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Global ubiquity and local endemism of free-living terrestrial protists: phylogeographic assessment of the streptophyte alga *Klebsormidium*

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Summary

Despite considerable research attention during the last 10 years, the distribution and biogeography of protists remain as highly controversial issues. The presumably huge population sizes and unlimited dispersal capabilities should result in protist ubiquity. However, recent molecular investigations suggest that protist communities exhibit strong biogeographic patterns. Here, we examined the biogeographic pattern of a very common green algal genus *Klebsormidium*. We evaluated the geographic distribution of *rbcL* genotypes for 190 isolates sampled in six sampling regions located in Europe, North America and Asia. Measures of correlation between genetic and geographic distance matrices revealed a differential distribution pattern on two geographic levels. Globally, the populations were genetically homogeneous; locally, the genotypes were patchily distributed. We hypothesized that a local fine-scale structuring of genotypes may be caused by various ecological factors, in particular, by the habitat differentiation of particular genotypes. Our investigations also identified a large number of new, previously unrecognized lineages. A total of 44 genotypes were identified and more than 66% of these were reported for the first time.

Introduction

Estimates of protist biogeography and diversity have become highly controversial topics during the last 10 years (Caron, 2009). It has been proposed that global protist species diversity is extremely high because most species have limited geographical distribution (Foissner, 1999; 2006). Conversely, there are arguments for ubiquitous distribution and unlimited dispersal of protists, which implies much lower total diversity compared with that of macroorganisms (Finlay *et al*., 1996; Finlay, 2002).

Supporters of the ubiquity theory argue that huge populations, small cell sizes, and almost unlimited dispersal of protists cause their cosmopolitan distribution (Fenchel, 1993; Finlay *et al*., 1996; 2004). The ubiquity model suggests that microbial taxa of typical protist dimensions (approximately 20 μm in diameter) are capable of global dispersal (Wilkinson *et al*., 2012). This dispersal is not limited by biogeographical barriers (Finlay, 2002), but it is driven by random events such as wind, ocean circulation, and transport on the bodies of migrating birds or animals (Fenchel and Finlay, 2004). Consequently, individual protist species occur wherever suitable environmental conditions are available.

The ubiquity model predicts a very low probability of local extinction in protist populations (Fenchel and Finlay, 2003). The consequence of very low local extinction coupled with extremely large population size is high local protist diversity and a low global diversity (Fenchel and Finlay, 2003; 2004). However, the reported low estimations of total protist species richness (Finlay and Fenchel, 1999) contradict the results of several molecular phylogenetic studies showing a large cryptic diversity in eukaryotic microorganisms (Von der Heyden *et al*., 2004; Šlapeta *et al*., 2006; Simon *et al*., 2008). Fenchel and Finlay (2006) postulated that the use of genetic data brought confusion into the estimations of real diversity in protists. They proposed that the variation in molecular markers reflects the accumulation of neutral mutations over historical time, rather than the existence of morphologically indiscernible, cryptic species.

Opponents of the ubiquity theory propose an extraordinarily high global diversity and endemicity of protist species. Foissner (2006) reviewed information that indicated restricted distributions for several protist groups and promoted the use of flagship species (i.e., species with conspicuous morphologies whose presence/absence could be easily demonstrated in samples) to demonstrate the endemism of several protists. For example, the morphologically distinct desmid species *Micrasterias hardyi*

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G. S. West and *Staurastrum victoriense* G. S. West have been reported only in Australia (Tyler, 1996). Accordingly, Foissner and colleagues proposed a moderate endemicity model, which considers that some protist species have cosmopolitan distributions, whereas other (perhaps rarer) protists have restricted distributions (Chao *et al*., 2006; Foissner, 2006). This model has been corroborated by a number of subsequent studies (Van de Vijver *et al*., 2005; Bass *et al*., 2007; Řezáčová and Neustupa, 2007; Robeson *et al*., 2011; Rangefords *et al*., 2012; Bates *et al*., 2013).

The vast majority of studies dealing with the biogeography of protists are based on aquatic organisms. However, terrestrial habitats could present more efficient migration barriers that lead to potentially high endemism in soil protists. Although Finlay and colleagues (2001) reported a cosmopolitan distribution for the majority of soil protists, several studies revealed significant biogeographical patterns in the distribution of terrestrial protist communities (Boenigk *et al*., 2005; Bass *et al*., 2007; Ragon *et al*., 2012; Bates *et al*., 2013). A recently published high-throughput pyrosequencing investigation of terrestrial microbial communities revealed that the community structure could be significantly influenced by environmental factors (Bates *et al*., 2013). Indeed, the terrestrial communities were strongly structured by climatic conditions that affect the annual soil moisture availability.

Investigating the community structure provides great insight into the spatial patterns of protist variation. However, focusing on narrow evolutionary lineages could provide a robust framework for evaluating biogeographical patterns at high phylogenetic resolution. In the present study, we investigated the global biogeography of the terrestrial cosmopolitan genus *Klebsormidium* (Silva *et al*., 1972). This genus occurs in a wide range of habitats and represents one of the most abundant autotrophic organisms found in various aeroterrestrial microbial communities (Deason, 1969; Handa *et al*., 1991; Nakano *et al*., 1991; Baldwin and Whitton, 1992; Rindi and Guiry, 2003; 2004; Smith *et al*., 2004; Barberousse *et al*., 2006; Rindi *et al*., 2008). *Klebsormidium* has widespread cosmopolitan distribution from polar to tropical regions (Ramanathan, 1964; Lee and Wee, 1982; Broady, 1996; Lokhorst, 1996; John, 2002; 2003).

Several molecular genetics studies demonstrated that diversity within the genus *Klebsormidium* is far greater than that expected on the basis of morphological features, and concluded that the traditional phenotypic species concept is insufficient (Rindi *et al*., 2008; 2011). For example, one of the most commonly identified species, *K. flaccidum*, is highly polyphyletic and occurring in five different clades. These cryptic lineages have been recently recognized to be ecologically differentiated (Škaloud and Rindi, 2013).

The general aim of this study was to examine the continental scale biogeographical pattern of a single evolutionary lineage of terrestrial protists, the green algal genus *Klebsormidium*. We used *rbcL* sequences for molecular screening of the isolated strains because they have better resolution than the rapidly evolving ITS rDNA (Rindi *et al*., 2011). We investigated whether the global dispersal of particular *rbcL* genotypes occurs faster than their divergence. We also assessed whether the overall genetic diversity of a molecularly well characterized protist lineage increases by a comprehensive worldwide sampling.

Results

The samples analysed in this work were collected in six regions located in Europe, North America and Asia. Within each region, terrestrial, epilithic and corticolous samples were collected at 2–4 sampling sites. A total of 190 *Klebsormidium* colonies were isolated from the samples, and the *rbcL* gene of each colony was sequenced (Supporting Information Table S1). The majority of sequences (87) were obtained from samples collected in Europe (Wales, 63; Czech Republic, 24); 65 sequences were obtained from samples collected in the USA (Washington, 21; Ohio, 22; Connecticut, 22); and the remaining 38 sequences were obtained from samples originating in Japan.

The sequences yielded a total of 44 unique *rbcL* genotypes, indicating very high genotype diversity in the dataset. Of the 44 unique genotypes, 23 were represented by a single sequence. The Bayesian phylogenetic analysis (Fig. 1) revealed a significant genetic divergence among the genotypes. They were recovered in almost all superclades sensu Rindi and colleagues (2011), with the exception of the superclades A and G. These represent a morphologically distinct genus *Interfilum* and an ecologically defined lineage of desert soil-crust inhabitants. Although several novel genotypes were recognized, none of them formed a new superclade. The majority of genotypes were inferred within superclade E, a heterogeneous assemblage of a number of morphologically and ecologically different lineages (Škaloud and Rindi, 2013). With the exception of the clade E6 (*K. subtillisimum*), the genotypes were inferred in all clades recognized within the superclade E by Rindi and colleagues (2011).

To achieve the best insight into the biogeography of particular *Klebsormidium* lineages, we constructed an additional phylogenetic tree based on only those sequences generated in this study (Fig. 2). In general, all inferred superclades were detected in at least two biogeographic regions. Within the superclade B + C, the sequences originated primarily from the USA (76%), whereas the European and Japanese sequences were less abundant (14% and 10% respectively). Superclade D

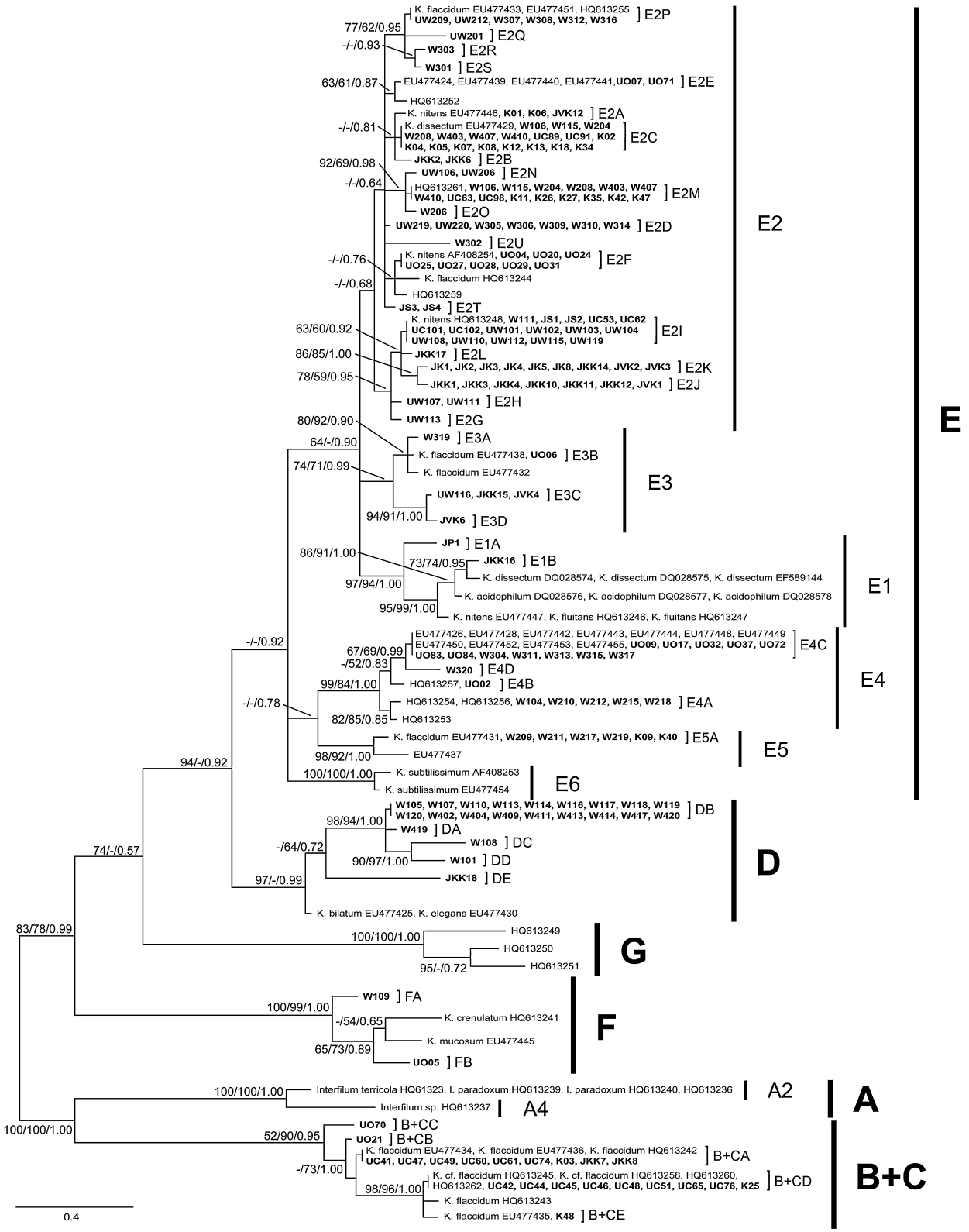

Fig. 1. Phylogenetic relationships among *Klebsormidium* lineages. The unrooted phylogenetic tree was inferred based on a Bayesian analysis of *rbcL* sequences. Values at the nodes indicate statistical support estimated by three methods: maximum parsimony bootstrap (left), maximum likelihood bootstrap (middle), and MrBayes posterior node probability (right). Sequences determined in this study are given in bold. Scale bar represents an expected number of substitutions per site.

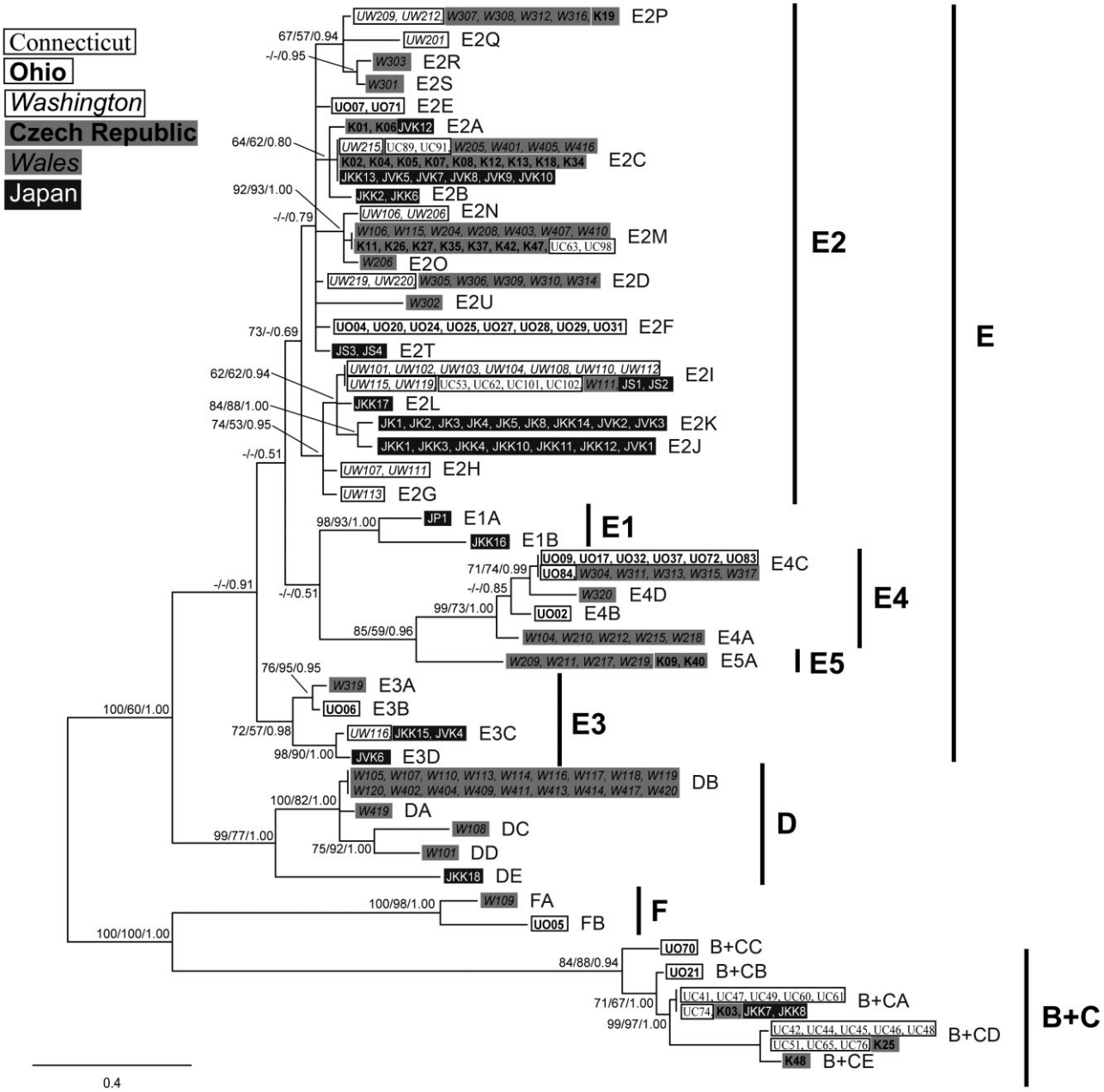

Fig. 2. Phylogenetic relationships among *Klebsormidium* genotypes obtained in this study. The unrooted phylogenetic tree was inferred based on a Bayesian analysis of *rbcL* sequences. Values at the nodes indicate statistical support estimated by three methods: maximum parsimony bootstrap (left), maximum likelihood bootstrap (middle) and MrBayes posterior node probability (right). Geographic origin of particular strains is indicated. Scale bar represents an expected number of substitutions per site.

contained strains isolated almost exclusively from Europe (96%), with a single, distantly related sequence that originated from Japan. Superclade E was present in all six investigated regions. It contained the majority of all obtained sequences (76%) and genotypes (73%). In general, superclade E did not predominate in any of the three continents; it represented 33%, 43% and 24% of North American, European and Asian sequences respectively. When focusing on individual nested clades, some biogeographical patterns were evident. For example, the clade E5 comprised solely of European sequences (E5A) found in both investigated areas, the Czech Republic and Wales. Previously, the E5A genotype has been found in Germany as well (Fig. 1). However, comparison with published sequence data often revealed broader distribution patterns of particular genotypes and clades. For example, we detected the clade E4 in USA and Europe only, though previously it has been reported from Australia, as well.

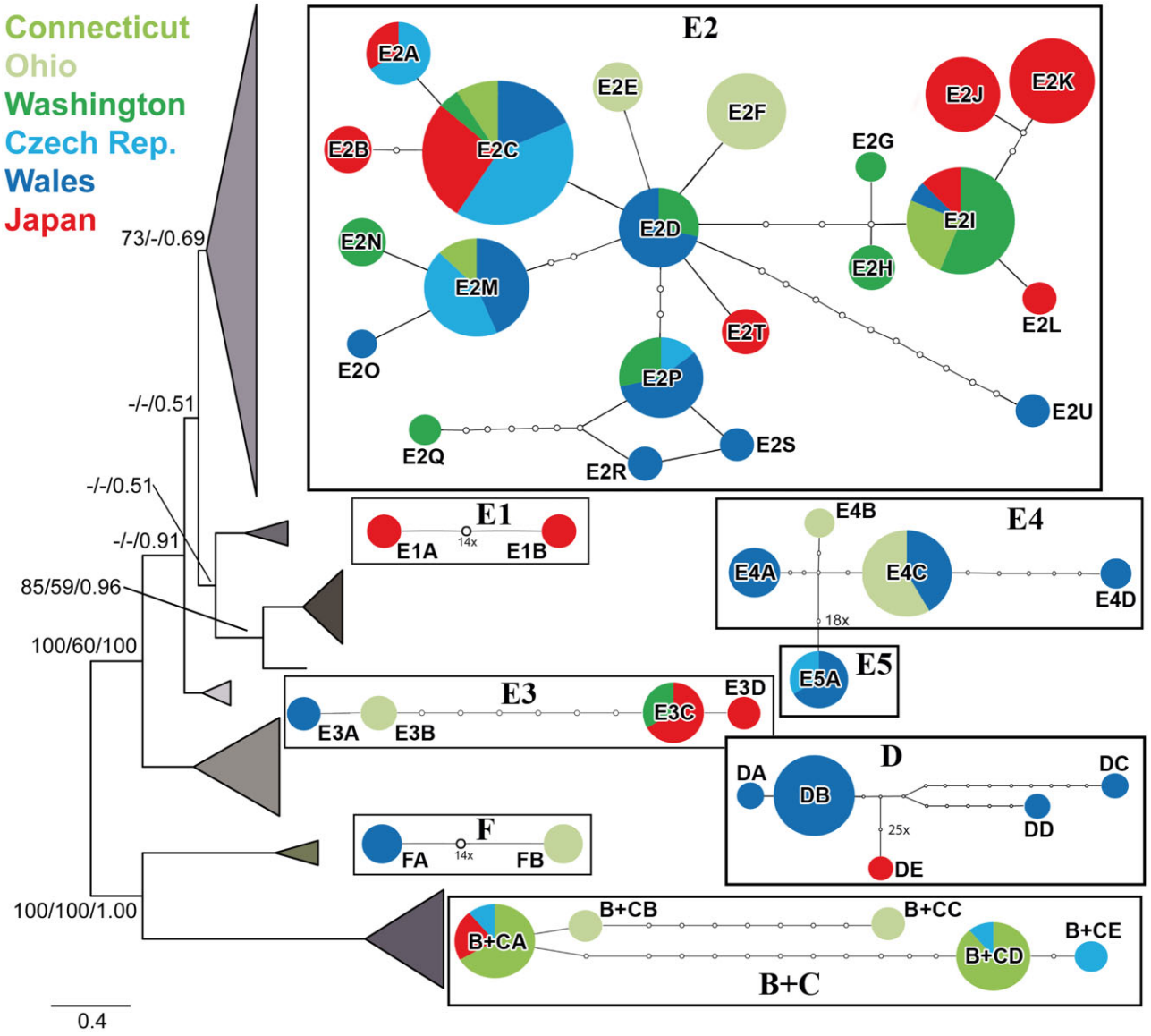

Fig. 3. Statistical parsimony haplotype network representing genealogical relationships among 44 *Klebsormidium* genotypes of the *rbcL* gene. Genotypes are coloured according to the respective sampling region (American and European regions are given in green and blue shades respectively). The sizes of circles representing genotypes reflect the number of sequences that share a genotype. Inferred intermediate haplotypes that were either not sampled or are extinct are represented by small non-coloured circles. Genotypes belonging to eight major inferred clades are outlined by the black frames. Each of the genotypes is coded by a unique identifier reflecting its clade affiliation.

Similarly, although the clade E1 was composed of two Japanese genotypes (E1A and E1B) only, it also contains another genotypes isolated in New Zealand and Great Britain (Fig. 1). The remaining two clades (E2 and E3) had cosmopolitan distributions. Finally, superclade F was formed by only two strains isolated from Europe and USA.

The regional distribution of the genotypes within each inferred clade is shown in Fig. 3. The highest genotypic diversity was found within clade E2, which also contained the most frequently occurring genotype E2C. This cosmopolitan genotype, represented by 22 genotypes, occurred in all, but one of the investigated regions. Almost all genotypes represented by more than eight *rbcL* sequences were shared between at least two continents, with the two exceptions, first of an endemic European genotype DB that was represented by 18 sequences, second of an endemic American genotype E2F represented by eight sequences (seven from Cleveland, and one from Barlow). In general, approximately half of 21 genotypes represented by at least two sequences had a cosmopolitan distribution. The genotypes were less shared among particular areas within a single continent than among continents. The data show that nine American genotypes were found in Europe and/or Asia, whereas only two of these

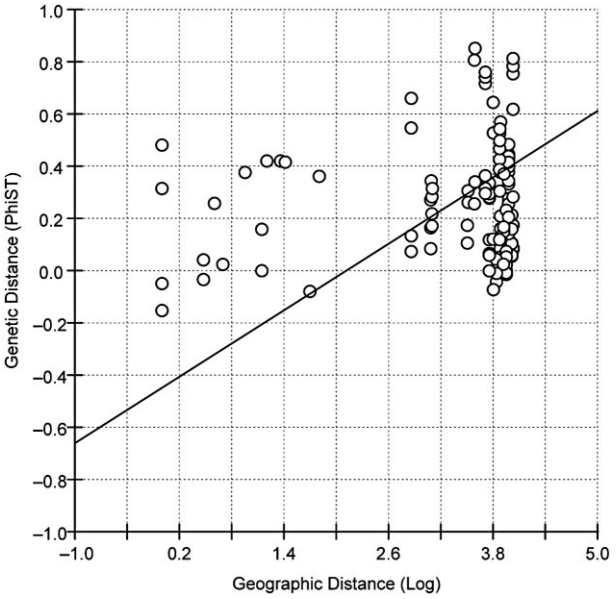

Fig. 4. Scatter plot of pairwise genetic distance (PhiST) versus geographic distance (log geographic distance in km) of 16 sampling sites. A significant correlation between the distances was detected using the Mantel test ($Z = 102.4818$, $r = 0.1188$, $P = 0.0382$).

genotypes were present at two different American regions. Indeed, no genotype was shared among the three American regions. Similarly, nine European genotypes were found in other continents, whereas only four genotypes occurred in both European regions.

To examine the effect of geographic distance on genetic structure and the relative contribution of gene flow to geographic structuring, isolation by distance (IBD) analyses were performed on the following two datasets: (i) the six sampling regions, comprising the Czech Republic, Wales, Washington, Ohio, Connecticut and Japan, and (ii) the 16 sampling sites. No significant correlation was detected among the six sampling regions ($P = 0.7261$). However, a positive, significant correlation between the genetic and geographic distances was detected among the 16 sampling sites (Fig. 4). The significance of the observed correlation was verified using the Mantel test ($Z = 102.4818$, $r = 0.1188$, $P = 0.0382$). Reduced major axis (RMA) regression analysis yielded a value of $R^2 = 0.0141$ (slope = 0.1123).

Discussion

Distribution of Klebsormidium *lineages*

Although the global biogeographic distributions of several aquatic protists have been documented (Finlay and Clarke, 1999; Sabbe *et al*., 2001; Montresor *et al*., 2003; Šlapeta *et al*., 2006; Neustupa and Řezáčová, 2007), the biogeography of aeroterrestrial organisms remained largely unclear. In this study, we analysed a relatively large number of aeroterrestrial algae belonging to the genus *Klebsormidium* to assess its distribution pattern in the Northern temperate zone. In total, we collected 190 strains originating from three different continents. The results demonstrated that a majority of genotypes represented by several isolates have a cosmopolitan distribution, whereas only a few genotypes were isolated in a single continent.

Our data indicate that the genus *Klebsormidium* has a generally cosmopolitan distribution with long-distance gene flow of the aeroterrestrial isolates. The small cell size and great abundance could result in unlimited dispersal that is unrestricted by geographical boundaries. Although filamentous, *Klebsormidium* species easily disintegrate into fragments containing a few cells (Škaloud, 2006). These fragments can spread easily because of random events, such as hurricanes or wind currents. Indeed, viable *Klebsormidium* cells have been detected in lower troposphere air samples (Van Overeem, 1937). Dispersal by wind (airborne dispersal) is a usual way of distribution for aeroterrestrial protists (Sharma *et al*., 2007). Crucial factor for airborne is to survive desiccation and UV radiation. Various physiological studies show that terrestrial algae provide mechanisms of protection and adaptation to high UV radiation (Lud *et al*., 2001; Holzinger and Lütz, 2006; Hughes, 2006; Karsten *et al*., 2007; Pichrtová *et al*., 2013) and desiccation (Haübner *et al*., 2006; Lüttge and Büdel, 2010; Karsten and Holzinger, 2012; 2014). Contrary to terrestrial protists, freshwater microorganisms are usually transported by birds (Schlichting, 1960; Atkinson, 1970; Figuerola and Green, 2002) and mammals (Maguire, 1963; Roscher, 1967). Dispersal among the water bodies typically involves significant changes of the environment (from water to air and back to water again), with a high danger of desiccation. If the transport takes place in the intestine of an animal, there is no danger of desiccation, but cells are exposed to digestion juices (Kristiansen, 1996; 2008). Therefore, freshwater protists should have much limited dispersal capacities in comparison with terrestrial microorganisms (Atkinson, 1971; Foissner, 2006; 2008). Indeed, dispersal limitation of freshwater protists, particularly diatoms, has been recently reported (Vyverman *et al*., 2007; Evans *et al*., 2009; Souffreau *et al*., 2010; Rangefords *et al*., 2012).

In a recently published phylogenetic investigation of this genus, Rindi and colleagues (2011) discussed a limited distribution of several identified clades, based on their investigation of 87 sequenced strains originating from five continents. However, our extended sampling revealed a cosmopolitan distribution of all of these presumably endemic lineages. For example, the proposed Eastern European superclade B has now been identified in North

America. Similarly, our sampling revealed the apparent cosmopolitan distribution of superclade C, which was proposed to be restricted to Western Europe. In fact, it is very likely that the presumably limited distribution of some *Klebsormidium* genotypes in our study may be due to their limited sampling. Similar trends were reported by Kristiansen (2008), who stressed that almost all *Mallomonas* taxa originally started as endemics but sooner or later lost this status because of more intensive research.

In our study, only 4 of the 16 most frequent genotypes were recognized to have limited geographical distribution. The two closely related genotypes E2J and E2K were restricted to the East Asian region, and the remaining two genotypes were identified only in North America (E2F) and Europe (DB). The distribution of those four lineages strictly contradicts a fundamental pattern of random spatial dispersal of ubiquitous organisms, characterized by correlated local and global species richness (Finlay and Clarke, 1999; Finlay *et al*., 2001). Actually, all genotypes were very numerous on a local scale, but very rare on a global scale. However, these atypical distribution patterns could be explained by strict ecological preferences for environmental conditions or habitat types. For example, the genotype DB was represented by 18 isolates sampled in a very restricted area of southern Wales (Supporting Information Table S1). Both sampling sites of this genotype were located on the southern edge of the Brecon Beacons National Park (Wales, UK), on the rocks of the Coal Measures (Barclay, 1989). Therefore, an ecologically restricted habitat preference of the DB genotype is the most plausible explanation of the observed distribution pattern. Recently, strong ecological differentiation of particular *Klebsormidium* lineages has been reported by Škaloud and Rindi (2013). Ecological differentiation of closely related lineages has been reported for many different protist taxa (Rindi and Guiry, 2003; Rindi *et al*., 2008; Peksa and Škaloud, 2011; Moniz *et al*., 2012).

The IBD analysis did not prove any significant correlation between the genetic and geographic distance among the six investigated regions. However, the IBD analysis of genetic sequence distributions among all 16 sampled sites revealed a weakly significant relationship between the genetic and geographic distances (Fig. 4). Dissimilar results of those two analyses imply a small-scale IBD patterns within each of the investigated regions. This indicates that the populations were homogeneous on a global scale, whereas the genotypes were patchily distributed on a local scale. Although this pattern might be viewed as an artifact of fragmentation of localities into smaller units, a fine-scale structuring of genotypes may be caused by various ecological factors, in particular by the habitat differentiation of particular genotypes (Škaloud and Rindi, 2013).

Genetic diversity

The data presented in this study expand our knowledge of the overall diversity and commonness of particular lineages within the genus *Klebsormidium*. Molecular investigations of nominal, morphologically defined protist taxa usually detect the presence of substantial genetic diversity and the presence of a large number of hidden species (Von der Heyden *et al*., 2004; Šlapeta *et al*., 2006; Simon *et al*., 2008). The genetic diversity within the genus *Klebsormidium* has been investigated in detail by Rindi and colleagues (2011) and Škaloud and Rindi (2013), who identified seven main superclades and 24 well-supported clades. Although we did not discover any novel superclade, our investigations led to the identification of a large number of new, previously unrecognized genotypes. From a total number of 44 identified genotypes, more than 66% were reported for the first time. Although the majority of published *Klebsormidium* sequences originated from various European isolates, we identified several novel lineages in Wales, including one within the most commonly sampled genotype DB.

We identified obvious differences in the frequency of genotype occurrences of particular *Klebsormidium* superclades. Consistent with the study of Rindi and colleagues (2011), we found that lineage E represents the most commonly sampled superclade. In particular, 76% of all investigated strains belonged within clade E2. Although we analysed approximately 200 *Klebsormidium* strains from three different continents, we did not sample a single genotype belonging to superclade G sensu Rindi and colleagues (2011). The superclade G could exhibit ecological preferences for specific environmental conditions or habitat types because it was originally isolated from biotic crusts of arid soils in South Africa (Rindi *et al*., 2011). This superclade seems to be ecologically restricted to arid soil environments, in particular to the soil crusts of warm and arid desert, and sub-desert areas (Rindi *et al*., 2011).

Experimental procedures

Sample collection and culturing

The samples were collected in six regions located in mixed forests of the Northern temperate zone on three continents: two in Europe (Wales and Czech Republic), three in North America (Washington, Ohio and Connecticut), and one in Asia (Japan). For each region, 2–4 different sampling sites were selected for collecting samples. At each sampling site, a number of samples were collected from a broad range of terrestrial, epilithic and corticolous substrates. Samples were mixed together and transferred in sterile plastic bags into the laboratory. Samples were inoculated on two agar plates with modified Bold's Basal Medium (Bischoff and Bold, 1963) at 20°C under a 14 h/10 h light/dark regime using photon irradiance of approximately 30–50 μmol photons m^{-2} s^{-1}

provided by 18W cool fluorescent tubes (Philips TLD 18W/33). After 5–10 weeks, the agar plates were checked for the presence of *Klebsormidium* microcolonies.

DNA isolation

For DNA isolation, identified *Klebsormidium* microcolonies were transferred to PCR tubes containing 100 μl of InstaGene matrix (Bio-Rad Laboratories, Hercules, CA, USA). The cells were then mechanically disrupted by shaking for 6 min in the presence of glass beads (3 mm diameter; Sigma-Aldrich) in Mixer Mill MM 400 (Retsch, Haan, Germany). Subsequently, the samples were incubated at 56°C for 30 min (under continuous mixing at 700 r.p.m.) and then heated to 99°C for 8 min. After vortexing, the tubes were centrifuged at 12 000 r.p.m. for 2 min, and the supernatant was directly used as a PCR template.

PCR and sequencing

Sequences of the *rbcL* gene were obtained by PCR amplification using a Touchgene Gradient cycler (Techne). The *rbcL* gene was amplified using the newly designed primer KF590 (5′-GAT GAA AAC GTA AAC TCT CAG C-3′) and the primer *rbcL*-KR2 (5′-GGT TGC CTT CGC GAG CTA-3′; Škaloud and Rindi, 2013). Each 20 μL PCR reaction contained 13.9 μL of sterile Milli-Q water, 2 μL of PCR buffer (Sigma), 2 μL of $MgCl_2$, 0.4 μL of dNTP (10 μM), 0.25 μL of primers (25 pmol ml^{-1}), 0.2 μL of AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 1 μl of DNA. The PCR amplification, purification and sequencing were performed as described in Škaloud and Rindi's (2013). Sequencing reads were assembled and edited by use of SeqAssem software (Hepperle, 2004).

Sequence analyses

For phylogenetic analyses, the newly obtained *Klebsormidium rbcL* sequences and the sequences available in NCBI GenBank database were used to generate the alignment. The final alignment of 606 base pairs (bp) was constructed by CLUSTALW (Thompson *et al*., 1994) using MEGA 5.05 (Tamura *et al*., 2011). The aligned dataset was analysed by maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) analyses as described previously (Škaloud and Rindi, 2013), using the GTR + I + G evolutionary model selected according to the Akaike Information Criterion computed in PAUP/Mr. Modeltest 2.3 (Nylander, 2004). Genotype networks were obtained by statistical parsimony analysis in TCS v1.21 (Clement *et al*., 2000), using the 95% plausible connection limit. Isolation by distance analyses were performed using the IBDWS 3.23 program (Jensen *et al*., 2005). Pairwise matrices of geographical distance (log geographical distance) and PhiST (incorporates sequence distance information) were compared using a Mantel test for matrix correlation (Mantel, 1967), with significance assessed by 10 000 randomizations of the genetic distance matrix.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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