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Fungal composition of lichen thalli assessed by single strand conformation polymorphism

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Abstract: Fungi that are unrelated to the mycobiont species frequently colonize lichens. Some of these fungal colonists are described lichenicolous fungi, lichen parasites and pathogens that produce recognizable morphological characters, while others apparently produce no noticeable structures. Here we apply the single strand conformation polymorphism (SSCP) technique to directly assess the abundance of different fungi in lichens. Twenty-eight lichen thalli were chosen, some with and some without externally visible symptoms of parasite infection, and these were subjected to total DNA extraction. PCR was conducted with fungal-specific primers for the ITS region of ribosomal DNA. Single strands of the products were separated on native acrylamide gels. The majority of lichen specimens, both infected and those without symptoms, displayed more than one band in the stained gels. In one case, 14 bands were detected using SSCP. Some of these bands apparently represent other neighbouring lichens in the habitat, but many are apparently non-lichen-forming. Since few lichen-associated fungi have been cultured and sequenced, it is difficult to know if SSCP bands represent obligate lichenicolous fungi, other asymptomatic lichen parasites, or fungi not obligately associated with lichens, but our results indicate that large numbers of non-lichen-forming fungi commonly co-occur with lichens in nature. For specimens of the filamentous lichens *Cystocoleus ebeneus* and *Racodium rupestre* we used cloned sequences to compare the number of sequences obtained by the SSCP method to the number obtained by direct sequencing of thallus extracts, and we generally found that more sequences could be detected by SSCP than could be seen by direct sequencing.

Key words: diversity, ITS, lichenicolous fungi, SSCP, symbiosis

Introduction

Lichens are generally considered a fungal-algal partnership representing "… an ecological obligate, stable mutualism between an exhabitant fungal partner (the mycobiont) and an inhabitant population of extracellularly located unicellular or filamentous algal or cyanobacterial cells (the photobiont)" (Hawksworth & Honegger 1994). Recent evidence indicates that a more complex situation prevails in nature, as lichens are usually inhabited by a diversity of other organisms, many of them fungi unrelated to the lichen mycobiont. In some cases, these lichen-associated fungi are recognizable taxonomically. For example, some 1000 lichenicolous fungi are named and recognized by phenotypic characters (Lawrey & Diederich 2003). The biology of these lichenicolous fungi is varied: some species are aggressive parasites, which can rapidly eradicate populations of different host species, whereas others are almost symptomless commensals and highly adapted to the biology of their host.

In addition to recognizable lichenicolous fungi, a large number of other fungi have been isolated from lichens by culture-based approaches (Petrini *et al.* 1990; Girlanda *et al.* 1997, Prillinger *et al.* 1997). A number of moulds can be isolated from the surface of thalli, with apparently unspecific relationships to lichens. These and similar fungi often grow faster than the lichen mycobiont on nutrient-rich media, and are usually referred to as "contaminants" of the axenic mycobiont cultures. Culture-based studies showed also the presence of "meristematic

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rock-inhabiting fungi" in lichens from seasonally arid habitats (Harutyunyan *et al.* 2008). The detected strains of these fungi belong to the genera *Capnobotryella*, *Coniosporium*, and *Rhinocladiella* and were related to those known from rocks (Ruibal *et al.* 2005). It is thus possible that strains of meristematic rock-inhabiting fungi opportunistically colonize lichens. A similar fungal consortium is also found in the intermixed populations of the two microfilamentous lichens *Cystocoleus ebeneus* and *Racodium rupestre* (Fig. 1). These species grow on acid rocks forming flat mats, which also entangle several other melanized fungi (Muggia *et al.* 2008).

The most recent culture-based study of diversity and specificity of lichen-associated fungi was conducted by Arnold *et al.* (2009), who concluded that isolated endolichenic fungi are similar to endophytic forms that live in vascular plant tissues.

Culture-based methods will continue to represent an important source of information about this hidden fungal diversity, especially as optimized culture conditions and medium composition increase the number of fungi retrieved from cultured lichen fragments. However, with a culture-based approach alone the diversity of these fungi in the original material will always be under-estimated. It is therefore useful to apply a direct method for convenient comparison of the fungal composition in lichen thalli.

Recent direct molecular approaches to obtain the mycobiont in DNA extracts from the lichens have used fungal-specific primers. Use of these methods occasionally yields ambiguous sequences suggesting presence of other fungi in the PCR products (Ekman 1999). Multiple fungi are also apparent as varying sizes of the amplified fragments obtained by PCR (Wolinski *et al.* 1999), unless they represent multiple size variants of the same species due to introns. These separated bands can be excised from the gel for sequence-characterization, and heterogeneous PCR products of equal sizes can be sequenced after subcloning of PCR products.

Research in microbial ecology routinely uses DNA-fingerprinting methods for characterizing bacterial communities. In the present contribution we show that single strand conformation polymorphism (SSCP; Orita *et al.* 1989) is a useful technique to assess fungal composition in lichen structures. The principle of this method is that single-stranded DNA fragments can be separated on a gel according to their nucleotide sequence variation. The separated bands can then be excised from the gels for sequencing. We apply SSCP to detect and preliminarily identify lichen-associated fungi from lichen specimens with and without noticeable fungal infections. We further test this method with PCR-cloning techniques in the analysis of *Cystocoleus ebeneus* and *Racodium rupestre* to see if similar sequences are obtained. We suggest that SSCP will prove a useful and reliable technique to detect non-lichen-forming fungi in environmental samples, such as lichenicolous and other, yet unculturable fungi.

Materials and Methods

Sampling

A total of 27 species of lichens was selected according to the availability of both fresh and infected material. The samples selected for the SSCP analyses were from nineteen lichens infected by known lichenicolous fungi (i) and from thirteen lichens without visible fungal infections (n) (Table 1). Because a greater number of thalli of *Cystocoleus ebeneus* (9) and *Racodium rupestre* (8) were available, we analysed them in more detail by cloning, direct sequencing of the PCR products, and SSCP. The material is deposited in the herbaria of the Institute of Plant Sciences Graz (GZU) and of the University of Trieste (TSB).

DNA extraction, PCR and sequencing

DNA was extracted from scraped lichen thallus parts according to Cubero *et al.* (1999). Samples from soil crust lichens also included a few attached fragments of the underlying soil substratum.

To detect the widest range of fungi putatively present in the lichen thalli, the DNA was amplified with the conserved fungal primers for the ITS region ITS1f (Gardes & Bruns 1993) and ITS4 or ITS2 (White *et al.* 1990). To produce single strand fragments for SSCP we used a phosphorylated ITS1f primer for exonuclease digestion.

PCR reactions were prepared for a 50 µl final volume containing double-distilled water, 1 mM *Taq* polymerase reaction buffer (Tris pH 8·3), 1·5 mM $MgCl_2$,

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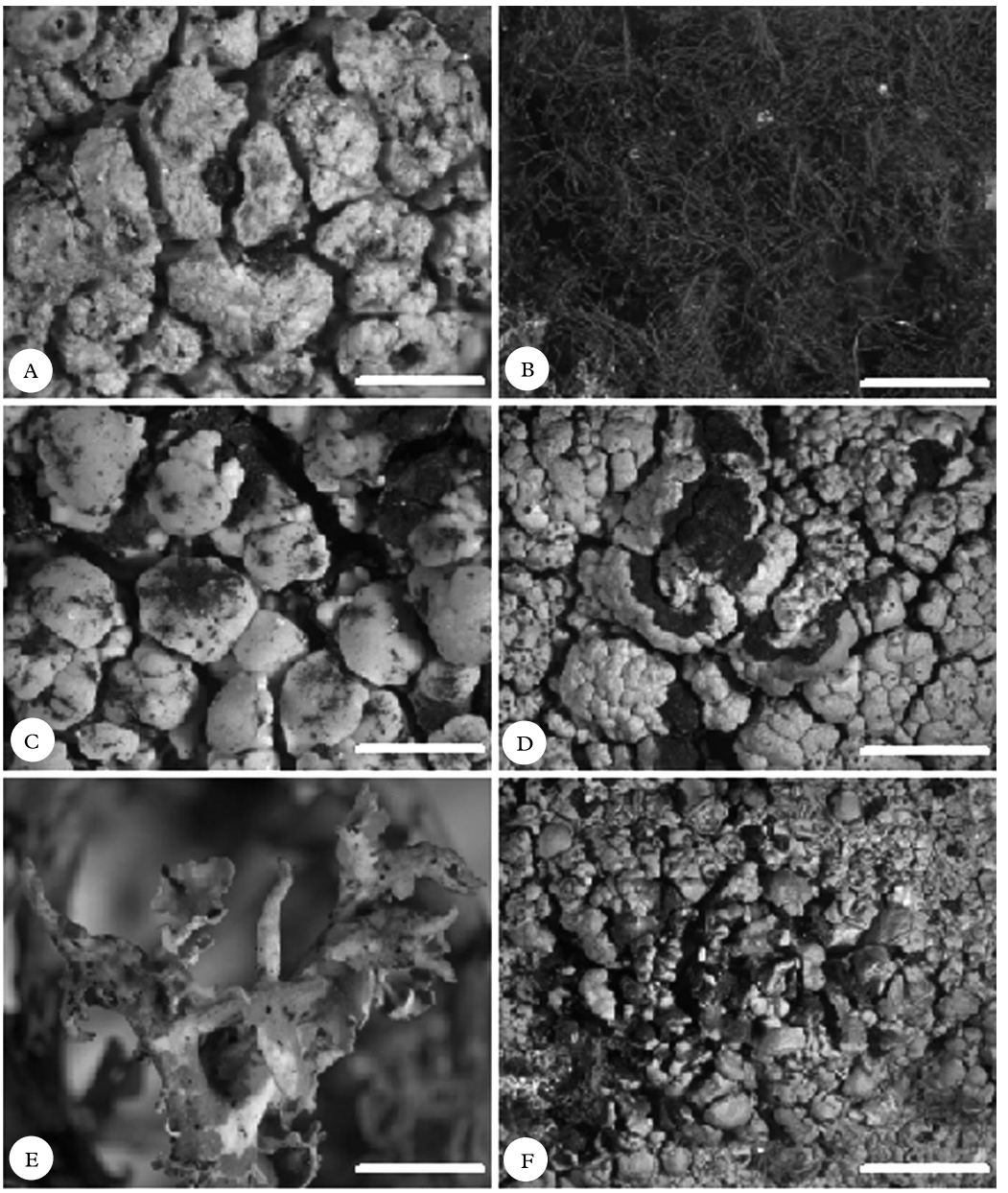

FIG. 1. Habit of some lichenicolous fungi and their lichen hosts. A, *Sagediopsis fissurisedens* on *Aspilidia myrinii*; B, intermixed filamentous thalli of *Cystocoleus ebeneus* and *Racodium rupestre*; C, an unidentified fungus on *Lecanora polytropa*; D, *Taeniolella atrocerebrina* on *Tephromela atra*; E, *Tremella* sp. on *Cladonia furcata*; F, *Sphaerellothecium atryneae* on *Lecanora swartzii*. Scales: A–C = 1 mm; D = 2 mm; E & F = 4 mm.

2·5 mM dNTPs, 5u/µl *Taq* DNA polymerase, 1 µM of each primer. PCR amplifications with the primer pair ITS1f/ITS4 were performed under the following conditions: an initial heating step of 2 min at 94°C, linked to 30 cycles of 1 min at 94°C, 1 min annealing at 53°C, 2 min extension at 72°C, and one final extension step of 7 min at 72°C, after which the samples were kept at 4°C. Touch-down PCR protocol was used for the primer pair

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TABLE 1. *Lichen specimens investigated in the DNA analyses. Samples are distinguished into infected (i) and not infected (n) by lichenicolous fungi*

Lichen species*	Specimen	Herbarium number (DNA extraction number)
Acarospora fuscata[3]	(i) Austria, Styria, Steinz, Höllgraben, Sporiroaofen, 2006, *Muggia & Hafellner*.	TSB 38839
Aspilidea myrinii[3]	(i) Austria, Carintia, Koralpe, Krakaberg, 2007, *Muggia & Hafellner*.	TSB 38842
Cladonia furcata[3]	(i) Austria, Carintia, Stubalpe, Lichtengraben 2006, *Muggia & Hctfellner*.	TSB 38840
C. pocillum[3]	(i) Austria, Styria, Stubalpe, Rappoldkogel, 2006, *Hafellner*.	GZU, Hafellner 66711
Lecanora polytropa[3]	(i) Austria, Carintia, Koralpe, Krakaberg, 2006, *Muggia & Hafellner*.	TSB 38837
L. polytropa[3]	(i) Austria, Styria, Stubalpe, Speikkogel, 2006, *Muggia & Hafellner*.	GZU
L. swartzii[3]	(i) Austria, Styria, Koralpe, Moschkogel, 2007, *Muggia & Hafellner*.	TSB 38841
Lobaria pulmonaria[3]	(i) Portugal, Madeira, 2008, *Berg* (N7).	GZU
L. pulmonaria[3]	(i) Portugal, Madeira, 2008, *Berg* (N6).	GZU
Lobothallia radiosa[3]	(i) Austria, Styria, Stubalpe, Gradenberg, 2006, *Hafellner*.	GZU, Hafellner 67051
Parmelia sulcata[3]	(i) Austria, Styria, Höllgraben, Sporiroaofen, 2006, *Muggia & Hafellner*.	TSB 38834
Pertusaria corallina[3]	(i) Austria, Styria, Stubalpe, Ameringkogel, 2005, *Hafellner*.	GZU, Hafellner 65226
Phaeophyscia orbicularis[3]	(i) Austria, Styria, Stubalpe, 2006, *Hafellner*.	GZU, Hafellner 67027
P. orbicularis[3]	(i) Austria, Styria, Stubalpe, Mitterberg, 2006, *Hafellner*.	GZU, Hafellner 66967
Physcia caesia[3]	(i) Austria, Styria, Seckauer Tauern, Hochreithhöhe, 2002, *Hafellner*.	GZU, Hafellner 58567
Pseudocyphellaria sp.[3]	(i) Portugal, Madeira, 2008. *Berg*.	GZU
Tephromela atra[3]	(i) Austria, Styria, Seckauer Tauern, Hämmerkogel, 2006, *Muggia & Hafellner*.	GZU
Toninia sedifolia[3]	(i) Italy, Trentino Alto Adige, Val Venosta, 2002, *Hafellner*.	GZU, Hafellner 61304
Xanthoparmelia conspersa[3]	(i) Austria, Styria, Stubalpe, Hirschegg, 2006, *Muggia & Hafellner*.	GZU
Arthrorhaphis citrinella[3]	(n) Austria, Carintia, Koralpe, Seespitz, 2008, *Muggia & Hafellner*.	TSB 38869
A. citrinella[3]	(n) Austria, Styria, Koralpe, Brandhöhe. 2008, *Muggia & Hafellner*.	TSB 38872
Aspicilia simoensis[3]	(n) Austria, Carintia, Stubalpe, Peterer Riegel, 2006, *Muggia*.	TSB 38595
Baeomyces placophyllus[3]	(n) Austria, Carintia, Koralpe, Seespitz, 2008, *Muggia & Hafellner*.	TSB 38868
B. rufus[3]	(n) Austria, Styria, Modriach, Großhofen, 2008, *Muggia & Hafellner*.	TSB 38873
Caloplaca erodens[3]	(n) Austria, Styria, Admont, Hanstein, 2007, *Muggia*.	TSB 38629
C. variabilis[3]	(n) Austria, Styria, Admont, Hanstein, 2007, *Muggia*.	TSB 38629a
Cystocoleus ebeneus[1]	(n) Austria, Styria, Edelschrott Jurikogel, 2005, *Muggia & Hafellner*.	GZU (L217)
C. ebeneus[1]	(n) Austria, Styria, Seckauer Tauern, Zinkenbachgraben, 2006, *Muggia & Hafellner*.	GZU (L315)
C. ebeneus[2,3]	(n) Slovenia, Pohorje, Sumik, waterfall Veliki Sumik, 2006, *Mayrhofer*.	GZU (L325)

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TABLE 1. *Continued*

Lichen species*	Specimen	Herbarium number (DNA extraction number)
Cystocoleus ebeneus[2]	(n) Austria, Carintia, Stubalpe, Höllgraben, 2006, *Muggia & Hafellner*.	GZU (L337)
C. ebeneus[1,3]	(n) Austria, Styria, Koralpe, Reinischkogel-Massiv, 2006, *Muggia & Hafellner*.	GZU (L343)
C. ebeneus[1]	(n) Austria, Styria, Koralpe, Reinischkogel-Massiv, 2006, *Muggia & Hafellner*.	GZU (L344)
C. ebeneus[2]	(n) Austria, Styria, Koralpe, Sommereben, 2006, *Muggia & Hafellner*.	GZU (L345)
C. ebeneus[2,3]	(n) Austria, Styria, Koralpe, Reinischkogel-Massiv. 2006, *Muggia & Hafellner*.	GZU (L348)
C. ebeneus[1]	(n) Austria, Carintia, Stubalpe, Lichtengraben, 2006, *Muggia & Hafellner*.	GZU (L361)
Dibaeis baeomyces[3]	(n) Austria, Styria, Großveitschtal, Veitsch, 2005 *Muggia & Hafellner*.	TSB 37288
Lecidoma demissum[3]	(n) Austria, Carintia, Koralpe, Seespitz, 2008, *Muggia & Hafellner*.	TSB 38867
Racodium rupestre[1]	(n) Austria, Styria, Steirisches Randgebirge, Koralpe, 2006, *Muggia & Hafellner*.	GZU (L335)
R. rupestre[1]	(n) Austria, Styria, Steirisches Randgebirge, Koralpe, 2006, *Muggia & Hafellner*.	GZU (L336)
R. rupestre[3]	(n) Austria, Styria, Koralpe, Sommereben, 2006, *Muggia & Hafellner*.	GZU (L339)
R. rupestre[1]	(n) Austria, Styria, Steirisches Randgebirge, Koralpe, 2006, *Muggia & Hafellner*.	GZU (L340)
R. rupestre[1]	(n) Austria, Styria, Steirisches Randgebirge, Koralpe, 2006, *Muggia & Hafellner*.	GZU (L341)
R. rupestre[1,3]	(n) Austria, Styria, Koralpe, Straußkogel, 2006, *Muggia & Hafellner*.	GZU (L346)
R. rupestre[1]	(n) United Kingdom, North Devon, Dartmoor National Park, 2006, *Hawksworth*.	GZU (L423)
R. rupestre[1]	(n) Italy, Trentino Alto Adige, Mt. Stelvio National Park, 2006, *Muggia & Hafellner*.	TSB 37932 (L424)
Sphaerophorus fragilis[3]	(n) Austria, Carintia, Stubalpe, 2006, *Muggia*.	TSB 38581

*Numbers indicate samples for which sequence results were obtained by direct sequencing[1], by cloning[2] or by SSCP analyses[3]

ITS1f/2 with the following conditions: an initial heating step of 2 min at 94°C, linked to 6 cycles of 30 s at 94°C, 30 s annealing at 54–48°C, 1 min 30 sec elongation at 72°C. The annealing temperature was decreased 1°C per cycle, followed by 30 cycles denaturation 30 sec at 94°C, annealing 30 sec at 49°C, extension 1 min 30 sec at 72°C, and one final extension step of 7 min at 72°C, after which the samples were kept at 4°C. PCR products were cleaned using Qiaquick spin columns (Qiagen, Vienna).

Both complementary strands of PCR products obtained with the primer pair ITS1f/ITS4 were directly sequenced with the BigDye Cycle Sequencing Ready Reaction Kit (Applera, Austria) according to the manufacturer's instructions. Primers used for the sequencing were ITS1F and ITS4. Sequences were run on an ABI310 automated sequencer (Applera, Austria) and their identity was checked by BLAST searches for similarity in GenBank (Altschul *et al*. 1997).

SSCP

The cleaned PCR products were digested with λ-exonuclease for 1 hour at 37 °C. The digested products were mixed with loading buffer (formamide 95%, 2·5M NaOH, 5% bromophenol blue in deionized H_2O), denatured for 5 min at 98 °C and then chilled in ice for 5 min. The products were run on a 8% SSCP gel (polyacrylamide 2 × MDE, 5 × TBE buffer, 0·1% TEMED, 10% ammonium persulphate, 26·64 ml distilled H_2O) for 24 hours at 400V and 26°C. Fragments were separated using the temperature gradient gel electrophoresis TGGE Maxi System (Biometra, Vienna, Austria), but no temperature gradient was used. The

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bands were visualized by silver staining with the following washing steps: 30 min with fix solution (300 ml 10% acetic acid), three washings in water for 5 min each, 30 min in silver-staining solution (0·1% $AgNO_3$, 37% formaldehyde), 10 sec washing in water, washing in developing solution (3% NaOH, 37% formaldehyde) until the bands become visible, 30 min washing in stop solution (10% acetic acid). A further washing step was performed with the conservation solution (10% EtOH, 13% glycerol).

The bands were cut out from the gel with a sterile razor blade and soaked in an extraction buffer (10mM magnesium acetate, 0·5M ammonium acetate, 1M EDTA, 0·1% SDS, water to 50 ml). The bands were stored at 4°C for 3 days before they were heated at 50°C for 3–5 hours. 40 µl of the solution was taken to clean the DNA: 40 µl isopropanol was added, the solution was stored at −20°C for 2 hours, then centrifuged for 10 min. The pellet was precipitated with further 40 µl EtOH 96% (10 min centrifugation), dried and resuspended in 40 µl of water or 1 mM Tris. An alternative extraction protocol was applied in cases in which the re-amplification of the rather long fragments generated by the primers ITS1f with ITS4 was not successful. In this alternative protocol, bands were soaked in 500 µl extraction buffer, frozen at −80°C for 30 min, heated for 1 hour at 65°C, and stored at 4°C for 4 days. The bands were centrifuged, the supernatant was retained and one volume of isopropanol was added. The supernatant was stored at −20°C overnight. The DNA was centrifuged and washed once with 70% ethanol, dried and re-suspended in 30 µl Tris HCl 10mM.

Re-amplification

The DNA purified from the SSCP bands was re-amplified with primers ITS1f/ITS4 and ITS1f/ITS2. Optimized conditions for re-amplification were as follows.The reaction mix final volume contained 2–5 µl of resuspended DNA from SSCP bands. Phusion polymerase (Finnzymes Oy) was used and the PCR amplifications were performed under the following conditions: an initial heating step of 30 sec at 98°C, linked to 30 cycles of 10 sec 98°C, 30 sec annealing at 53°C (or at 58°C for primers ITS1f/2), 30 sec extension at 72°C, and one final extension step of 7 min at 72°C, after which the samples were kept at 4°C. PCR products were cleaned (as above) and both complementary strands were sequenced as described above.

Clone library construction

PCR products obtained from DNA extracts of *Cystocoleus ebeneus* and *Racodium rupestre* (Table 1) were cloned into pGEM-T Easy Vector System (Promega, Vienna) following the manufacturer's instruction. The ligation was performed overnight and desalted on a 0·025 µm millipore membrane (Bartelt, Graz). Competent *Escherichia coli* cells were transformed using electroporation (Micropulser system; Biorad, Vienna). The transformed cells were re-suspended in 1 ml of 2 × TY liquid medium and plated on LB/Amp^+/IPTG/X-Gal medium. Positive clones were selected by standard blue-white screening. Up to eight positive clones were picked and used for colony-PCRs. PCR reactions were prepared to 30 µl total volume: 3 µl *Taq* polymerase buffer (10 mM Tris pH 8·3/ 50 mM KCl/ 1·5 mM $MgCl_2$/ 50 mg gelatine), 0·1 units of *Taq* DNA Polymerase (Amersham Pharmacia Biotech Inc.), 0·2 mM of each of the four dNTPs, 0·5 µM of each primer. Colony PCRs and sequencing were performed either with the same primer pair used in the original PCR, or with the promoter primers SP6 and T7 of the plasmid, when the amplification with the original primers failed.

Phylogenetic analysis

All the ITS sequences that we obtained were subjected to BLAST searches for similarity in GenBank (Altschul *et al.* 1997). Those sequences recovered for the large set of *Cystocoleus ebeneus* and *Racodium rupestre* covering about 500–600 bp of the ITS fragment were used to construct small phylogenetic hypotheses. These sequences were obtained from clones, direct sequencing of PCR product and from the re-amplified SSCP bands. The most similar sequences retrieved from NCBI were included in the analyses. The alignments were produced using ClustalW (as implemented in BioEdit 5.0.6, Hall 1999) and manually adjusted. The General Time Reversible substitution model (Rodriguez *et al.* 1990) with estimation of invariant sites and assuming a gamma distribution with four categories (GTR+I+G) was used for likelihood calculations. The optimal nucleotide substitution model was estimated with the program MrModeltest v3.7 (J. A. A. Nylander, http://morphobank.ebc.uu.se/mrbayes/) using the Akaike Information Criterion and the hierarchical likelihood ratio test (Posada & Crandall 1998). The Bayesian phylogenetic analyses were performed with the program MrBayes 3.1.2 (Huelsenbeck & Ronquist 2003). The MCMC analysis was run for one million generations, with 6 chains starting from a random tree and using the default temperature of 0·2. Every 100th tree was sampled, and the first 100 000 generations were discarded as burn-in. The burn-in period was determined after testing for stationarity of likelihood values, i.e. by plotting numbers of generation vs. the log probability and checking for the convergent diagnostic PSRF, which approached 1 (Ronquist *et al.* 2005). The resulting consensus trees were drawn with the program TreeView (Page 1996).

Results

A total of 44 DNA extracts (Table 1) were successfully used for the three molecular analyses performed, cloning, direct sequencing and SSCP. Two samples among the fungal-infected lichens (*Parmelia sulcata* TSB 38834, *Phaeophyscia orbicularis* Hafellner

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58567) were excluded due to failure in PCR reaction. For the SSCP analyses we performed DNA extractions from 17 visibly fungal-infected lichens and from 13 lichens that lacked any symptoms of infection (listed in Table 1, the identifications of lichenicolous fungi are given in Table 2). Samples used for SSCP were prepared with the primer pair ITS1f/ITS4 and ITS1f/ITS2 (Table 2). Of these, four samples presented only one single thick band, which we assume belongs to the lichen mycobiont. All other extracts from lichen thalli displayed more than one band. The number and the thickness of the bands varied among the samples. Two to seven bands were recovered for each extract with ITS1f/ITS4, whereas up to 14 bands were obtained with ITS1f/ITS2. Up to nine bands among the thickest and clearest ones were cut from the gel (Fig. 2; Table 2).

Re-amplification of bands extracted from gel pieces was problematic with long fragments of the entire ITS fragments (500–600 bp), but re-amplification of the ITS1 region alone was usually possible in 70% of cases. Preliminary results show that longer soaking of the excised SSCP gel pieces could increase this percentage. The sequences were easily obtained from the strongest bands: in 15 extracts we retrieved the lichen's mycobiont according to BLAST searches, whereas 16 sequences confirmed the presence of other fungi. Among the latter, 12 sequences are related to fungi of poorly determined taxonomic position in Dothideomycetes (Ascomycota) or the Basidiomycota. Four correspond to other lichen species, which could have been present in the lichen thallus as numerous contaminant spores or tiny fragments (Table 2). These usually correspond to lichen species that can be found in the same habitat as the specimens.

Because a large number of specimens of *Cystocoleus* and *Racodium* were available, we were able to obtain additional sequences using direct sequencing of thallus extracts and by sequencing of cloned PCR products. This gave us a way to compare the resolution of SSCP to that of direct sequencing. For these samples, up to seven SSCP bands per sample could be seen, and we successfully re-amplified and sequenced eight separate SSCP bands in total. For three specimens sequences obtained by SSCP were the same as those detected by direct sequencing. This was true for the samples L325, L343 (*Cystocoleus*) and L346 (*Racodium*). In the samples L343 and L346 the same ITS fragments were retrieved by direct sequencing and by sequencing of SSCP bands. However, for the specimen L325 direct sequencing underestimated the number of sequences, and additional sequences were detected by SSCP. For the sample L348 (*Cystocoleus*) the same ITS sequence was found in one SSCP band and in five clones, and a different ITS sequence resulted from sequencing of a further SSCP band. Further ITS fragments of different identities were sequenced from clones of the two *Cystocoleus* samples L337 (3) and L345 (2). In these last three samples, L348, L337 and L345, the SSCP method resolved decisively better than the results obtained by direct sequencing of their PCR products, where no sequence could be obtained due to double peaks in the chromatograms (indices of multiple amplified fragments in the PCR products). These results indicate that SSCP can detect fungi in lichens not observable using direct sequencing of thallus DNA extracts. All sequences of full length ITS fragments, irrespective of the method used to obtain them, were included in the phylogenetic analyses.

Phylogenetic analysis

A total of 37 complete ITS sequences (including those of *Cystocoleus* and *Racodium* mycobionts) obtained from the SSCP (8), cloning (15), or direct thallus PCR (14) experiments exclusively from samples of *Cystocoleus ebenus* and *Racodium rupestre* were analysed. Thirty fungal ITS sequences were added to the dataset after BLAST searches for similar sequences. After a first general analysis including all the sequences obtained (not shown), we identified three main groups of fungal sequences. Each of these were then analysed separately with smaller phylogenetic analyses (Fig. 3). The three smaller phylogenetic trees represent a) uncultured

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TABLE 2. *The single strand conformation polymorphism (SSCP) DNA analyses: the total number of SSCP bands, the number of extracted ones and the identity of the sequences obtained for each sample are reported for those samples analysed by SSCP.*

Lichen species (host)	Known lichenicolous fungus	DNA extraction number	Total Number SSCP bands (no. extracted)		Sequences obtained from SSCP bands.
			ITS1f/4	ITS1f/2	
Acarospora fuscata	*Polycoccum microstictum*	L505	4(4)	7(4)	host; uncult. fungus
Aspilidea myrinii	*Sagediopsis fissurisedens*	L508	4(4)	5(3)	host; other lichen (*Umbilicaria*)
Cladonia furcata	*Tremella* sp.	L506	5(3)	–	host; uncult. Basidiomycetes/ *Filobasidium*
C. pocillum	*Sphaerellothecium cladoniae*	O67	1(1)	7(5)	–
Lecanora polytropa	black fungus	L503	6(3)	11(8)	host
L. polytropa	*Muellerella pygmaea*	O69	4(3)	7(5)	host
L. swartzii	*Sphaerellothecium atryneae*	L507	5(4)	–	melanized Ascomycetes
Lobaria pulmonaria	*Arthonia* sp.	O59	3(2)	7(5)	host; *Tremella* sp.; other lichen (*Lecanora*)
L. pulmonaria	*Tremella* sp.	O61	3(2)	8(6)	host
Lobothallia radiosa	*Lichenostigma elongata*	L510	1(1)	6(2)	host
Pertusaria corallina	*Sclerococcum sphaerale*	O66	4(2)	13(9)	uncult, fungus (Dothideomyc.); other lichens (*Tephromela atra*, *Brodoa intestiniformis*)
Phaeophyscia orbicularis	*Taeniolella phaeophysciae*	O56	3	5(4)	–
Physcia caesia	*Stigmidium pumilum*	O47	1	10(5)	–
Pseudocyphellaria sp.	*Arthonia* sp.	O60	4(1)	9(5)	host
Tephromela atra	*Taeniolella atrocerebrina*	O72	2	–	–
Toninia sedifolia	*Stigmidium tabacinae*	L509	1(1)	13(6)	host
Xanthoparmelia conspersa	*Lichenostigma cosmopolitanum*	O68	2(1)	12(7)	–
Arthrorhaphis citrinella	~	L661	4(3)	–	–
A. citrinella	~	L664	3(2)	–	–
Aspicilia simoensis	~	L676	–	7(5)	host
Baeomyces placophyllus	~	L660	4(4)	–	host; uncult. Sporidiobolales/ Basiodiomycetes
B. rufus	~	L665	3(3)	6(3)	*Rodutorula*; Ascomycetes
Caloplaca erodens	~	L446	2(2)	6(3)	host; *Mycosphaerella*
C. variabilis	~	L447	3(3)	–	–
Cystocoleus ebeneus	~	L325	3(2)	12(8)	host: Dothideomycetes
C. ebeneus	~	L343	3(1)	–	host
C. ebeneus	~	L348	7(2)	11(7)	Dothideomycetes; *Fellomyces* spp. (basidiomyc.)
Dibaeis baeomyces	~	L667	2(1)	–	–
Lecidoma demissum	~	L659	6(5)	–	host; *Capronia*/ uncult. Herpotrichiellaceae
Racodium rupestre	~	L346	3(1)	14(8)	Dothideomycetes
Sphaerophorus fragilis	~	L684	–	14(6)	host; other lichen *Ramalina*

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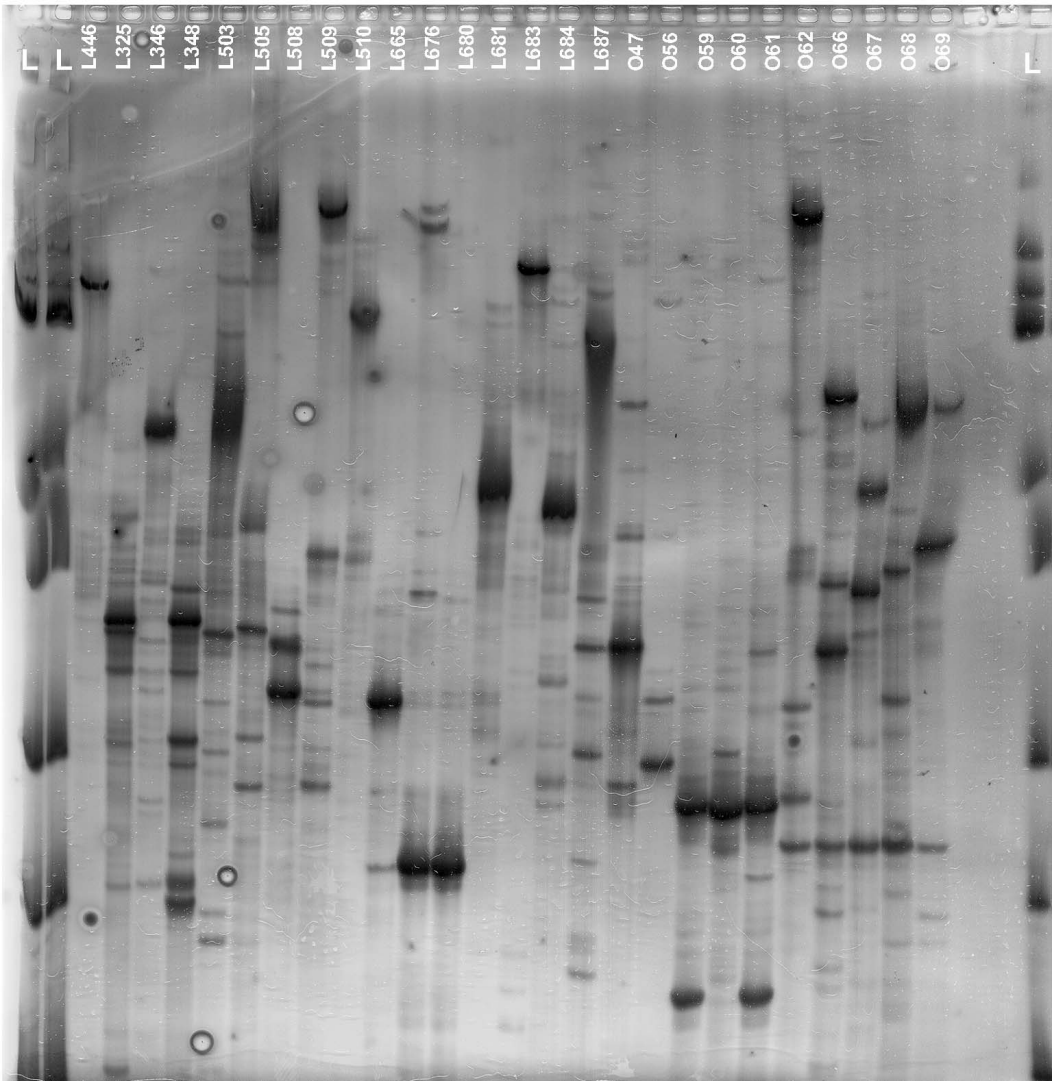

FIG. 2. Single strand conformation polymorphism (SSCP) analysis of fungal ITS of lichen samples. Samples were amplified with the primer pair ITS1f/ITS2. Samples are identified with the DNA extraction numbers. The 1kb molecular marker (L) is used as control of the straightness of the runs.

black fungi and mycorrhizal fungi (Fig. 3A), b) Chaetothyriomycetidae (Fig. 3B), and c) Dothideomycetidae (Fig. 3C). In the last tree, the majority of the ITS sequences was apparently from mycobionts of *Cystocoleus* (Fig. 3C, clade IV) and *Racodium* (Fig. 3C, clade II). The phylogenetic hypothesis of Fig. 3C is consistent with the results recently presented by Muggia *et al.* (2008), who showed the phylogenetic position of the lichenized filamentous fungi *C. ebeneus* and *R. rupestre* in the Dothideomycetes. Four sequences (L348D and L325E Fig. 3C clade I, and L217 and L361, Fig. 3C clade III) obtained from SSCP and directly from amplification and sequencing of two DNA extractions of *Cystocoleus* are rather distinct from the main clades II and IV, and likely

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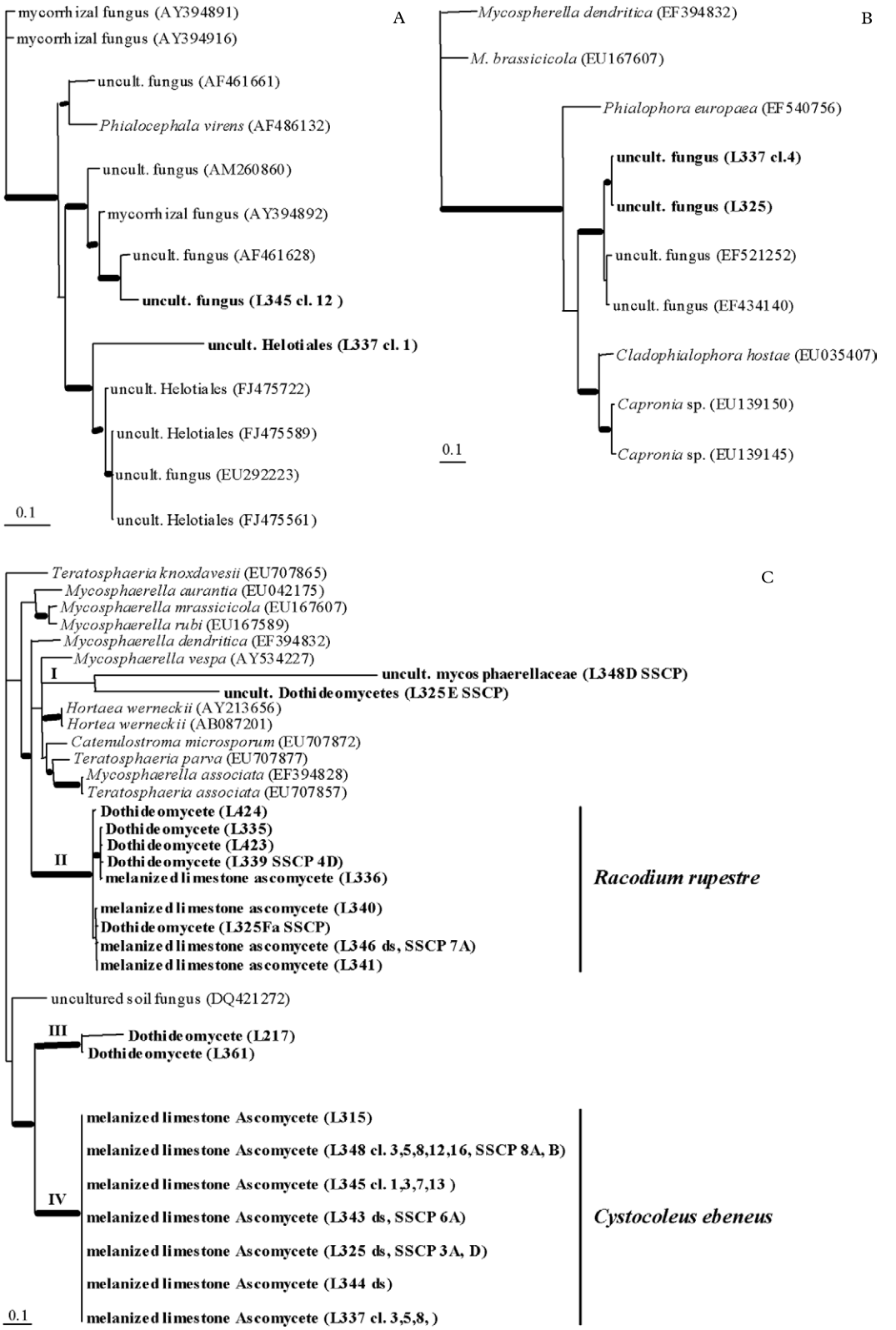

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represent other Dothideomycetes. One additional sequence obtained from an SSCP band of a *C. ebeneus* sample shows high similarity with the basidiomycetous yeast genus *Fellomyces* (Table 2).

Discussion

The microbial composition of complex fungal communities can be investigated by a variety of culture-independent methods (Mitchell & Zuccaro 2006). More recently, multiple throughput sequencing using 454-technology is also becoming popular for this purpose, although this approach requires access to expensive equipment. We have shown here that fragment separation methods such as SSCP are useful for convenient comparisons of multiple samples and for semi-quantitative visualization of abundances in fungal communities. The same ITS fragments can indeed be obtained from SSCP bands or by sequencing of cloned PCR products, but SSCP appears to provide more resolution than direct sequencing. Separation of bands by SSCP permits direct targeting of specific fragments for sequencing.

Large numbers of lichens can be efficiently screened for fungal composition using SSCP. Because SSCP can also provide a direct picture of fungal composition, this approach can also evaluate the effort needed for diversity studies using clone library analyses (Anderson & Cairney 2004). Unlike the short sequences used in SSCP (up to the length of the ITS region), clone libraries can host longer sequences, which are required for higher level phylogenetic studies. Although they are too short for concise phylogenetic studies above the genus level, sequenced SSCP-fragments represent tags for recognition of species or strains. Such short tags are becoming popular in the area of DNA-barcoding approaches, which aim towards a DNA based recognition of species. The resolution power of fungal ITS sequences and the amount of data already available suggest this locus as a prime candidate for a fungal barcode (Seifert 2009). Using SSCP, DNA barcoding approaches can be extended to studying specificity in fungal communities in an ecological or geographical context ("community barcoding"), and also variation at different stages of lichen thallus development.

Previous studies using culture-based methods have shown that lichens host a wide diversity of non-lichen-forming fungi, even when there is no externally visible evidence of them (Harutyunyan *et al*. 2008). Arnold *et al*. (2009) showed that phylogenetically diverse fungi can be cultured after isolation from surface-sterilized lichen thalli, indicating that internal parts of lichens could represent "cradles" of fungal diversification. These fungi seem to be associated preferentially with the algal layers, which somehow recall the biology of higher plant endophytes. The phylogenetic position of most of the fungi suggests that they are neither closely related to known lichenicolous fungi nor to lichen-forming fungal lineages.

These approaches may preferentially detect fungi that grow well under the culture conditions (media, physical parameters), but a significant fraction of uncultivatable biotrophic fungi beside the mycobionts may remain undetected. This is certainly true for lichenicolous fungi, most of which have never been cultured or sequenced.

The presence of more than two fungal SSCP bands from lichens that are infected by a lichenicolous fungus complicates the

FIG. 3. Fungal diversity among the filamentous lichens *Cystocoleus ebeneus* and *Racodium rupestre*. Phylogenetic analysis of ITS sequences from lichen associated fungi; 50% majority-rule consensus trees based on 19001 sampled trees from Bayesian analyses. A, mainly unknown uncultured fungi, B, fungi belonging to Chaetothyriomycetidae, C, fungi belonging to Dothideomycetes closely related to *C. ebeneus* and *R. rupestre*. Thick branches denote Bayesian posterior probability support exceeding 95%. Sequences in bold are those obtained in this study, accession numbers are reported for those sequences retrieved from GenBank. The same sequences obtained from SSPC bands, clones, and direct thallus PCR products are grouped under the same name. Samples are named after the highest similarity match in GenBank.

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assignment of a sequence to the lichenicolous species. The phylogenetic position of most of these fungi has not yet been well studied by molecular data (e.g. Sikaroodi *et al.* 2001). Isolation of lichenicolous fungi was presented for a broad range of lichens (Crittenden *et al.* 1995), but re-infection of the original lichen was rarely tested (e.g. Lawrey 1993, Torzilli *et al.* 2002). Our data on fungal complexity in lichens suggest that lichenicolous fungi may not always form recognizable structures and therefore remain undetected by the collector. Since many lichenicolous fungi are highly adapted to their host biology, we expect that they could be difficult to culture. On the other hand, lichenicolous fungi could perhaps reside in other than their typical host lichens, without expression of fertile structures, or undergoing an otherwise different lifestyle (cf. 'symbiotic lifestyle switching'; Redman *et al.* 2001). Generally the direct sequencing of total DNA extracts from lichen thalli neglect the presence of a large number of other fungi in lichens, unless these are dominating and obscure the sequence signal.

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