

## Fungal Diversity in Rock Beneath a Crustose Lichen as Revealed by Molecular Markers

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### Abstract

Lichen-forming fungi have been assumed to be more or less restricted to the surface of the substrate on which they grow. Conclusive identification of hyphae or an assessment of the fungal diversity inside lichen-covered rock has not been possible using methods based on direct observation. We circumvented this problem by using a DNA sequencing approach. Cores were drilled from a Devonian arcotic sandstone rock harboring the crustose lichen *Ophioparma ventosa* (L.) Norman on the surface. The cores were cut vertically, and DNA was extracted from the pulverized rock slices. A series of polymerase chain reactions using fungal-specific primers as well as *Ophioparma ventosa* specific primers were employed to amplify the internal transcribed spacer region of the nuclear ribosomal DNA. The results show that hyphae of *O. ventosa* penetrate approximately 10–12 mm into the rock. Consequently, the hyphal layer formed by the lichen fungus inside the rock could be 7–12 times as thick as the symbiotic thallus at the surface of the rock. In addition, eight non-lichenized fungal taxa and five that could not be identified to species were encountered. One fungal species in the order Helotiales occurs in six of the eight cores. The significance of these results to the colonization and weathering of rock by lichenized fungi is discussed.

### Introduction

Lichen-forming fungi are particularly successful in habitats that are inhospitable to many other groups of organisms, and they are found in abundance on, e.g., tree bark, bare rock, or various anthropogenic substrates [2, 20]. In fact, nearly all rock surfaces, at least in northern temperate and

Arctic Europe, are more or less covered in lichens. The vast majority of the known lichen species are crustose in growth form, i.e., the symbiotic thallus forms a more or less continuous crust on the substrate. Most of the remaining lichens either take a foliose (“leafy”), fruticose (“shrubby”), or an intermediate growth form. Until now, it has been assumed that crustose lichens either cover the surface of the substrate (“epilithic,” “epiphloeodic,” or “epixylic” lichens depending on the substrate) or that they are restricted to the uppermost few millimetres of the substrate on which they grow (“endolithic,” “endophloeodic,” or “endoxylic”) [14, 15]. However, there are a number of observations of fungal hyphae occurring inside rock covered by epilithic or endolithic lichens [3, 4, 6–8, 11, 13, 18]. These observations come from applications of scanning electron microscopy (SEM), but using this technique alone, it has not been possible to ascertain whether the hyphae belong to the lichen fungus or to any other fungi potentially present in the rock, largely because individual fungal hyphae are difficult to trace through rock. The purpose of this study was (1) to measure the depth to which an epilithic, crustose lichen fungus can penetrate into rock, using an accurate identification method, and (2) to assess the extent of the fungal diversity inside the rock beneath a lichen thallus. We circumvented problems in direct observations of fungal hyphae by the use of molecular markers.

### Methods

**Preparation.** We selected a crustose epilithic lichen, *Ophioparma ventosa* (L.) Norman (order: Lecanorales *sensu lato*), as a model [16]. Analyses of the lichen–rock interface of different lichen taxa in Norway have shown that fungal hyphae are more abundant and penetrate deeper (max 3 cm) into the weathering beneath *O. ventosa* compared to other crustose epilithic lichens growing on the same bedrock [8]. Furthermore, *O. ventosa* has no close relatives in the area, which is an advantage when

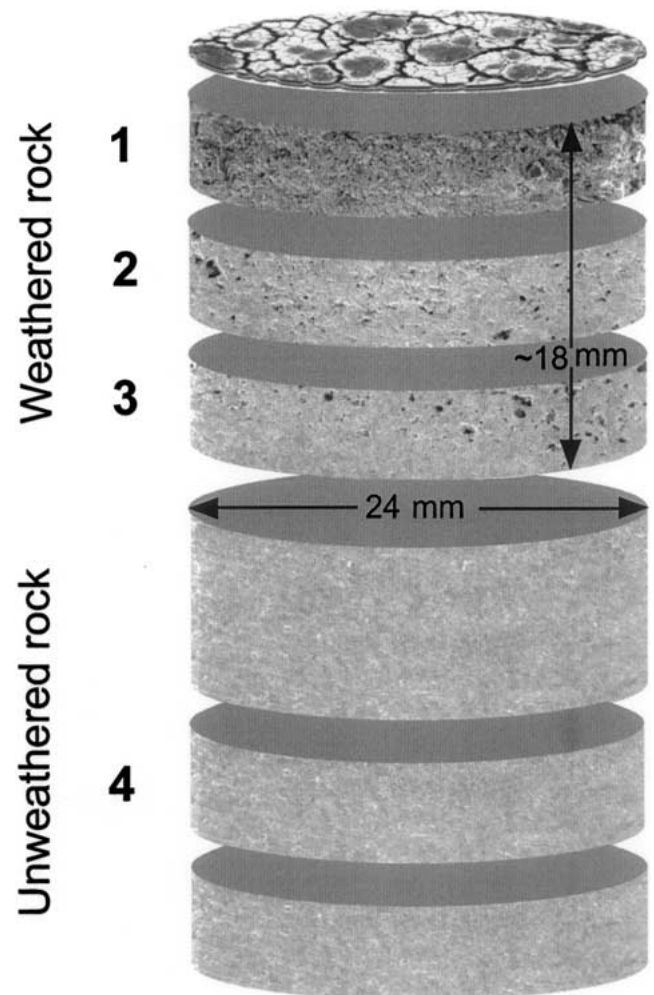
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attempting to identify it by means of molecular markers. *O. ventosa* should thus be a suitable model species for this project. This competitive species is common on hard, acidic, windswept rocks in northern Europe. Samples of rock harboring *O. ventosa* on the surface were collected from the west coast of Norway (Vingen, 61° 50'N 05° 20'E). The bedrock in this area is a Devonian arcose sandstone, a metamorphic sandstone occurring in scattered locations along the coast of Norway. The unweathered sandstone has zero porosity and consists mainly of quartz (45–55%) and feldspar (20–45%), and, in addition, muscovite, chlorite, epidote, and other accessory minerals [8]. The grains, which are cemented by calcite, vary in size from medium (500–250  $\mu\text{m}$ ) to fine (250–125  $\mu\text{m}$ ). Weathered sandstone has a porosity between 14% and 20% in the upper part, decreasing downward in the rind to 5–12%. The porosity reflects the degree of dissolution of minerals in the rock (total dissolution of calcite, partly dissolution of chlorite and plagioclase).

Eight cores (diameter 24 mm) were drilled, each from a single lichen thallus. The cores were air dried and then stored in a freezer prior to DNA extraction. The *O. ventosa* thallus, which had an average epilithic thickness of 1.0–1.5 mm, was removed from the top of the cores. Each weathering rind (~20 mm) was cut vertically into 5 mm thick slices (Fig. 1). The cores were wet sawn in distilled water. Approximately 1.0–1.5 mm of rock was lost between each slice during sawing. The three slices from the weathering rind from each of the cores were used for subsequent DNA extraction.

**DNA Extraction, PCR Amplification, Sequencing, and Editing.** Total DNA was extracted from apothecia (fruiting-bodies) of an *O. ventosa* lichen thallus (for reference DNA) with the DNeasy Plant Mini kit (Qiagen) and from the pulverized rock slices with the FastDNA SPIN Kit for Soil (BIO 101). The apothecia were not from any of the core samples but from another thallus in the area. We followed the manufacturer's protocol, except that for the DNeasy Plant Mini kit the extraction buffer (AP1) was allowed to work for 20 min instead of 10 min at 65°C and that DNA was eluted with 50  $\mu\text{L}$  instead of 200  $\mu\text{L}$  of water. The FastDNA SPIN Kit for Soil was used in accordance with the manufacturer's protocol, except that we used a sodium phosphate buffer (0.1M  $\text{Na}_2\text{HPO}_4$ , 5% SDS [pH 8.7]) instead of the buffer in the kit, that we used freeze-thaw cycles to lyse cells [28], and that we used a vacuum centrifuge to remove [ $\text{H}_2\text{O}$ ] remnants instead of air drying the SPIN<sup>TM</sup> FILTER.

To avoid problems with contamination between slices arising from the drilling process, the cores were first treated with UV radiation, then cut into slices. We used a new saw blade for this project. Each core was cut from the bottom (unweathered rock) toward the surface (li-



**Figure 1.** Core of Devonian arcose sandstone from western Norway covered by the lichen *Ophioparma ventosa* at the top. The numbers 1–4 indicate slice number in each core, from the surface down. Each slice is 5 mm thick. Approximately 1.0–1.5 mm of rock was lost between each slice during sawing.

chen–rock interface). Between each slice, we cut an unweathered rock sample and then cleaned the saw blade with distilled water. Peripheral parts of the core were removed; i.e., only the central parts of the slices were used. Each slice was ground in a sterile mortar. All tools for rock grinding were flame-sterilized between each sample. In addition, a piece of unweathered rock from the base of each core was extracted and used as an additional negative control. Altogether 32 crushed rock samples were analyzed (inclusive of the 8 negative controls from unweathered rock). Approximately 0.50 g of pulverized rock was used for each extraction.

Each sample was subjected to polymerase chain reaction (PCR) amplification of the complete nuclear ITS1–5.8S–ITS2 ribosomal DNA, which was performed using (1) the primers ITS1F [12] and ITS4 [29] and (2) newly designed *O. ventosa*–specific primers. The internal tran-

scribed spacer (ITS) region of the nuclear ribosomal DNA was selected because it is variable enough in sequence and in length to separate even closely related species [9]. *O. ventosa*-specific primers were used, presumably to identify even small amounts of *O. ventosa* in the samples. Available *O. ventosa* sequences were used to generate the specific primers, and those sequences were performed using the online version of the software GeneFisher 1.22 [http://bibiserv.techfak.uni-bielefeld.de/genefisher]. The forward primer Ov-F is situated in the ITS1 sequence (5'-CCG-AAC-CTC-CCA-CCC-TCT-GCG-TA-3'), and the reverse primer Ov-R is situated the ITS2 sequence (5'-GTT-GTC-TGG-CAG-GCC-CGA-CCT-GA-3').

The PCR cocktail, the total volume of which was 50  $\mu$ L, contained extracted DNA, 2.5 mM Mg<sup>2+</sup> free buffer in the concentration recommended by the manufacturer, 200  $\mu$ M of each of the four dNTPs, 0.7  $\mu$ M of each primer, and 1.5 U of a DNA polymerase, Herculanase (Stratagene). The following PCR cycling parameters were used: a 4-min hold at 95°C followed by six cycles including denaturation at 95°C for 60 s, annealing at 62°C (decreasing 1°C each cycle) for 60 s, and extension at 72°C for 105 s, then 34 cycles with denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 105 s, plus 3 s for each cycle, and a final 10-min hold at 72°C, after which the reaction was soaked at a constant 4°C. The PCR products were electrophoresed in a 1% agarose gel and visualized with ethidium bromide. Single PCR products were cleaned with the QiaQuick Spin kit (Qiagen), multiple products using the QiaQuick Gel Extraction kit (Qiagen). Cleaned PCR products were sequenced with PCR primers using the Big Dye Terminator sequencing kit version 3.1 (Applied Biosystems) according to the manufacturer's recommendations, PCR yield allowing. The final extension products were subjected to automatic sequencing on an ABI 3700 sequencing robot (Applied Biosystems). Sequence fragments were assembled and edited using the software Sequencher 3.1.1 (Gene Codes Corp.) or SeqMan II 4.05 (DNASTAR). All the different sequences were submitted to GenBank, see Table 1 for a detailed list.

**Sequence Comparisons.** Sequences obtained from fungal hyphae inside the rock were compared with the reference sequence obtained from the *O. ventosa* apothecium and with GenBank sequences obtained through a standard nucleotide-nucleotide BLAST (blastn) with default settings [1, 26].

## Results

The PCRs resulted in a total of 34 products, including one from the *O. ventosa* apothecium (Table 2). Three of these products (from samples C2, E2, and F2) were

**Table 1. Species and specimens from which new nucleotide sequences were obtained**

Species	GenBank accession number internal transcribed spacer
<i>Ophioparma ventosa</i>	AY011013, AY612326, AY612327, AY612328
Helotiales I	AY011014
Helotiales II	AY612332
<i>Cladosporium</i> sp.	AY612333
<i>Malassezia globosa</i>	AY612334
<i>Malassezia restricta</i>	AY612335
<i>Mycosphaerellaceae</i>	AY612331
<i>Penicillium commune</i>	AY612329
Saccharomycetes	AY612330

obtained by use of the *O. ventosa*-specific primers. The specific primers always amplified a single product. All negative controls (the extracted rock slices from unweathered rock and the actual negative controls of the PCR reactions) were negative.

Five of the 34 PCR products were too weak for sequencing (from samples A3, F2, and H3), *O. ventosa* sequences were obtained from the apothecium and from 13 of the 24 weathered rock slices. These sequences were 0.4–1.6% divergent.

Six sequences were completely identical (100.0%) to sequences in GenBank. Of these, three were from basidiomycetes: *Malassezia globosa* (Ustilaginomycetes, one PCR product) and *Malassezia restricta* (Ustilaginomycetes, two PCR products). Three sequences belonged to ascomycetes: *Debaryomyces hansenii* (Saccharomycetes), *Penicillium commune* (anamorphic, probably Eurotiomycetes), and *Cladosporium* sp. (anamorphic Mycosphaerellaceae, Dothideomycetes et Chaetothyriomycetes incertae sedis), represented by one PCR product each.

Nine of the sequences were not 100% identical with existing sequences in GenBank. One of the sequences is likely to belong to a member of the Mycosphaerellaceae, as all the top 50 BLAST hits were in the Mycosphaerellaceae. The top 50 hits for each of the remaining eight sequences belonged to species in the order Helotiales (Leotiomycetes). Seven sequences (here named Helotiales I) were identical except that three of them had one extra nucleotide compared to the other four. We chose a conservative approach and considered this difference as variation within a single species. One sequence was very different from Helotiales I and was considered another species (Helotiales II).

## Discussion

Our results demonstrate that most of the fungal hyphae within the rock below thalli of *O. ventosa* belong to this species, and that its hyphae penetrate through, the upper part of the weathering rind to a depth of 10–12 mm into

**Table 2. Fungal diversity hidden inside eight cores (A–H) of Devonian arcotic sandstone rock covered by the lichen *Ophioparma ventosa* from western Norway**

	A	B	C	D	E	F	G	H
1	<i>Ophioparma ventosa</i> (1 + O)	<i>Ophioparma ventosa</i> (1 + O)	<i>Ophioparma ventosa</i> (1 + O)	<i>Ophioparma ventosa</i> (1 + O)	<i>Ophioparma ventosa</i> (1 + O)	<i>Ophioparma ventosa</i> (1 + O)	<i>Ophioparma ventosa</i> (1 + O)	<i>Ophioparma ventosa</i> (1 + O)
2	Helotiales I	<i>Malassezia restricta</i> (O) Mycosphaerellaceae Helotiales I	<i>Ophioparma ventosa</i> (O) Mycosphaerellaceae Helotiales I	<i>Ophioparma ventosa</i> (O) Helotiales I Helotiales II	<i>Ophioparma ventosa</i> (O) Helotiales I Helotiales II <i>Cladosporium</i> sp.	<i>Ophioparma ventosa</i> (O) Helotiales I 1 unidentified <i>Penicillium commune</i>	<i>Ophioparma ventosa</i> (1 + O) Helotiales I	<i>Ophioparma ventosa</i> (1 + O) Helotiales I
3	3 unidentified	Saccharomycetes <i>Malassezia globosa</i>	<i>Malassezia restricta</i>				Helotiales I	Helotiales I 1 unidentified

Results from PCRs with ITS primers (1) and *Ophioparma ventosa* specific primers (O). 1–3 indicates slice number in each core, from the surface down.

the rock. However, the amount of lichen fungus seems to decrease with increasing depth, as subjectively judged from the strength of the PCR products. This means that the thickness of the hyphal layer formed by the lichen fungus inside the rock is at least 7–12 times as thick as the symbiotic (green algal containing) thallus that is visible at the surface of the rock. In other cores carrying *O. ventosa* (not included in this study), the weathering rind containing fungal hyphae is up to 30 mm deep [8].

Although hyphae of *O. ventosa* appear to dominate, an additional 13 species of fungi were encountered in the total of c. 54.3 cm<sup>3</sup> of weathered rock that was investigated. One of the unidentified species of the ascomycete order Helotiales, Helotiales I, occurs in six of the eight cores. This suggests that this is either a common endolithic species or a symbiont or parasite associated with *O. ventosa*. The other unidentified Helotiales species, Helotiales II, occurred in only one of the samples. The BLAST search for similar sequences in GenBank indicates that there is an interesting (but admittedly speculative) possibility that the helotialen fungi are related to groups forming ectomycorrhiza with members of the angiosperm family Ericaceae [19]. As the vegetation in the sampling area includes several species in the Ericaceae, this seems possible.

Two different species within the studied cores have been identified as belonging to the ascomycete family Mycosphaerellaceae (Dothideomycetes et Chaetothyrionmycetes incertae sedis). Many of the species in this family are plant pathogenic fungi [10].

Species in the Saccharomycetes and *Penicillium commune* occur in a very wide range of habitats, and it is consequently possible that they could also occur endolithically. The two basidiomycetes found in the cores were 100% identical with two *Malassezia* sequences in GenBank. Both *Malassezia globosa* and *Malassezia restricta* are known to cause skin diseases, but they have recently also been found in association with soil nematodes in Europe [25].

Recognizing the possibility that the inside of an ordinary rock can sustain a diversity of non-lichenized and lichenized fungi opens up a series of intriguing possibilities. First, rock-dwelling lichen communities, often forming spectacular, complex mosaics on rock, may be shaped by more taxa than previously understood, i.e., not only by competition between lichens, but between lichens and non-lichenized fungi. Furthermore, competition between lichens may extend deeper into the rock than has been thought. It is interesting to note that, in this case, the weathering rind contained no detectable trace of fungal hyphae from neighbouring lichen thalli belonging to *Fuscidea cyathoides* (Ach.) V. Wirth & Vězda, *Lecidea* sp. Ach., *Pertusaria corallina* (L.) Arnold, *Porpidia macrocarpa* (DC.) Hertel & A. J. Schwab, or *Rhizocarpon geographicum* (L) DC. Second, we normally assume that lichen colonization takes place from above, either through the relichenization of ascospores of the lichen

fungus or by lichenized vegetative propagules of the lichen thallus, e.g., soredia, isidia, or simple thallus fragments. However, with hyphae of the lichen fungus firmly settled at considerable depths, the possibility of colonization (or regeneration) from below cannot be ruled out, assuming that cells of the correct photobiont species become available at the rock surface. Lichen mycobionts are known to temporarily associate with suboptimal photobionts in the absence of the optimal photobiont [21]. As poikilohydric microorganisms, lichen mycobionts and photobionts can survive extreme temperature stress periods unharmed in a state of dormancy [17]. Third, biologically mediated weathering of rock [5], caused by, i.e., lichen-forming fungi [22–24 and references therein], and other fungi [27], is likely to be a more complex phenomenon than expected because of the number of fungal species involved and the depth to which they can penetrate.

This study has some limitations. For example, it is not possible to compare the fungal diversity in weathered rocks covered by lichens and those without lichens, as there are no lichen-free reference surfaces in the study area. Furthermore, in the experiment presented here, a single species was investigated, and we do not know whether the fungi encountered in this study are restricted to *O. ventosa*. Finally, the PCR method we used allows for positive identifications only. A negative PCR result does not necessarily indicate absence; it either means that fungi are absent or that they occur in amounts too small to be detected. In addition, quantification is not possible. Further studies of the biodiversity of rocks with molecular markers are needed to assess the generality of our observations. In particular, a quantitative PCR approach would allow a more exact quantification of the amounts of DNA present in the rock.

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