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ORIGINAL ARTICLE

Lichen-associated fungi of the *Letharietum vulpinae*

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Abstract In the present study, a well-defined lichen community was screened for associated fungi for the first time. The photophilous lichen community *Letharietum vulpinae* was chosen because its character species, *Letharia vulpina*, was expected to host rather specialized fungi due to the presence of antimycotic secondary compounds. A considerable number of the associated fungi that were isolated were probably selective for lichens, because they appeared to be distantly related to fungi known from other substrates. The majority of these obligatory, lichen-associated fungi were only isolated in the course of the present study and represent hitherto unknown phylogenetic lineages. Parts of the lichen-associated fungi overlapped those colonizing rock surfaces or were closely related to endophytic fungi, but the lichen-associated and endophytic fungi still represented separate lineages.

Keywords Lichen-associated fungi · Endolichenic fungi · *Letharietum vulpinae* · *Letharia* · Substrate preference

Introduction

Fungi living asymptomatically in thalli of lichenized fungi are commonly referred to as 'endolichenic fungi' (Paranagama et al. 2007). Initial studies on this ecological group revealed a comparably high diversity from plant-inhabiting or 'endophytic' fungi, (Petrini et al. 1990; Girlanda et al. 1997). The endolichenic fungal communities present in corticolous lichens showed only marginal overlap with fungal communities colonizing the bark on which the lichens grew (Suryanarayanan et al. 2005). While U'Ren et al. (2010) suspected that endolichenic fungi might be more selective in their habitat preferences compared to endophytic fungi, they found a high similarity between endolichenic and endobryophytic communities. Evidence for a certain degree of host selectivity of endolichenic fungi (Li et al. 2007) suggested that several unknown fungal taxa may be hosted by previously uninvestigated lichen taxa.

We investigated the fungal community associated with the *Letharietum vulpinae*. Since the lichen community is photophilous (Barkmann 1958), the associated fungi are subjected to strong fluctuations of water content and temperature like rock-colonizing fungi, some of which were shown to be also capable of growing on or within lichens (Harutyunyan et al. 2008). Further, it can be assumed that antimycotically active substances of the character species (Kowalski et al. 2011), *Letharia vulpina*, potentially inhibit some fungal inhabitants. All these extreme microhabitat conditions supposedly favor more specialized strains of endolichenic fungi.

The aim of this study was to investigate whether the *Letharietum vulpinae* hosts a fungal community which partly overlaps with (1) endophytic as well as (2) rock-colonizing communities, but also includes (3) fungi exclusively growing associated with lichens.

Materials and methods

Sampling sites

Foliose and fruticose lichens of the *Letharietum vulpinae* were sampled at three sites in the European Alps (G, A, I)

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and two in California (Ca+b, Cc). At the Californian sites, only lichen genera also present at the European sites were sampled. Although additional lichens were present they were not sampled, but are listed for each sampling site:

- A: Austria. Kärnten. Turracher Höhe. 46°55′N, 13°53′ E. 1,880 m alt. On bark of *Larix decidua* Mill. Lichens sampled*: Bryoria implexa* (Hoffm.) Brodo & D. Hawksw., *Cetraria chlorophylla* (Willd.) Vain., *Hypogymnia physodes* (L.) Nyl., *Letharia vulpina* (L.) Hue, *Parmeliopsis ambigua* (Wulfen) Nyl., *Pseudevernia furfuracea* (L.) Zopf, and *Usnea* cf. *hirta* (L.) Weber ex F. H. Wigg.
- G: Germany. Bayern, Nationalpark Berchtesgaden, Stuhlgrabenkogel Mtn. 47°29′N, 12°57′E. 1,820 m alt. On bark of *Larix decidua*. Lichens sampled: *Bryoria fuscescens* (Gyeln.) Brodo & D. Hawksw., *Evernia divaricata* (L.) Ach., *Hypogymnia austerodes* (Nyl.) Räsänen, *H. physodes*, *L. vulpina*, *P. ambigua*, *P. furfuracea*, and *Usnea filipendula* Stirt. Additional lichen present: *Hypocenomyce scalaris* (Ach. ex Lilj.) M. Choisy.
- I: Italy. Piemonte, Province of Torino, Alta Valle di Susa, slope of the mountain La Selletta. 45°03′N, 6°43′ E. 2,060 m alt. On bark of *Larix decidua*. [Sampling relevés on two trees (Ia, Ib) standing 3 m apart were selected.] Lichens sampled: *B. fuscescens*, *L. vulpina*, *P. ambigua*, *P. furfuracea*, and *U. filipendula*.
- Ca + b: U.S.A. California. Riverside County, James Reserve. 33°48′N, 116°47′W. Ca: 1,820 m alt. On wood of *Arctostaphylos* cf. *pringlei* Parry. Lichens sampled: *Letharia columbiana* (Nutt.) J. W. Thomson, *Letharia vulpina*, and *Pseudevernia intensa* (Nyl.) Hale & W. L. Culb. Additional lichens present: *Lecanora hagenii* (Ach.) Ach., *Lecanora* sp., cf. *Trapeliopsis* sp. Cb: Alt. 1800 m. Bark of *Pinus ponderosa* Dougl. Lichens sampled: *L. vulpina*.
- Cc: U.S.A. California. San Diego County, Sky Oaks Field Station. 33°22′N, 116°37′W. 1,400 m alt. On *Adenostoma fasciculatum* Hook. & Arn. Lichens sampled: *Kaernefeltia merrillii* (Du Rietz) A. Thell & Goward, *L. columbiana*. Additional lichens present: *L. hagenii*, *Lepraria* sp., and *Rinodina* cf. *herrei* H. Magn.

Isolation of fungal strains

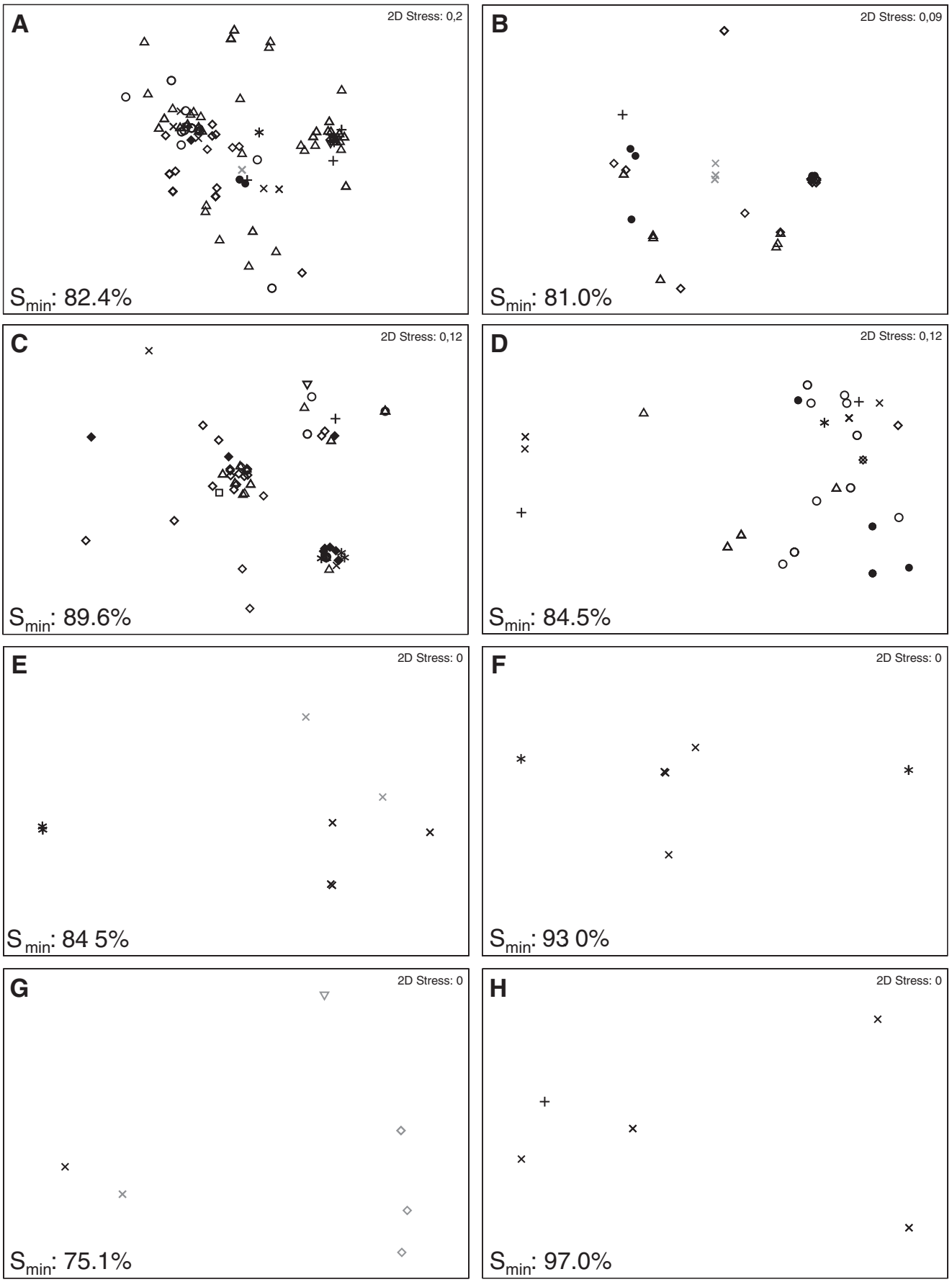
Fungal strains were isolated by inoculating fragments of approximately 1 mm^3 of the apices or margins of the lichen thallus to the culture media (malt–yeast agar, MYA). Once moistened, the fragments were further dissected using sterile tweezers into as many fragments as possible, and these were dispersed on the agar plates. The isolation was performed under semi-sterile conditions in the field, using a plastic tarpaulin as underlay and tweezers, both of which had been sterilized with 70% EtOH before processing each new sample. Starting 24 h after isolation, the plates were examined daily with optical equipment (×50 magnification). Developing mycelia were separated and transferred to fresh media immediately after detection, until pure cultures were obtained. Dried cultures were deposited in the fungal collection of the Botanische Staatssammlung München (M).

Fig. 1 a–m Sequence similarities among groups of *Letharietum vulpinae*-associated fungal strains and all published sequences showing at least 90% similarity to the respective group. Similarities among ITS rRNA gene sequences are visualized by non-metric multidimensional scaling (NMDS). Symbols code the origin of the corresponding strains: Lichens of the *Letharietum vulpinae* (×), other lichens (✻), fungi (□), rock (●), surface sterilized living plant leaves or stems (○), non-surface sterilized living or plant material decaying above-ground (◊), plant roots (▽), soils (△), arthropods (♦), and other substrates (+). Undifferentiated substrates (+) include dung (**a**), water (**a**, **c**), air (**b**), skin (**b**), and particles in house dust (**d**, **h**). Isolates from the European Alps are marked in *black*; those from California are marked with gray asterisks. Other symbols in gray (**g**) indicate related sequences with less than 90% similarity to the isolates. The similarity among the least similar sequences of each clade (S_{min}) is given for each cluster for comparison. Letters indicate the different clusters according to the names of clusters used in the text. The groups include sequences deposited as members of the taxa *Pseudeurotium* (cluster A), *Recurvomyces* (**b**), *Fusarium* (**c**), *Celosporium* (**d**), Ascomycota (**e**, **f**, **h**, **k**), Chaetothyriales (**g**), *Sarea* (**i**), *Phaeomoniella* (**j**), *Sorocybe* (**l**), and *Cladophialophora* (**m**). A separate NMDS analysis of the subset from group M indicated by an arrow (→) is provided under **m**’

Sequence analyses

The ITS rRNA gene was sequenced for a subset from all isolates. This subset was randomly chosen, but with a focus on isolates obtained from *Letharia*. Such isolates are over-represented among the sequenced isolates by a factor of ca. 2. Data of double-stranded sequences were obtained and analyzed as described by Peršoh and Rambold (2002). Sequence data were deposited under the accession numbers JN053061–JN053174 at GenBank (www.ncbi.nlm.nih.gov).

The GenBank database (status: Feb. 2011) was searched for sequences most similar to those obtained using ‘Mega BLAST’ (Zhang et al. 2000). The ten best matches for each query sequence were downloaded and SSU as well as LSU rRNA sequence parts were deleted. Pairwise similarities among the sequences were calculated using Local BLAST (ftp://ftp.ncbi.nih.gov/blast/executables/release/2.0.10/blast-2.0.10-ia32-win32.exe) with the parameter selection ‘-m 8 -r 2 -G 5 -E 2’. The function ‘simMatrix’ (Flessa et al. 2010), written in R (R Development Core Team, 2008), was applied to transform these initial pairwise similarities into a similarity matrix, with similarity values calculated as follows: similarity=number of matching positions per length of queried sequence. Hierarchical cluster analyses of the similarity matrix were conducted by using the R function ‘hclust’. Sequences with minimal


A
2D Stress: 0,2
S$_{min}$: 82.4%
B
2D Stress: 0,09
S$_{min}$: 81.0%
C
2D Stress: 0,12
S$_{min}$: 89.6%
D
2D Stress: 0,12
S$_{min}$: 84.5%
E
2D Stress: 0
S$_{min}$: 84 5%
F
2D Stress: 0
S$_{min}$: 93 0%
G
2D Stress: 0
S$_{min}$: 75.1%
H
2D Stress: 0
S$_{min}$: 97.0%

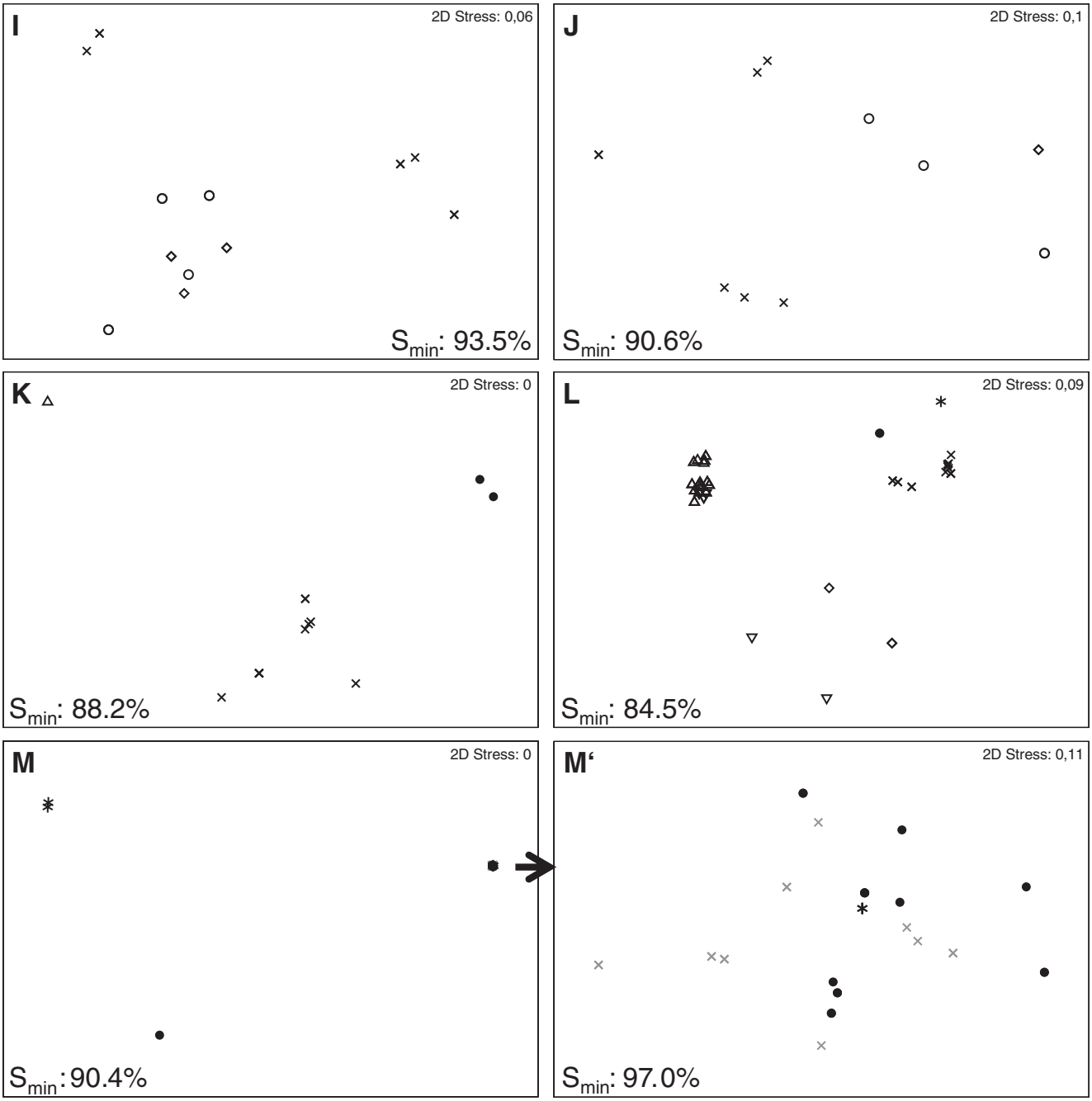


Fig. 1 (continued)

similarities of 90% according to the average linkage method were grouped, using the R function ‘cutree’. Groups including only isolates from a single sampling site were regarded as singletons and not considered for further analyses.

Separate BLAST searches for each non-singleton-group in GenBank were conducted to obtain all sequences showing at least 90% similarity to any query sequence of each group. [For group G (Fig. 1g), a lower threshold was applied, because no sequences with similarities above 90% were found.] Similarity matrices were calculated for each group independently, but otherwise as described above. The matrices were imported into Primer 6 (Plymouth Routines, v.6.1.6), to conduct non-metric multidimensional scaling (NMDS) analyses. Substrate data of all isolated strains including available ones of published reference sequence data were mapped on the NMDS graphs (Fig. 1) and are provided as supplementary information (Supplementary Table 2).

The most likely trees and 500 bootstrap replicates were calculated using RAxML (Stamatakis 2006) for groups I–L, applying the GTRCAT approximation of substitution. In addition, trees were calculated using DNAML and DNAPARS (Felsenstein 1993).

Taxonomy to genus level follows the three global databases: Species Fungorum and Dothideomycetes (both http://www.speciesfungorum.org/) for non-lichenized fungal species, and LIAS names (http://liasnames.lias.net/) for lichenized taxa. Fungal classification above genus level was based on that of LIAS with inclusion of current versions of the "Outline of Ascomycota" (http://archive.fieldmuseum.org/myconet/outline.asp).

Results

The 197 strains selected for sequencing associate into 76 90%-similarity groups according to the hierarchical cluster analysis. Twenty-three of the groups (comprising 114 strains) include fungal genotypes isolated from at least two sampling sites. The remaining 83 strains represent singletons and are not considered further. One group (group N, Supplementary Table 1) comprises five strains with ITS sequences matching the sequences of the host taxa. The remaining 22 groups include isolates from different sites and these may be further grouped into four categories according to published data on substrate preference of the closest relatives within each group (Supplementary Table 2). The majority of 13 groups (i.e. groups A–D and O–W) includes fungi from a wide range of substrates, four groups (E–H) include exclusively or predominantly strains isolated from lichens, two groups (I–J) comprise endophytic and otherwise plant-associated fungi, while three groups (K–M) of *Letharietum vulpinae*-associated strains are most closely related to rock surface colonizers. The following detailed presentation of the results is structured according to the predominant substrate preference of each group.

The isolates of several strains show the highest ITS sequence similarities to **ubiquitous taxa**, i.e. to representatives of the genera *Aureobasidium*, *Celosporium*, *Cladophialophora* (2 clusters), *Cladosporium*, *Fusarium*, *Hypholoma*, *Leucostoma*, *Oidiodendron*, *Penicillium*, *Phlebiopsis*, *Pseudeurotium*, and *Recurvomyces*. The substrate of origin of the most closely related strains is mapped for four of these clusters (A–D) in Fig. 1. Six strains of group A were isolated from *L. vulpina* at four sampling sites (A, I, G, and Ca) and one each from *B. implexa* (site G), *P. ambigua* (A), and *U.* cf. *hirta* (A). The isolates are predominantly related to soil fungi and fungi detected on non-surface-sterilized plant material, several of which were identified as members of the genus *Pseudeurotium.* The three strains of group B (aff. *Recurvomyces*) originate from *L. vulpina* and *L. columbiana* specimens from two Californian sites (Ca, Cc). They cluster among strains from various substrates. Group C (aff. *Fusarium*) includes two strains isolated from *L. vulpina*. The strain from site G clusters among isolates from insects, soil and various lichens, while the one from site I is more distantly related to the other members of group C. The isolates of group D (aff. *Celosporium*) are also split into two groups. A strain isolated from *B. implexa* in Austria and one from *L. vulpina* in Italy are well separated from the majority of members of group D. Sequences from mostly endophytic fungi cluster together with isolates from thalli of *L. vulpina* from Italy (site I, 3 isolates) and isolates of *E. divaricata* and *H. physodes* from Germany (site G). The latter sequence is identical to two of the sequences from *L. vulpina*-associated strains. These are not distinguished in Fig. 1d.

Groups E–H (Fig. 1e–h) include, almost exclusively, **lichen-associated strains**. These groups account for 17% of all non-singleton groups, including 8% of all strains isolated. While the sequences of groups E (6 isolates) and G (2 isolates) derived from the samples from both continents, those of groups F (4 isolates) and H (4 isolates) were only isolated from sites in Europe. Group E includes lichen-associated strains from both species of *Letharia*, one from *H. austerodes*, and further two endolichenic strains from *Tuckneraria laureri*. The most similar sequences (85% similarity) not deposited as endolichenic fungi originate from voucher specimens of *Verrucaria funckii* (Spreng.) Zahlbr. (Verrucariaceae, Verrucariales). Members of group F were isolated from *L. vulpina* and *U. filipendula* at the sites in Germany and Italy. They show an ITS sequence similarity of >90% to two unidentified endolichenic fungi ('Ascomycota sp.': EF373561, EF373586) isolated from *Arctocetraria andrejevii* (Oxner) Kärnefelt & A. Thell and *Tuckermannopsis chlorophylla* (Willd.) Hale. A more precise taxonomic affiliation of members of group F was not possible, because all other sequences with similarity values above 50% were obtained from uncultured isolates from environmental samples. Group G includes fungi from both *Letharia* species with 84% and 75% ITS similarity respectively, to sequence EU030275 from the resinicolous *Sorocybe resinae* (Fr.) Fr. (Herpotrichiellaceae, Chaetothyriales). Group H includes four strains from *B. fuscescens*, *H. physodes*, and *P. ambigua*, isolated at the sites in Germany and Italy, together with a sequence isolated from house dust in Finland. The closest match (85% similarity) among well-referenced sequences (GQ266144, GQ266146) refers to *Phaeotheca fissurella* Sigler, Tsuneda & J. W. Carmich. (Capnodiales inc. sed.).

The two groups of strains exclusive to plant material and lichens (Fig. 1i–j), were detected at the European sampling sites in this study, for each of *L. vulpina*, *P. furfuracea*, and *U. filipendula*. In addition, group I includes an isolate from *U.* cf. *hirta* and group J one from *H. physodes*. In group I, the isolates form two clusters distinct from the sequences obtained from plant material (Fig. 2a), all indicative of species of *Sarea* (Leotiomycetes inc. sed.). The three clusters of isolates within group J are only distantly related to an epiphytic species of the genus *Phaeomoniella*

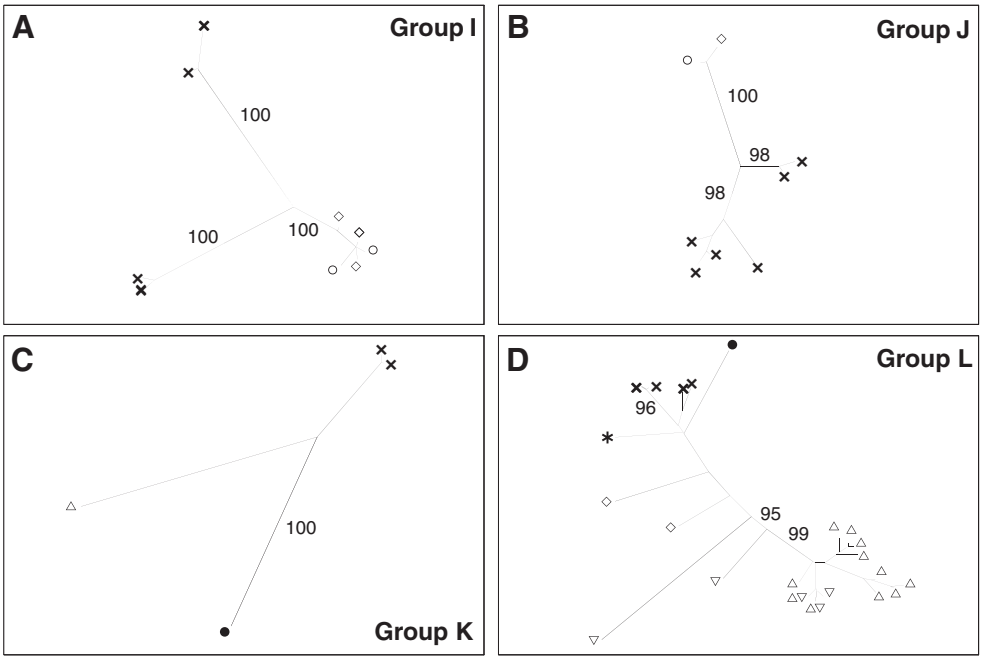


Fig. 2 **a–d** Phylogenetic relationships between the sequences of groups I–K. The best scoring trees found by the RAxML analysis are shown, the topology of which is identical to the topology found by DNAML and DNAPARS. Bootstrap support values above 90% are noted for the respective branches. Symbols code the origin of the corresponding strains: Lichens of the *Letharietum vulpinae* (×), other lichens (✻), rock (●), surface-sterilized living plant leaves or stems (○), non-surface-sterilized living or plant material decaying above-ground (◊), plant roots (▽), and soil (△). A symbol may code multiple identical sequences

(Herpotrichiellaceae, Chaetothyriales) and unidentified endophytic strains (Fig. 2b).

The lichen-associated strains in groups K and L (Fig. 1k–l) were only isolated from European sites whereas those of group M derived from Californian sites. Seven of the strains in group K were isolated from *L. vulpina* and one from *U. filipendula*. They constitute a distinct cluster of their own, separate from soil-inhabiting and **rock-colonizing fungi** (Fig. 2k). In group L, the isolates from *H. physodes*, *L. vulpina*, *P. furfuracea*, and *U. filipendula* cluster with a rock surface-colonizing strain and another lichen-inhabiting strain isolated from *Tuckermannopsis chlorophylla*. This cluster is clearly separated from predominantly soil-inhabiting fungi and isolates from roots and other plant material (Fig. 2d). Nine isolates from *L. columbiana* (from California, present study) and two from *Lasallia rossica* (from China) cluster among the rock-colonizing fungi (from Central Spain and Mallorca) within cluster M (Fig. 1m'). Two further isolates from the lichens *Cetraria australiensis* W. A. Weber ex Kärnefelt and *Tuckermannopsis ciliaris* (Ach.) Gyeln. and one from a rock surface are well separated (Fig. 1m).

Discussion

As in preceding studies on lichen-inhabiting fungi (Petrini et al. 1990; Li et al. 2007), we refrained from sterilizing the surface of the host lichens, because of suction effects on the antibiotic sterilizing agents by the thalli of the foliose and fruticose species of *Letharietum vulpinae*. However, the isolation technique utilized permitted recovery of the lichen mycobionts (group N), which are supposedly among the slowest-growing fungi. Nevertheless, fungal strains germinating from spores adhering to or being enclosed in the lichen thalli had to be expected along with the obligate lichen-associated fungi. In part, the strains originating from coincidental associations were eliminated during data analysis, by discarding strains derived from a single sampling site. Still, the majority of the observed strains clustered among **ubiquitous taxa**, noted for their lack of substrate selectivity. The occurrence of these strains (Fig. 1a–d) on or in lichen thalli is therefore considered to be incidental, due to attached air-borne spores.

The proportion of isolates considered as being **obligate inhabitants of lichens** (Fig. 1e–h) was remarkable. The transatlantic distribution of some clusters in conjunction with the lack of closely related published sequences from other substrates indicates a preference of these fungi for lichen thalli as predicted. Moreover, within two groups (E and G), the strains from the *Letharietum vulpinae* obviously represent hitherto unknown phylogenetic lineages. However, host selectivity seems to be low, because all the groups of potentially obligate lichen inhabitants were found in more than one host species within the lichen communities investigated. Group H included isolates from various host taxa, but not from *Letharia* spp. The antimycotically active vulpinic acid produced by the *Letharia* spp. (Kowalski et al. 2011) may possibly suppress these strains. However, strains of group H may be rare since they have only been isolated from four lichen thalli. Their absence in *Letharia* spp. is therefore not statistically significant. Since all other strains isolated multiple times from lichens of the *Letharietum*

vulpinae were detected on *Letharia* spp., a general inhibitory effect of vulpinic acid seems unlikely.

Interestingly, the only matching GenBank reference sequence for fungi of non-endolichenic origin in these groups originated from particles in house dust (Pitkäranta et al. 2008). Other sequences from the same sample indicated the presence of the sorediate lichens *P. ambigua* and *H. physodes* (Pitkäranta, personal communication), being conspecific with the hosts for 75% of members of group H. As indoor growth can be excluded for lichens, their DNA in a dust sample can be explained with some certainty by the presence of soredia or thallus fragments. In the case of soredia, the observation may be regarded as the first indication for vertical transmission of endolichenic fungi, and should be a subject for further study.

Several **endophytic fungi** were considered to have evolved in part from endolichenic fungi (Arnold et al. 2009). The neighboring but separated clusters of endolichenic and endophytic strains in groups I and J (Figs. 1i–j and 2a–b) are indeed in agreement with a common evolutionary ancestry for these two ecological groups. Furthermore, it is in accordance with the finding that endolichenic fungi are rare colonizers of higher plants (Suryanarayanan et al. 2005). Congruencies between bryophyte- and lichen-inhibiting fungi, as found by U'Ren et al. (2010), can neither be confirmed nor rejected by the present data, due to the scarcity of published molecular data for bryophilous fungi.

Similar environmental conditions like high radiation and low nutrient supply were suggested as possible reasons for the co-occurrence of certain fungi on rocks and in lichens (Onofri et al. 2007; Harutyunyan et al. 2008). Moreover, it has been shown that **rock-colonizing** and endolichenic **fungi** may form haustorial and/or appressorial structures for interacting with their photobionts similarly to lichen mycobionts (Gorbushina et al. 2005; Brunauer et al. 2007). Based on these findings, it seems plausible to assume that the strains of group M may form similar symbiotic hyphal structures or interfaces with algae, whether they grow on rock or in association with lichens. The results also indicate the possibility of a common ancestry for rock-colonizing and endolichenic fungi within group K (Fig. 2c), as discussed above for endophytic and endlichenic fungi.

A closer look at group M, however, underlines our limitation in interpreting the results in further detail. While the endolichenic fungi seem to be selective for *L. columbiana* among the lichens of the *Letharietum vulpinae*, they obviously colonize other lichens outside this community, as, e.g., *L. rossica*. Furthermore, the rock-colonizing members of group M exclusively originate from European sites, while all the endolichenic strains were isolated at localities outside Europe. Accordingly, the strains in group M may be grouped by host preference, substrate preference and/or geographical distribution, but neither of these groups corresponds to phylogenetic groups (Fig. 1m').

The study revealed that the foliose and fruticose lichen species of the *Letharietum vulpinae* are colonized by fungal strains of several phylogenetic lineages. While ubiquitous fungi were most frequent, several strains appeared to be closely related to plant-inhabiting and rock-colonizing fungi. The overlap with rock-colonizing fungi was striking in number and phylogenetic proximity. However, a considerable number of sequences from non-ubiquitous lichen-associated taxa were only distantly related to fungi from other substrates and may represent hitherto unknown phylogenetic lineages.3

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