RESEARCH ARTICLES

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A protocol for PCR in situ hybridization of hyphomycetes

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Summary A protocol for application of Polymerase Chain Reaction (PCR) in situ hybridization for the detection of hyphomycetes is presented. The experiments are exemplary carried out with strains of the genera *Penicillium* and *Cladosporium*. The small ribosomal subunit is amplified in situ by PCR using fungal specific primers. The amplicon is used as target region for a fluorescein-marked probe. The permeability of the fungal cell wall for the primers and the probe can be successfully achieved by enzymatic treatment with β-glucanase. The protocol can be used as a basis for further development of in situ hybridization with taxon specific probes.

Key words Hyphomycetes · In situ hybridization · In situ PCR · Rock inhabiting fungi · Fungal ecology

Introduction

Within the last years the focus of mycological studies has shifted emphasis from mere taxonomic studies to more ecological questions. To understand the mechanisms of fungal interactions with other organisms and their pathogenicity [4, 6], as well as their influences on geological processes as e.g. their potential to destroy rock material [11], or to develop brightly colored patinas on monumental surfaces [14], it is necessary to analyze fungal communities in their natural habitats. Such investigations are often greatly hampered by the following factors: (i) isolation and viable cell count does not allow a reliable statement about the quantitative abundance of a fungal species in the natural habitat because they are multicellular and sometimes heavily sporulating organisms; (ii) not all fungal species can easily be isolated by the currently available methods; and (iii) in culture, slow growing fungi are often overgrown by fast growing ubiquitous species with minor ecological importance. The last point is of special interest, because some important black yeasts, including human pathogenic species as e.g. *Hortaea werneckii*, the causative agent of human tinea nigra [4, 5], and those deteriorating monuments and works of art [10], belong to this slow growing group of fungi [15]. For this reason, in addition to the classical approach of isolation and cultivation, there is increasing need for methods that allow analysis of fungi in their environment. Currently applied fluorescent staining techniques [9] and immunochemical techniques [3] have only limited specificity, and are thus inappropriate to distinguish different taxa in field samples.

Bacteriologists developed the method of in situ hybridization as a technique that allows DNA and RNA targeted and thus taxon-specific staining of bacteria in field samples (for an extensive reference list, see [1]). This technique so far was only applied to bacteria and some yeasts [7], but was not appropriated for the special requirements of hyphomycetes, with rigid cell walls hampering probe entry and with a wide range of secondary metabolites possibly masking the probe signal by their own fluorescence.

In this study we develop a protocol for in situ hybridization of hyphomycetes as a basis for further application of this method in the field of mycological and ecological studies.

Materials and methods

The variables for in situ hybridization and for PCR were changed several times throughout the experiments. However, only the variables that yielded best results are documented here. In situ hybridization was also tested with paraffin embedded mycelia, but because this procedure did not yield satisfactory results [10] it is not documented here.

Organisms and cultivation 30 isolates of hyphomycetes and black yeasts, including species of the genera *Penicillium*, *Aspergillus*, *Cladosporium*, *Scytalidium*, *Paecilomyces*, *Aureobasidium*, *Trichoderma*, *Alternaria* and *Exophiala*, were used for testing the suitability of the probe and its specificity for fungi. To exclude probe specificity for prokaryotes and algae, PCR reactions were additionally carried out with one strain of *Arthrobacter nicotinae* and the green alga *Chlorococcum* sp. Hybridization experiments were carried out with *Cladosporium cladosporioides*, *Cladosporium herbarum*, *Penicillium citrinum* and *Penicillium frequentans*. All fungal strains had been isolated

from marble and sandstone surfaces in Berlin (Germany) and are maintained in the culture collection of the Geomicrobiology division of the University of Oldenburg. Fungi were grown in liquid cultures with 2% malt-extract medium (pH 7.0) at 18.5°C, filtered on Schleicher and Schüll No. 589 (70 mm) and washed with buffer (20 mmol Tris-HCl, 150 mmol NaCl, pH 7.5). At this point, mycelia may be stored at –20°C until further processing.

Primer and probe design Primers for amplification of 18S rDNA were designed by comparative analysis of 18S rDNA sequences of fungi from the European Molecular Biology Network and synthesized by Roth (Karlsruhe, Germany). The primers can also be used for 18S amplification for subsequent sequencing of the product [12]. For in situ hybridization the forward primer-sequence was labeled with fluorescein (excitation wavelength: 490 nm; emission wavelength: 520 nm) at the 5'-end (Roth).

Primer pair for PCR reactions:
    Forward: 5'>3'   AccTggTTgATccTgccAg
    Backward: 5'>3'   ATccTTccgcAggTTcAcc

Probe for in situ hybridization:
                 5'>3'   *AccTggTTgATccTgccAg

Glucanase treatment To facilitate primer and probe entry 50 mg of mycelium were mixed with 6 units of β-1,4 glucanase from *Bacillus subtilis* (Fluka Chemie AG, Buchs, Switzerland) (5 mg in a total volume of 200 µl, pH 6.0) and incubated over night at 55°C.

PCR reactions To test the specificity of primers, PCR reactions were carried out as described below but with purified DNA. DNA was extracted as described previously [12]. Reactions were done with 30 different fungal species, with one strain of the bacterium *Arthrobacter nicotinae* and with one strain of the green alga *Chlorococcum* sp. In situ PCR was performed in 100 µl volumes containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.1% gelatin, 100 pmol of each unlabeled primer, 200 pmol of each deoxynucleotide triphosphate, 2.5 U *Taq* DNA polymerase and 15 µl glucanase treated mycelial suspension. *Taq* DNA polymerase was added directly before starting the first denaturation step. The mixture was amplified by 30 PCR-cycles with the following amplification-cycle profile: denaturation at 94°C for 1 min, annealing of primers at 45°C for 1 min, extension of primers at 72°C for 1 min 30 s. First denaturation step was 94°C for 4 min, last extension was 72°C for 10 min. PCR products were determined on 1% agarose gels (Biozym DNA Agarose) with TAE buffer and visualized with ethidium bromide stain (0.5 mg/ml). Lambda-DNA digested with *Pst*I was used as molecular-weight marker.

In situ hybridization In situ hybridization was carried out in 0.5 ml tubes with 15 µl of glucanase-treated mycelium after in situ PCR. DNA was denatured at 94°C for 5 min. For prehybridization 18 µl of prewarmed (48°C) hybridization buffer were added (5 × SSC; 1% w/v of a 10% stock solution of blocking reagent [Boehringer, Mannheim]; N-lauroylsarcosine, 0.1% w/v; SDS, 0.02% w/v) and the tubes were incubated for

60 min at 55°C. Denaturation was repeated. Hybridization reaction was processed with 0.5 µl of labeled primer at 62°C for 6 h. All reactions were carried out in a Biometra OV1 hybridization oven. Two different control reactions were carried out: samples treated by the same procedure but without adding fluorescent probe and in situ hybridization without previous PCR. All experiments were done in triplicate.

Fluorescent microscopy Fluorescent samples were analyzed using a Zeiss Axioplan with fluorescent light and documented using a Leica M35 camera and Fuji-Chrome Sensia 400 film. A 450–490 nm filter was used and exposure time for photographs was 60 s.

Results and Discussion

From the beginning of this study it was the aim to target the probes against DNA, instead of the more frequently targeted rRNA, because the method was especially developed for rock-inhabiting and medically important fungi, most of which are extremely slow growing, especially in their natural environment. Organisms with low metabolic activity have a rRNA content insufficient for being detected with fluorescent probes [1]. Thus, targeting towards DNA, amplified with in situ PCR, was more promising in the case of those organisms.

PCR reactions with purified DNA from 30 fungal strains, yielded a 1800 bp fragment for all fungi and no amplification product for *Arthrobacter nicotinae* and *Chlorococcum* sp. The results of PCR reactions are shown in Fig. 1. Where amplification
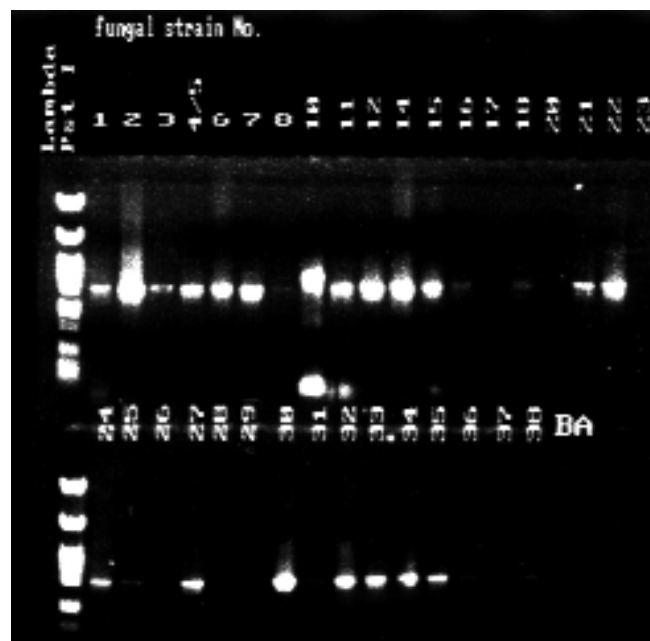

Fig. 1 PCR reactions of purified DNA of 34 fungal isolates, *Arthrobacter nicotinae* (indicated by "B") and *Chlorococcum* sp. (indicated by "A"). The amplification product has a size of 1800 bp for all fungi tested. Repeated amplification did not yield any product for the bacterium or the alga

failed in the first PCR, reactions were repeated and resulted in a 1800 bp product for all fungi. From these results it was concluded that the amplification primers designed with the help of the EMBL databank are specific for a wide range of fungal species. An example of in situ hybridization without previous PCR is shown in Fig. 2A. Mere hybridization without amplification of DNA did not yield any fluorescent signal. From this, the following conclusions were drawn: (i) no signal is caused by autofluorescence of fungal hyphae; (ii) the stringency of hybridization temperature and washing procedures was high enough and no signal is caused by unspecific binding of probe material; (iii) as is was assumed before the experiment, the intracellular content of target DNA or RNA is too low to allow detection without previous PCR.

In situ PCR followed by hybridization with a fluorescent probe resulted in brightly green fluorescent mycelia, examples of which are shown in Fig. 2B–D. Where the signal is especially strong due to overlaying hyphae, the fluorescence appears brightly yellow on the glossy prints presented here. The fluorescence of mycelia is not homogenous throughout the hyphae, which can be explained by local accumulation of DNA in the relatively large fungal cells.

For successful in situ hybridization it is of special importance to achieve entry of the probe, on the one hand, and to preserve the morphology of the organisms on the other hand [2]. As can be seen in Fig. 2A–D, the morphologies of the mycelia of *Penicillium* sp. and *Cladosporium* sp. were not affected by the glucanase treatment. In this context, it is important to notice that the spores of *Penicillium* sp. have a quite good fluorescent signal after in situ hybridization (Fig. 2D), whereas for the *Cladosporium* sp. tested here no signal of the spores was observed. This can be explained by the fact that glucanase treatment was sufficient for the thin walled *Penicillium* spores, but not for the rigid *Cladosporium* spores. From this it can be concluded that the right time and concentration of the glucanase treatment has to be evaluated experimentally for a wide range of fungal species with different cell walls and (because the constitution of fungal cell walls depends greatly on environmental conditions [8]) also for fungi grown under different conditions. The last point is of special importance if the method is applied to field samples with fungi grown under environmental stress conditions.

Using the basic protocol presented here the method can be further developed for genus and species specific detection of
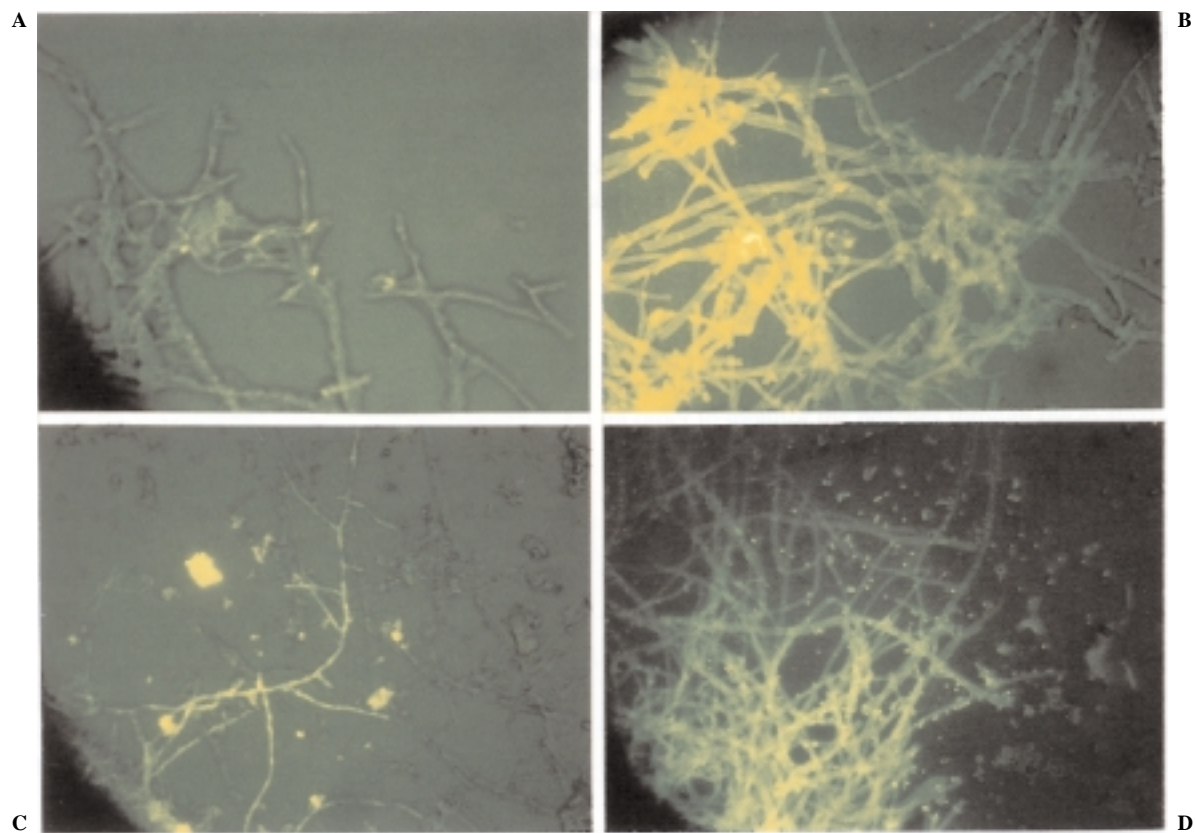
Fig. 2 (A) In situ hybridization without previous amplification of the target region did not give any fluorescent signal. (B) Result of in situ hybridization after amplification of the target region. The hyphae of *Penicillium* sp. show a brightly green to yellow fluorescence. (C) The morphology of hyphae and branching patterns of *Cladosporium* sp. is still clearly visible after the in situ hybridization procedure. (D) In situ hybridization of *Penicillium* sp. showing fluorescence of hyphae and spores

fungi in natural samples as animal tissue, rock, soil and sediment material. Taxon specific probes will be designed based on sequences of medically important black yeasts [4] and microcolonial rock fungi, the 18S rDNA of selected strains was recently sequenced in collaboration with the Centraalbureau voor Schimmelcultures (CBS, Baarn, the Netherlands) [13].

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