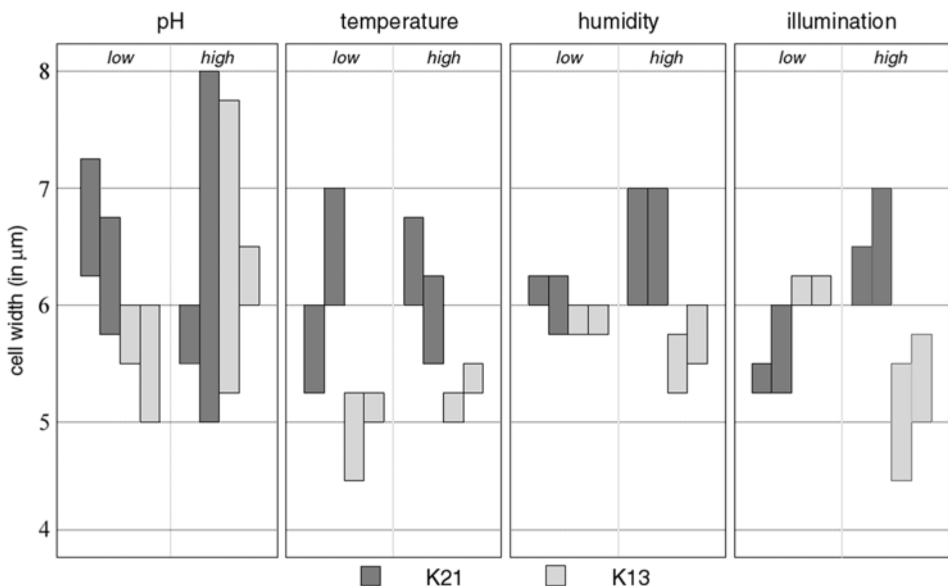

Fig. 15. Influence of four physical-chemical parameters on the cell width in strains CAUP J302 and K13. The values of two parallel measurements are shown.

Table 3. Cell dimensions of two selected strains (CAUP J302 - *Klebsormidium flaccidum* and K13 - *K. nitens*), cultivated in different environmental conditions. Results of both parallel measurements are listed in the table. During the experiments, only one of the physico-chemical parameters was varied while the others remained standard (temperature 25°C, humidity - 15 ml of sterile water in 5 g soil, illumination of about 200 µmol m^{-2} s^{-1}, pH 6.5).

Strain	Conditions		Cell width (µm)		Cell length (µm)	
CAUP J302	pH	Low (4.5)	6.25-7.25	5.75-6.75	5-14	6-20
		High (8.5)	5.5-6	5-8(-9)	5-22	5-17
	Temperature	Low (8°C)	5.25-6	6-7	5-18	5-17
		High (26°C)	6-6.75	5.5-6.25	5-15	5-18
	Humidity	Low (2.5 ml H_2O)	5.9-6.25	5.75-6.25	5-15	6-15
		High (50 ml H_2O)	6-7	6-7	3.5-14	5-15
	Illumination	No	5.25-5.5	5.25-6	7-16	6-15
		High (200 µmol m^{-2} s^{-1})	6-6.5	6-7	7-19	6-15
K13	pH	Low (4.5)	5.5-6	5-6	5-10	7-18
		High (8.5)	5.25-5.75(-8)	6-6.5	6-19	7-11
	Temperature	Low (8°C)	4.5-5.25	5-5.25	5.5-17	5-12
		High (26°C)	5-5.25	5.25-5.5	5-10	5-13
	Humidity	Low (2.5 ml H_2O)	5.75-6	5.75-6	6-15	4-16
		High (50 ml H_2O)	5.25-5.8	5.5-6	5-13	5-10
	Illumination	No	6-6.25	6-6.25	5-13	7-17
		High (200 µmol m^{-2} s^{-1})	4.5-5.5	5-5.75	5-15	6-15

influence of temperature on the cell width was noted. In both experimental conditions, cells of strain CAUP J302 were distinctly wider than those of strain K13. Another change in cell dimension was, however, observed in strains cultivated at high or low humidity. The effect of high humidity was similar to the effect of low pH - mild increase of the cell width of strain K13. Interestingly, at low humidity both strains showed a cell width of about 6 µm. Cell dimensions and overall morphology were alike in both populations. Under continuous high illumination, cell dimensions remained identical: the cells of strain CAUP J302 were distinctly wider in comparison to the cells of strain K13. A dissimilar behavior was noticed in strains which were cultivated in permanent darkness. Despite slow growth, the filaments were in good condition, without signs of ageing or degeneration. However, the cell width in both cultures changed considerably. The cells of strain CAUP J302 became thinner, whereas the cells of strain K13 became distinctly wider. Furthermore, the cells of K13 exceeded the cells of CAUP J302 in width, although in standard laboratory conditions they were clearly thinner.

The results of statistical analyses

The correlation matrix, shown in Table 4, describes the relationships among the characters used for strain description. No high correlation was detected. The highest correlation ($R = 0.71$) was noticed between the cell length/width ratio and length of the cells. Nevertheless, the l/w ratio was used in following analyses (e.g. in the cluster analysis or PCA, the use of this character gets the same weight to cell dimension variables and habitat variables). The characteristics of zoosporangia and zoospore

Table 4. Correlation matrix of the characters used for strain differentiation. The correlation between reproductive characters and habitat type is highlighted. Positive values of "Aperture" mean inconspicuous margins of apertures, positive values of "Germin" mean bipolar type of zoospore germination.

	Width	Length	Aperture	Germin	Rock	Soil	Moss	Water	L/w ratio
Width	1.00								
Length	0.25	1.00							
Aperture type	0.20	0.23	1.00						
Germination type	0.13	0.19	0.42	1.00					
Rock	-0.16	-0.15	**-0.46**	**-0.44**	1.00				
Soil	0.17	0.07	-0.05	0.04	-0.41	1.00			
Moss	0.14	0.07	0.19	**0.46**	-0.37	-0.19	1.00		
Water	-0.08	0.06	**0.42**	0.10	-0.49	-0.26	-0.23	1.00	
L/w ratio	-0.44	0.71	0.03	0.00	0.08	-0.13	-0.11	0.11	1.00

germination were only moderately correlated ($R = 0.42$). Interestingly, these characters were also influenced by environmental factors. Inconspicuous apertures in the empty zoosporangial cell walls occurred mainly in strains from aquatic microbiotopes ($R = 0.42$), whereas the distinct ones appeared in the strains from rock ($R = -0.46$). Similarly, the bipolar germination of the zoospores was correlated with moss microbiotope ($R = 0.46$), whilst the unipolar germination corresponded with microbiotope rock ($R = -0.44$).

No distinct large groups of strains were created in a cluster analysis of the 34 reproductive isolates, as illustrated in Fig. 16. Only several smaller groups were divided by means of cluster analysis. However, these groups correspond well with the microbiotope of the habitat. For example, all strains isolated from the rock surface formed one separate cluster. The strains with the same aperture type formed mostly in the separate sub-clusters within the microbiotope-defined clusters. The high occurrence of inconspicuous apertures in "water" strains and the low occurrence of this aperture type in "rock" strains are clearly visible in the dendrogram.

The ordination diagram in Fig. 17 shows the result of the principal component analysis. The first and second principal component axis explained 27.1 and 20.2% of the total variability, respectively. Rock microbiotope, type of aperture and the zoospore germination contributed to the first axis, whereas cell dimensions (especially length/width ratio) accounted for the second axis. The strains were evenly distributed in the ordination diagram, without formation of specific clusters. Moreover, none of the delimiting characters strictly separate the strains into two groups.

The distinction of strains by means of responded variables (character of zoosporangia, zoospore germination, type of microbiotope and the cell width) was tested via a two-group permutation test and canonical discriminant analysis (see Table 5). Three analyses significantly divided the strains into two separate groups: Analysis of aperture type (p-values 0.013 and 0.012) and the analyses of differences between the strains from microbiotopes "rock" and "moss" (p-values 0.013 and 0.009) and "rock" and "water" (p-values 0.035 and 0.042).

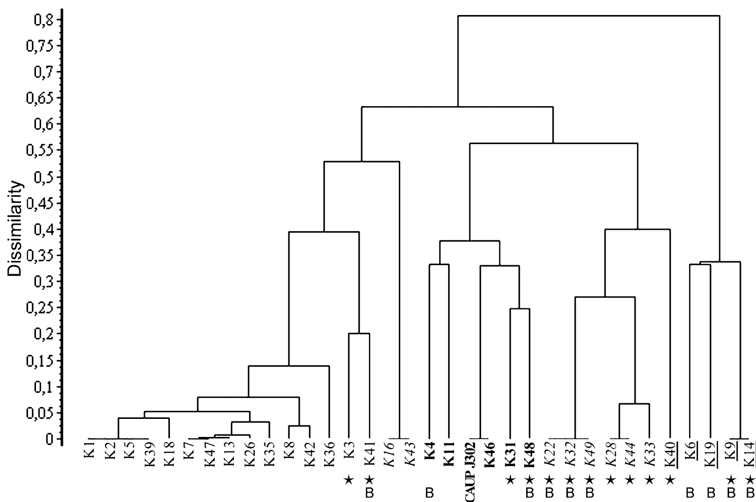

Fig. 16. Cluster analysis of 34 reproductive strains. The microbiotopes are distinguished by different font type (regular - rock, italic - water, bold - soil, underlined - moss). ★ - strains with inconspicuous aperture margins of empty zoosporangial cells. B - strains with bipolar zoospore germination.

The significant influence of habitat type led the author to interpret the variability in morphology of strains by means of habitat humidity (the values of humidity are listed in Table 1). This hypothesis was tested via redundancy analysis (RDA). The influence of humidity was significant both with aperture type (p-value 0.004) and the type of microbiotope (p-value 0.013) used as a covariable. In contrast, the test of apertures (with microbiotopes used as covariables) was not significant (p-value 0.358). Moreover, the effect of habitat humidity to the type of apertures in empty zoosporangial walls was significant in the two-sample t-test (p-value 0.022).

Table 5. Results of statistical analyses, tested the distinguishing of isolated strains by means of responded variables (listed in the left column). Separating of strains into two clusters by means of aperture types and microbiotopes rock vs. moss and rock vs. water was significant in both analyses (highlighted).

Tested pairs	Two-group permutation test	Discriminant analysis
Aperture type	**0.013**	**0.012**
Gernination type	0.067	0.068
Microbiotopes rock, soil	0.644	0.665
Microbiotopes rock, moss	**0.033**	**0.009**
Microbiotopes rock, water	**0.035**	**0.042**
Microbiotopes soil, moss	0.527	0.597
Microbiotopes soil, water	0.391	0.525
Microbiotopes moss, water	0.599	0.611
Cell width (boundary 6 μm)	0.471	0.492

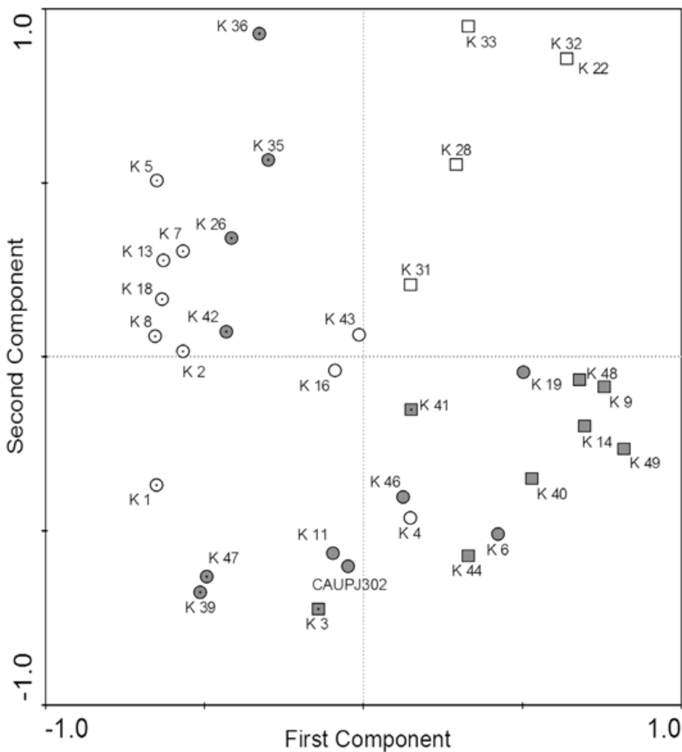

Fig. 17. PCA diagram showing the position of 34 isolated strains in the space of the first two ordination axes. The final shape of the symbols is characterized by the combination of three variables: CELL WIDTH - less than 6 µm (white colour), more than 6 µm (grey colour); APERTURE TYPE - distinctive aperture margins (circle), inconspicuous aperture margins (square) and MICROBIOTOPE - rock (symbols with dot), other microbiotopes (symbols without dot).

Discussion

For the morphological separation of thin *Klebsormidium* species, many authors mentioned a number of morphological features. Usually, the species have been differentiated on the basis of cell dimensions, especially cell width (Kützing 1843, Klebs 1896, Farooqui 1969, Lokhorst 1996). Lokhorst (1996) furthermore distinguished the species for the different habit of apertures in empty zoosporangial cell walls and occurrence of bipolar type of zoospore germination. However, the most of these morphological features seems to be taxonomically irrelevant when exposed to culture conditions.

The length and disintegration of filaments were significantly dependent on population age (in culture conditions, on nutrient content). The short cells occurred in well-

growing, mature populations, whereas the long ones were produced in very young or old cultures, where the nutrient depletion affects the disintegration of filaments into few-celled fragments. Despite the identical cultivation conditions, the onset of filament disintegration, equally as the rate of life cycle, was specific for each strain. In strain K49, several centimeters long filaments, containing more than 1000 cells, were observed during almost the whole life cycle (see Table 2). These long filaments were observed not only in strain K49 and not only in the culture conditions. For example, the longest filaments were noticed in strain K10, found in a fountain. There, *Klebsormidium* threads up to 2 meters long were found, consisting of several bundled filaments.

Contrary to cell length, the width of the cells varied only minimally during the life cycle. The cell width is considered to be one of the significant interspecific features in the genus *Klebsormidium* (Ettl & Gärtner 1995, Lokhorst 1996). However, the results of the above-mentioned experiment indicate the variability of cell width in dependence on some environmental conditions, especially humidity and intensity of illumination (Fig. 15). For example, cells cultivated in standard cultivation conditions differed from the cells of the same strain cultivated in darkness and low humidity. Similar results were presented by Poulíčková et al. (2001) who observed cell variability of a *Klebsormidium* strain obtained from a desert habitat cultivated under different culture conditions. They showed a high variation of cell width under different light, temperature and nutrient conditions.

On the other hand, the conditions used in present study are extreme and unnatural in biotopes where the *Klebsormidium* species are found (especially permanent darkness or pH 8.5). Under standard, non-extreme cultivation conditions the cell width observed was constant during the whole life cycle and therefore could be considered as a valuable strain (or species) feature (Table 2). Moreover, a boundary in cell width could be created to separate all 40 studied strains. During the whole life cycle, the cell width of 18 strains never exceeded 6 μm, whereas the cell width of the remaining 22 strains was always greater than 6 μm (Fig. 18). Even though a cell width of 6 μm was observed in some strains from both groups, no strain exhibited cell width fluctuation around this value (e.g. 5.5-6.5 μm).

Lokhorst (1996) pointed at the taxonomic value of zoospore germination and characteristics of apertures in the zoosporangial cell wall. In the present study, the unipolar germination was observed in all reproductive studied strains, whereas the bipolar type was present only in a few of them. Zoospore germination behavior characterized each strain, but not in a unique way. In addition, each reproductive *Klebsormidium* strain exhibited one of two types of apertures. In the majority of species the apertures were quite clearly visible, with distinct margins. In the rest of the studied strains the margin of aperture was inconspicuous. Following Lokhorst (1996), the type of zoospore germination and the aperture type are in coherence, i.e. the bipolar germination should be observed only in strains, where apertures with indistinctive margins are produced. Even though the present study indicated the correlation between these two characters (Fig. 17, Table 4), this link was not observed in all strains: in seven strains with bipolar germination the apertures were inconspicuous, in the remaining three strains they were obvious (Table 1). However,

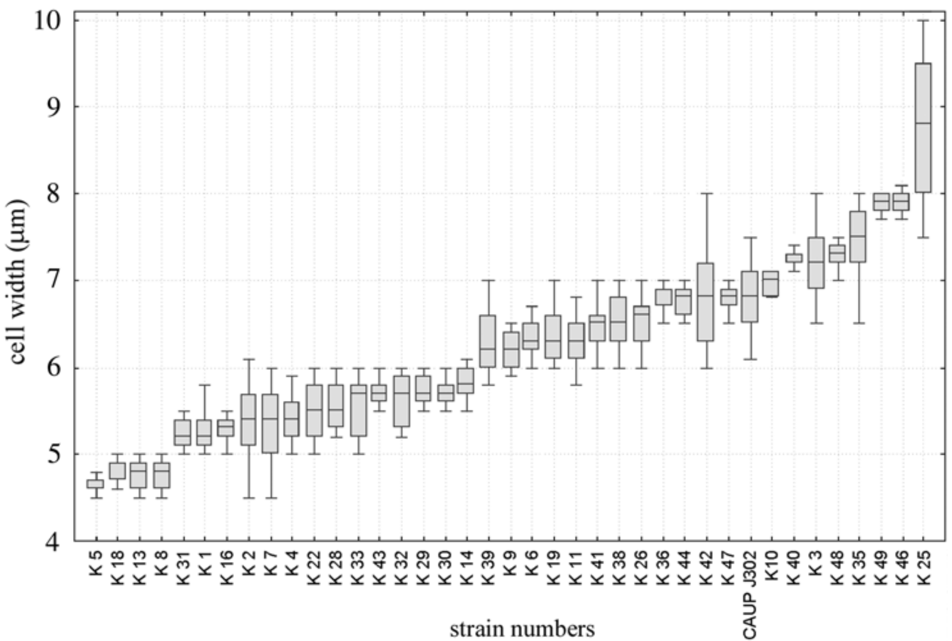

Fig. 18. Cell width range of all studied strains. The strains are ordered according to average cell width.

when only the aperture types were used as a delimiting character, the strains were successfully distinguished into two clusters (Table 5).

The most common species of the genus, *Klebsormidium nitens* and *K. flaccidum*, are traditionally differentiated by cell width - the cells of *K. nitens* should be thinner than the cells of *K. flaccidum* (Klebs 1896, Printz 1964, Starmach 1972, Lokhorst 1996). Considering the most studied strains as representatives of one of these two species, it is possible to divide the strains into two groups, using cell width of 6 mm as a boundary (Fig. 18). With the exception of three outlying cultures (K25, K46, K49, probably another species), the strains with cell width 6 and below 6 µm should represent *K. nitens*, whereas the other ones should belong to *K. flaccidum* (cell width 6 and above 6 µm). However, it is also possible to significantly separate the strains according to aperture type (but not germination behaviour). According to Lokhorst (1996), strains with distinctive margin of apertures should represent *K. nitens*, whereas strains with inconspicuous margin should characterize *K. flaccidum*. This delimitation is confirmed by the two-group permutation test and discriminant analysis (Table 5), equally as the strain separating by means of microbiotope of the habitat (Table 5, Fig. 16) or habitat humidity, respectively. In strains isolated from water biotopes, the inconspicuous apertures predominated. The type of habitat, and more precisely, habitat humidity seems to have a high impact on strain morphology.

Thus, it is possible to significantly divide the strains according to three attributes - cell width, character of zoosporangia and microbiotope of habitat. However, the

clusters of strains created on the basis of one delimiting character do not correspond with clusters constructed on account of other characters (Fig. 17). Although the cell width is suggested to be the main discriminant character, it is impossible to decide which feature is the most suitable for proper differentiation of *Klebsormidium nitens* and *K. flaccidum*. The present study, based on morphological features observed in culture conditions, indicates that the question of species concept in *Klebsormidium* can possibly be solved only by an appropriate combination of morphological and molecular data.

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