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DIPLOMARBEIT

Titel der Diplomarbeit

The role of fungal nitrogen cycling in soil systems: assessment of fungal diversity and development of a sequence set of fungal nitrate reductases

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„Der beste Weg, sich selbst eine Freude zu machen: zu versuchen, einem anderen eine Freunde zu bereiten.“
Mark Twain

„Der Acker freue sich mit allem, was auf ihm wächst!
Auch die Bäume im Wald sollen jubeln, wenn der Herr kommt.“
Psalm 96 Vers 12

Danksagung

„Von ganzem Herzen will ich dir danken, Herr, mein Gott; dich will ich preisen.“
(Psalm 86 Vers 12)

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Abstract

Fungi fulfil a range of important ecological functions. However, there is poor understanding of soil fungal community diversity and the specific roles of individual phylogenetic groups present in the environment.

Fungal diversity of four different agricultural soils (Maissau, Niederschleinz, Purkersdorf, Tulln) and one grassland soil (Riederberg) was examined by a culture-dependent and a culture-independent approach. Identification of fungi was accomplished by DNA sequence analysis of the ITS/LSU of the ribosomal RNA gene region. A diverse set of 61 different species of mainly *Ascomycota* was cultivated, including *Fusarium* spp., *Penicillium* spp., *Trichoderma* spp., as well as previously undescribed species. In the course of the culture-independent approach, clone libraries were constructed followed by RFLP-analysis. Again, *Ascomycota* predominated all libraries, whereas a highly different list of species was obtained compared to the cultivation approach. *Basidiomycota* occured more often and were distributed over all libraries. Generally, all clone libraries differed in their fungal community composition from each other. Species richness estimator Chao2 analysis of all clone libraries revealed a good coverage of the expected fungal “species” richness from agricultural soils. Only 8.6 % of the identified “species” were detected by both methods. The majority of “species” (62.6 %) was detected by the culture-independent procedure, while 28.8 % were exclusively found by the culturing-approach. The analysis clearly demonstrates that both methods are complementary rather than overlapping. The clone library derived from the Riederberg grassland soil harboured the highest diversity and exclusively contained the newly described ascomycetous subphylum Soil Clone Group I (SCGI), maybe suggesting a preference of SCGI-fungi for undisturbed sites. The Maissau clone library showed the lowest species richness, and other specific characteristics contrasting with the other clone libraries. The second part of the study dealt with the exploration of the role of fungi in N-transformation processes. Therefore, fungal nitrate reductase genes (*niaD*) were investigated by the generation of a set of partial *niaD* sequences from isolates as well as from uncultured soil fungi. This knowledge will facilitate the monitoring of transcriptional activities of fungal populations expressing the *niaD* gene under different environmental settings. Additionally, fluorescence *in situ* hybridisation (FISH) was performed using the model organism *Aspergillus nidulans*. During a preliminary test, the optimisation of FISH conditions for the detection of *niaD* mRNA was attempted. Our promising results form the basis for future studies, dedicated to the investigation of fungal *niaD* expression under different conditions.

Preface

The study presented here is part of the “Nitro-Genome”-project, aiming at opening the microbial “Black-Box“ in respect to nitrogen (N) cycling in agricultural soils to finally improve N fertiliser efficiency. Soil still represents a “Black-Box“, since processes (in regard to N-transformation) within biotic soil components (plants, fungi and bacteria) and their interaction with the geochemical parameters are still under questions, and are targeted by an interdisciplinary team of researchers within this project. Accordingly, a better knowledge about these processes will provide the chance of a targeted manipulation of microbial communities. New insights into the mechanisms how N distribution and compartmentalisation are regulated will help to develop strategies to optimise N fertiliser efficiency in the model-plant barley.
In order to study these relationships and processes, a diverse set of analyses is required. The analyses involve chemical measurements of fluxes[1] and gas emissions as well as biological parameters like transcription of functional genes. For this, a variety of chemical, biochemical, genetic and molecular biological tools are applied to investigate the different aspects of microbes, plants, water, air and soil (organic and inorganic fractions) (Inselsbacher et al. 2007).

The work performed in the course of this diploma thesis deals with the examination and comparison of fungal biodiversity in five agricultural soils representing contrasting textures, pH, carbon- and N contents. Furthermore, the exploration of fungal nitrate reductases and their transcriptional activity under certain conditions were of particular interest for the project.

[1] Movements of elements and compounds within and among the biospheric, hydrospheric, pedospheric, atmospheric, and lithospheric fractions (for more information on biogeochemical cycles see Bolin and Cook 1983 or Winstanley et al. 2000)

1. Introduction

1.1. The nitrogen cycle

Nitrogen (N) can be found in nearly all of the macromolecules that are essential for structure and function of all living organisms. In nature, nitrogen exists in a variety of oxidation states. Transformations of nitrogen compounds via redox reactions are mainly carried out by microorganisms (Bock and Wagner 2006).

The N-cycle is defined by the biogeochemical series of conversions of nitrogen compounds. The cycle mediated by the biosphere is summarised in Fig.1.1.

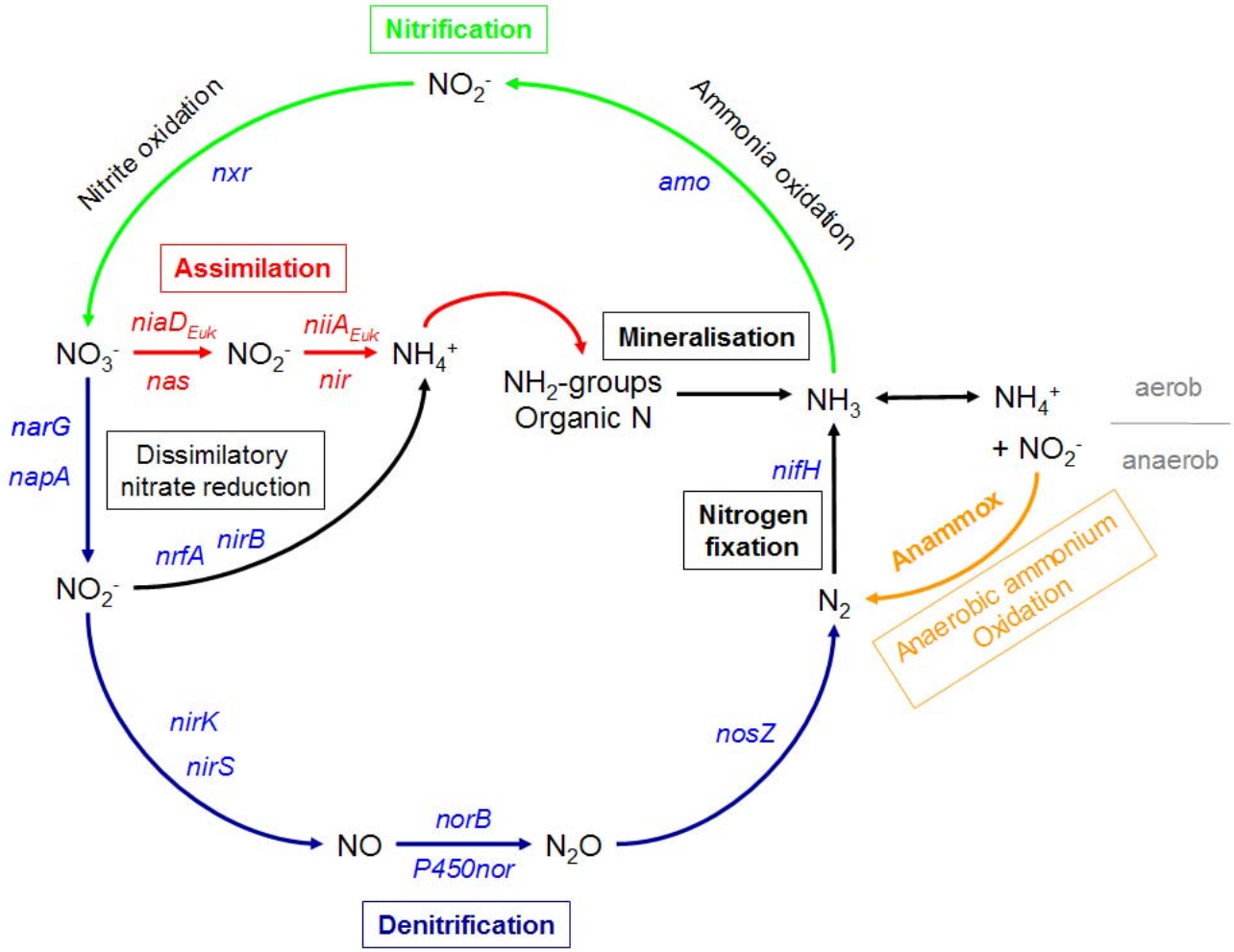


Fig.1.1. Biological nitrogen transformations. Crucial genes and reactions are described in the text below.

1.1.1. Nitrogen reservoirs

Dinitrogen gas (N_2) is the most stable form of nitrogen due to its triple bond. It makes up ~78 % by volume of the earth's atmosphere. Atmospheric N also represents the largest pool of N accounting for 3.9 x 10^{15} metric tons N (~83 % of total global N). Another major N reservoir is the earth's crust (including the entire lithosphere found in either terrestrial or ocean environments), which contributes ~16 % of total N as bound, nonexchangeable ammonium (NH_4^+). Neither of these reservoirs is actively cycled; the N in the earth's crust is unavailable and the atmospheric N_2 must be fixed before it is available for biological use. Other N reservoirs with minor contributions to the total global N pool include the organic N found in living biomass and dead organic matter and soluble inorganic N-salts. These small reservoirs tend to be actively cycled (Maier et al. 2000; Winstanley et al. 2000).

1.1.2. Nitrogen fixation

Diazotrophic bacteria are capable of **nitrogen fixation**, which is the reduction of dinitrogen to ammonia (NH_3) and its further conversion into organic nitrogen. Nitrogen-fixing organisms can exist as independent free-living organisms or in association with other microbes, plants and animals. The best studied system of nitrogen-fixing associations is the rhizobia-legume symbiosis due to its beneficial effect for agricultural crop production. But also many free-living bacteria are known to fix nitrogen: both aerobic (*Azotobacter*, *Beijerinckia*) and anaerobic (*Clostridium*), as well as some actinomycetes and cyanobacteria (Maier et al. 2000).

The energy-intensive reaction (16 – 40 mol of ATP per mol of dinitrogen fixed) is catalysed by the nitrogenase complex consisting of dinitrogenase and dinitrogenase-reductase, which are encoded by the *nif*-genes.

Since the nitrogenase enzyme is irreversibly inactivated by molecular oxygen, obligate aerobic diazotrophs have developed strategies to protect the nitrogenase enzyme: For example, by increasing respiration rate, thereby maintaining low levels of intracellular oxygen (*Azotobacter*) (Fay 1992). Furthermore, many filamentous cyanobacteria (like *Anabaena*), which produce oxygen as a byproduct of photosynthesis, protect their nitrogenase in differentiated cells called heterocysts (specialised cells that create a microoxic environment for nitrogen fixation) (Golden and Yoon 2003).

Industrial nitrogen fixation is achieved by the Haber-Bosch process, whereby dinitrogen is converted together with hydrogen gas (H_2) into ammonia, which is the basis material for the production of nitrogen fertilisers.

1.1.3. Nitrification

Nitrification is the biological oxidation of ammonia to nitrite (NO_2^-) and the subsequent oxidation of nitrite to nitrate (NO_3^-). These aerobic reactions, coupled to ATP synthesis, are performed by two groups of nitrifying organisms: 1. ammonia-oxidising microorganisms (including ammonia-oxidising bacteria, AOB as well as ammonia-oxidising archaea, AOA) and 2. nitrite-oxidising bacteria (NOB). These two types of nitrifiers are generally found together in the environment due to their mutual dependence on each other. The key enzyme involved in bacterial ammonia oxidation is the ammonia monooxygenase (Amo), which oxidises ammonia to hydroxylamine (NH_2OH). The intermediate hydroxylamine is further oxidised to nitrite by hydroxylamine oxidoreductase (Hao). The reactions are given in Fig.1.2. So far it has not been shown whether ammonia or ammonium is the substrate for archeal Amo. Due to chemical equilibrium both compounds are present in the environment, whereby the equilibrium being dependent on pH and temperature (ammonia proportion rises with increasing pH and ascending temperature). Recent studies have shown that AOA are numerically abundant and transcriptionally active in terrestrial and marine environments (Könneke et al. 2005; Treusch et al. 2005; Leininger et al. 2006). However, the relative contribution of AOA to nitrification is still under debate.

Amo: $NH_3 + O_2 + 2\ H^+ + 2\ e^- \rightarrow NH_2OH + H_2O$

Hao: $NH_2OH + H_2O \rightarrow HNO_2 + 4\ H^+ + 4\ e^-$

Fig.1.2. Reactions catalysed by Amo and Hao enzymes of AOB.

Nitrite oxidation, the second step of nitrification, is catalysed by the nitrite oxido-reductase (Nxr[1]) according to the equation in Fig.1.3 (Bock and Wagner 2006). Up to now there are no hints that archaea might be involved in this process.

[1] In the past, the nitrite oxidoreductase enzyme has been abbreviated as NOR, an abbreviation that has also been used for nitric oxide reductase. To eliminate this confusion, a new abbreviation for *n*itrite oxido*r*eductase, NXR was proposed by Starkenburg et al. (2006).

$$\text{Nxr:} \quad NO_2^- + H_2O \rightarrow NO_3^- + 2\,H^+ + 2\,e^-$$

Fig.1.3. Reaction catalysed by the Nxr enzyme of NOB.

Chemo-lithoautotrophic[1] nitrifiers use the energy produced by the oxidation of ammonia (*e.g. Nitrosomonas*) or nitrite (*e.g. Nitrobacter*) to fix carbon dioxide (CO_2). In addition to autotrophic nitrifiers, various heterotrophic bacteria, fungi and algae are capable of heterotrophic nitrification (Bock and Wagner 2006). **Heterotrophic nitrification** is the oxidation of reduced nitrogenous compounds (either ammonium or organic N) while organic carbon is utilised as carbon and energy sources (Castignetti and Hollocher 1982; McLain and Martens 2006). Many heterotrophic nitrifiers also release N_2O aerobically, whereby nitrite and nitrate are used as electron acceptors and reduced to N_2O (McLain and Martens 2006). The environmental importance of heterotrophic nitrifiers is controversial in the literature (Bock and Wagner 2006).

Nitrification in agricultural soil may lead to the loss of mobile soil nitrogen through leaching. Unlike ammonium, which adsorbs well to clay particles of soil owing to its positive charge, nitrate is highly mobile and transferred to the groundwater (Bock and Wagner 2006).

1.1.4. Anammox

Ammonium oxidation was for a long time considered a strictly aerobic process. Recently, the microbiology of anaerobic ammonium oxidation (anammox) was discovered (Strous et al. 1999). Thereby ammonium is oxidised by nitrite forming dinitrogen gas (Fig.1.4). This kind of chemical reaction, in which two compounds consisting of the same element with different oxidation states are reduced and oxidised, respectively, is referred to as comproportionation. Anammox is of particular importance in the marine environment and may be responsible for up to 50 % of the global removal of fixed nitrogen from the oceans. The anammox process takes place in a unique cell compartment, the anammoxosome, which contains ladderane lipids in its membrane. These lipids confer unusual impermeability to the membrane, therefore preventing diffusion of the reactive intermediate hydrazine from the anammoxosome. Anammox bacteria have been found in marine sediments and

[1] obtain energy from the oxidation of inorganic compounds, and carbon from the fixation of CO_2

wastewater treatment plants, including three described genera belonging to the *Planctomycetales*: *Brocadia*, *Kuenenia* and *Scalindua* (Dalsgaard et al. 2005; Strous et al. 2006).

$$NO_2^- + NH_4^+ \rightarrow N_2 + 2\ H_2O$$

Fig.1.4. Equation of the anammox process.

1.1.5. Denitrification

The elimination of fixed nitrogen from soil is achieved by denitrification, the reduction of nitrate via nitrite, nitric oxide (NO) and nitrous oxide (N_2O) to dinitrogen maintaining the global environmental homeostasis. Denitrification is initiated by the nitrate reductase. In contrast to assimilatory nitrate reduction, the respiratory transformation of nitrate or nitrite to a gas species occurs concurrently with energy conservation.

The genes for denitrification encoding functions for nitrate respiration (*nar*), nitrite respiration (*nir*), nitric oxide respiration (*nor*), and nitrous oxide respiration (*nos*) are often assembled in clusters. Of environmental importance is the constant increase of nitrous oxide in the atmosphere over the past few decades. In addition to other anthropogenic nitrous oxide sources, fertiliser denitrification is thought to contribute significantly to this increase (Zumft 1997; Fan and Haruo 2004; Groffman et al. 2006).

Until 1991, denitrification was thought to be a sole bacterial process. Nevertheless, over the years, many fungi of the group of *Fusarium* and its teleomorphs were shown to be capable of partial denitrification (Ferguson 1998).

Kobayashi et al. (1996) demonstrated that nitrate respiration occurs in fungal mitochondria, where the reduction of nitrate via nitrite to nitric oxide is catalysed by the membrane-bound nitrate reductase (Nar) and nitrite reductase (Nir). These enzymes are distinct from assimilatory nitrate and nitrite reductases (Takaya 2002). Denitrification by fungi is coupled with ATP synthesis through the respiratory chain at low oxygen (O_2) conditions (Yanai et al. 2007). Nitric oxide produced by mitochondrial Nir is further reduced to nitrous oxide by cytochrome P450nor. Depending on the P450nor isoform, this reaction is either catalysed in the mitochondria (P450norA) or in the cytosol (P450norB) (Takaya 2002; Zhang and Shoun 2008). Whereas nitrous oxide was the major product of the reduction of nitrate or nitrite by fungi in a number of studies (Usuda et al. 1995; Tsuruta et al. 1998;

Laughlin and Stevens 2002; Crenshaw et al. 2008), the formation of dinitrogen was also observed in some fungi species (Shoun et al. 1992; Tanimoto et al. 1992).
Fungal denitrification is of ecological significance since nitrous oxide, which is a potent greenhouse gas along with carbon dioxide (CO_2) and methane (CH_4) (Zumft 1997), is the dominant gaseous end product. Furthermore, due to the ability of fungi to perform denitrification and oxygen respiration simultaneously in a range of oxygen-stress conditions, there is the potential for fungi to produce nitrous oxide in a wider range of soil aeration conditions than bacteria, which in general need anaerobic conditions to denitrify (Laughlin and Stevens 2002). In addition, a denitrifying bacterium which reduced nitrates micro-aerophilically has been reported by Robertson and Kuenen (1992).
Fungi are widely distributed and often dominate the microbial biomass in soils (Crenshaw et al. 2008). Therefore, the potential for fungi to contribute significantly to the global nitrous oxide budget may not be neglected. Studies have been conducted to determine and compare the contributions of fungal versus bacterial communities to nitrous oxide production in soils and revealed a fungal dominance (Laughlin and Stevens 2002; Yanai et al. 2007; Crenshaw et al. 2008).

Loss of P450nor in *Fusarium oxysporum* mutants does not effect cell growth under denitrifying conditions (Takaya 2002), which is striking, because the reactive nitrogen radical nitric oxide needs to be removed. NO diffuses across cell membranes and through the cytoplasm, reacting rapidly with diverse targets, particularly iron centres, thiols and superoxide. These toxic effects are therefore suggesting another NO detoxification system. Microbes have evolved a number of mechanisms for coping with nitrosative stress. Enzymes associated with bacterial NO detoxification have been reviewed by Poole, whereby the best understood mechanism for NO detoxification involves the enterobacterial flavohaemoglobin (Hmp) of *E. coli* (Poole 2005). Recently, the fungal gene encoding flavohemoglobin denitrosylase (fhb) has been identified in *Cryptococcus neoformans*. Fhb converts NO to nitrate via a bound nitroxyl (NO^-) intermediate across a broad range of physiological oxygen concentrations (de Jesus-Berrios et al. 2003). As well as there are different pathways for NO disappearance, there are also various ways of NO generation besides denitrification. For example, oxidation of L-arginine results in the formation of NO and L-citrulline, a reaction catalysed by nitric oxide synthase (NOS) described in fungi

and plants (Ninnemann and Maier 1996). Furthermore, during nitrate assimilation (1.1.7), when electrons and oxygen are transferred, NO can be formed as a by-product of the enzymatic steps. The radical is then detoxified via fhb (Thorsten Schinko, personal communication).

1.1.6. Ammonification

Generally, ammonification refers to any chemical reaction that generates ammonia as an end product (or its ionic form, ammonium). In the ecological context, ammonification means the processes by which organic nitrogenous compounds are transformed during decomposition of dead organic matter, releasing ammonia and ammonium (mineralisation). Furthermore, the generation of ammonia/ammonium by respiratory nitrate (or nitrite) reduction is also termed ammonification (Simon 2002; Klotz and Stein 2008). In this way, an electrochemical proton potential across the membrane is generated to gain energy from the proton motive force. The term ‘respiratory’ has therefore a different definition compared to ‘dissimilatory’, whereby the latter refers to the regeneration of reducing power without the generation of a proton motive force (Simon 2002).

In contrast, assimilatory nitrate and nitrite reduction serves in the production of ammonia/ammonium which is incorporated into cell material thus allowing growth with nitrate or nitrite as a nitrogen source (Simon 2002). For detailed definitions and distinction between assimilatory, dissimilatory and respiratory nitrate reduction see (Moreno-Vivian and Ferguson 1998).

The designated assimilatory processes are carried out under both aerobic and anaerobic conditions, while the respiratory and dissimilatory processes of nitrate reduction, nitrite ammonification and denitrification are typical anaerobic processes.

1.1.7. Nitrate assimilation

Nitrate assimilation is the process by which inorganic nitrogen is converted to ammonium, which is finally incorporated into the amino acids glutamate and glutamine that act as primary nitrogen donors for all subsequent biochemical syntheses. The process by which ammonium is incorporated into amino acids is called amination.

Nitrate is a significant nitrogen source for plants and microorganisms. The initial reaction in nitrate reduction is catalysed by the enzyme nitrate reductase (NR).

Prokaryotic NRs belong to the dimethylsulfoxide reductase family with a cofactor distinct from eukaryotic NRs. As in eukaryotes, prokaryotic NR involved in nitrate assimilation (Nas) is localised in the cytoplasm. The expression of the *nas*-gene is induced by a lack of ammonium and the presence of nitrate (Stolz and Basu 2002).
The nitrite produced by NR is further converted to ammonium, which is finally incorporated into biomolecules. The nitrate assimilation pathway of fungi is described in more detail below.

1.2. The role of fungi in soil

Fungi are ubiquitous in nature and play a major role in the re-cycling of nutrients and mineral weathering (Jongmans et al. 1997; Hoffland et al. 2004). They are heterotrophic organisms that obtain their nutrients by absorption. Moulds, which are the central organisms examined in this study, are filamentous fungi, consisting of tubular cells in long, branched structures called hyphae, which form a network referred to as mycelium.
Overall, fungi often dominate in terms of soil biomass and can represent a significant portion of the nutrient pool, particularly in oligotrophic soils. Regarding growth rate, there is high variability among fungi, but as a group they are not as capable of rapid growth as the bacteria. However, some soil genera including *Aspergillus*, *Geotrichum*, and *Candida* have a doubling time of about one hour in pure culture (Maier et al. 2000).

Many regulatory steps in ecosystems are controlled by fungi:
(i) Saprotrophs are decomposers and control the rate at which complex organic matter is returned as simple organic and inorganic nutrients available for uptake by other organisms.
(ii) Fungi act as competitors to bacteria and archaea for simple nutrients (nitrogen, carbon, phosphorus).
(iii) Fungi form mutualistic associations with other organisms resulting in beneficial cooperations due to the exchange of resources. Interactions with algae or cyanobacteria are known as lichens. Symbiotic associations formed with plant roots are called mycorrhizae. Fungi provide nitrogenous compounds, phosphorous, mineral nutrients and water to plants which increases net primary productivity,

whereas the plant provides carbohydrates to the fungus in return. Furthermore, mycorrhizal plants are often more resistant to diseases and to the effects of drought. Mycorrhizae of different types are present in the majority of plant families in a wide variety of habitats including agricultural systems.

(iv) Pathogenic fungi cause diseases and mortality affecting community composition and turnover. Some fungi are used for biological control of unwanted plants (*e.g.* weed), fungi or insects.

(v) Fungi also attend biological soil crusts, meaning soil surface communities within or immediately on top of the uppermost millimetres of soil. Soil crusts consist of cyanobacteria, algae, mosses, microfungi and lichens. Microbiotic crusts have been found throughout the world and in most habitats. They play important roles in the ecosystem, including the modification of soils (influencing *e.g.* roughness, fertility, hydrology, stability). Therefore soil crusts influence soil food webs, nutrient cycling rates, plants and faunal components (Dighton et al. 2005).

(vi) Finally, fungi influence soil structure and aggregation. Through hyphal inter-connection of soil particles and extracellular polysaccharides on the surface of hyphae, soil particles are stabilised into aggregates (Wright and Upadhyaya 1998; Duiker 2006).

1.3. Nutrient acquisition in fungi

For the soil habitat the hyphal/mycelial growth form of fungi is advantageous compared to the unicellular body form of bacteria. Since nutrients are unevenly distributed in soil, fungal hyphae are better adapted to bridge nutrient-poor spots than bacteria. Furthermore, to gain access to recalcitrant substrates *e.g.* vascular plants, penetration is required for efficient decomposition. The hyphal growth form allows penetration and is therefore a crucial feature of filamentous fungi to colonise and utilise the well-protected organic matter (Boer et al. 2005). Moreover, owing to the hyphal organisation, the translocation of nutrients, including nitrogen, is facilitated from spatially separated soil microsites enriched in mineral nitrogen to the place of where it is needed (Dighton et al. 2005).

Decomposition of litter is a major source of nutrients. Essential chemical elements such as nitrogen or phosphorus (P) are often limited, since free inorganic nitrogen or phosphorus undergoes a fast turnover in the soil. This highlights the role of decomposers in recycling of these elements and making them available for primary

producers. For example, fresh leaf litter is first colonised by microfungi, which make use of easily degradable carbohydrates, before decomposition is continued by saprotrophic basidiomycetes. They are responsible for decomposition and mineralisation of recalcitrant compounds. This task is achieved by the activity of a whole set of extracellular enzymes (Colpaert and Tichelen 1996). Since soil is an enzyme-hostile environment, these enzymes need to be very resistant. Proteinase K, for instance, derived from the mold *Tritirachium album* possesses a more efficient proteolytic activity relative to other proteases. It has a wide pH optimum and even works in the presence of strong detergents.

To gain access to the more readily available nutrients inside of plant material, the degradation of cell walls (consisting of lignin, hemicellulose and cellulose) is essential, in the first place. Lignin embedded with cellulose fibrils forms a robust structural framework, which is both physically and chemically resistant to degradation even after death of the plants. In addition to their penetration abilities, fungal decomposers have therefore developed new pathways to degrade recalcitrant structural compounds and in this way gaining access to the nutrient pool waiting inside (Boer et al. 2005).

1.4. Nitrogen metabolism in fungi

Nitrogen is present in all living organisms. It is a component of proteins, nucleic acids and other molecules. Consequently, a constant nitrogen supply is vital and requires control mechanisms to ensure this. Mechanisms regarding nitrogen metabolism and its regulation have been studied extensively in fungi, such as *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Neurospora crassa* (Marzluf 1997).

Fungi can utilise a broad range of nitrogen sources, *e.g.* ammonium and amino acids, which are preferentially used (primary nitrogen sources). However, other compounds, *e.g.* nitrate, nitrite, purines, amides, most amino acids, and proteins (secondary nitrogen sources) can be used when primary nitrogen sources become limited. Is this the case, synthesis of a set of pathway-specific enzymes and permeases is required. The expression of genes of a particular pathway is regulated at the level of transcript steady state. In the presence of preferred nitrogen sources these genes are repressed, whereas utilisation of any of the secondary nitrogen sources involves de-repression and specific induction of genes encoding the

corresponding enzymes. The activation of a particular pathway not only requires this global signal, but also a pathway specific signal which indicates the presence of a substrate (Marzluf 1997). The mechanism of how nitrate assimilation is regulated is best understood in *A. nidulans*.

1.5. The model organism *Aspergillus nidulans*

The filamentous fungus *A. nidulans* belongs to the phylum *Ascomycota* which are the most ubiquitous fungi worldwide. *A. nidulans* is amenable to both classical and molecular genetics. A wide range of mutants suffering from alterations in metabolism, signalling and development is available. The relative easy to manage *A. nidulans* is a favoured model organism to study fundamental physiological processes such as pH regulation, regulation of secondary metabolite production (*e.g.* mycotoxins, antibiotics), regulation of the eukaryotic cell cycle, or nitrogen assimilation (Bernreiter 2005). In addition, already five *Aspergilli* and one *Eurotium* (a close relative of *Aspergillus*) genome sequences are available.

1.5.1. Nitrate assimilation in *Aspergillus nidulans*

Nitrate assimilation is a key process in the global nitrogen cycle with enormous ecological and agricultural significance. Both nitrate and ammonium N serve as good N sources for the whole N assimilating soil “community” (i.e. plant roots, fungi and bacteria). One key problem of efficient nitrogen assimilation by plants and many soil microbes is the fact that ammonia represses the utilisation of nitrate leading to nitrate leaching and loss by volatile N compounds.

The nitrate assimilation pathway is composed of three components: two nitrate-specific transporters (CrnA/B), and two enzymes catalysing the step-wise reduction of nitrate via nitrite to ammonium (NR and NiR).

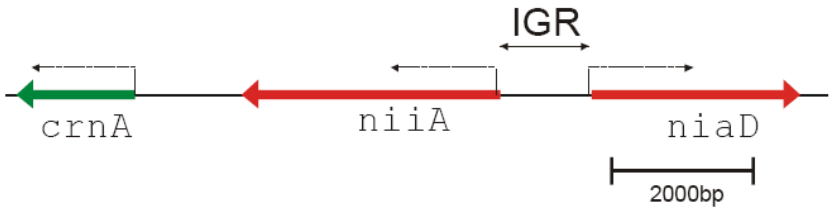


Fig.1.5. The nitrate assimilation cluster in *Aspergillus nidulans*. The genes *crnA*, *niiA* and *niaD* are in close proximity on chromosome VIII. *niiA* and *niaD* are transcribed from the same promoter region (IGR) in divergent directions (Bernreiter 2005).

The genes which code for the components stated above are located in a gene cluster on chromosome VIII (Fig.1.5), except *crnB* (also known as *nrtB*) which is located outside of this cluster on chromosome VIII. Nitrate reductase (*niaD)* and nitrite reductase (*niiA)* genes are transcribed divergently from a common intergenic region (IGR) that serves as a promoter for both genes. The *crnA/B* genes are closely linked to the *niiA* and *niaD* genes; they have, however, their own promoter.

Transcription is induced by the presence of nitrate itself, and de-repressed by the absence of a primary nitrogen source. Two functional regulatory proteins are responsible for the expression of *crnA/B*, *nii*A and *niaD*. In *Aspergillus nidulans* the gene products of these regulatory genes, NirA and AreA are both acting as positive transcription factors by binding defined sequences in the promoter region of their target genes. For further details on the function of the GATA-factor AreA and the nitrate-specific Zn-cluster regulator NirA see (Berger et al. 2006; Bernreiter et al. 2007; Berger et al. 2008).

Nitrate assimilation is well characterised in filamentous fungi. In contrast, the ability of nitrate and nitrite assimilation in yeast is restricted to few species (*e.g. Hansenula polymorpha*) (Siverio 2002), whereas species such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lack the enzyme system to assimilate nitrate or nitrite and are therefore unable to utilise these nitrogen sources (Siverio 2002; Bernreiter 2005).

1.6. The nitrate reductase

Encoded by the *niaD* gene, the nitrate reductase (NR) catalyses the reduction of nitrate to nitrite via the reaction depicted in Fig.1.6.

$$NO_3^- + 2H^+ + 2e^- \longrightarrow NO_2^- + H_2O$$

Fig.1.6 Reaction catalysed by the NR enzyme.

The fungal NR obtains its reducing equivalents from reduced nicotinamide adenine dinucleotide phosphate (NADPH); electrons are then transferred stepwise to flavin adenine dinucleotide (FAD), a cytochrome and a molybdopterin cofactor (Marzluf 1997).

Eukaryotic NR is a multimer and occurrs in either a homodimeric or homotetrameric form with monomers of about 100 kDa (Stolz and Basu 2002).

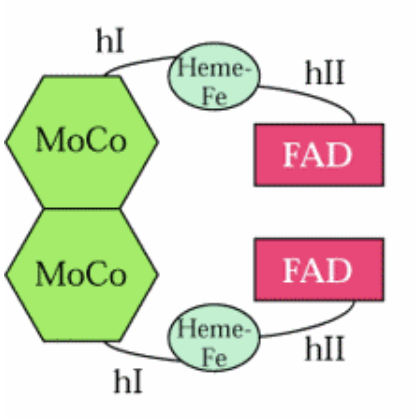


Fig.1.7. Scheme of the domain structure of a nitrate reductase dimer.[1]

The NR monomer is comprised of three functional domains: a flavin (FAD) domain, a heme (Fe) domain and a molybdenum cofactor (MoCo) domain. Short linker sequences, depicted as hinge 1 (hI) and hinge 2 (hII), separate the N-terminal Moco fragment from the cytochrome b5 domain and the cytochrome b5 domain from the C-terminal FAD fragment, respectively (Fig.1.7). In the course of catalysis, electrons are transferred from NADPH to FAD and then shuttled via reduction of the cytochrome b5 heme-Fe to Moco. The reduced Mo atom in the Moco domain then transfers two electrons to nitrate, reducing it to nitrite and water (Eckardt 2005).

Eukaryotic NR belongs to the sulfite oxidase family of mononuclear molybdenum enzymes, whereas prokaryotic NRs are structurally distinct and belong to the dimethylsulfoxide (DMSO) reductase family. Assimilatory NRs of both eukaryotes (NR) and prokaryotes (Nas) are localised in the cytoplasm. However, NRs involved in respiration (dissimilatory nitrate reduction) are membrane-bound. In prokaryotes, NRs are either facing into the periplasm (Nap) or into the cytoplasm (Nar) (Stolz and Basu 2002), while fungal nitrate respiration occurs in mitochondria (Takaya 2002).

1.7. Taxonomy of fungi

Fungi commonly reproduce via spore formation, either in an asexual or a sexual manner, depending on the conditions in the environment. Fungi that reproduce both sexually and asexually often show only one means of reproduction at a specific time point or under specific circumstances (pleomorphic life cycle).

[1] http://www.uky.edu/~dhild/biochem/23/lect23.html

Asexual reproduction via vegetative spores (conidia, mitospores) or through fragmentation of a mycelium is common in many fungal species and allows more rapid dispersal than sexual reproduction. The asexual reproductive stage is called anamorph, whereas the sexual reproductive stage, which is typically a fruiting body, is designated as teleomorph. In sexually reproducing fungi, compatible individuals unite by cell fusion of vegetative hyphae leading to the process of meiosis and formation of meiospores.
According to Article 59 of Chapter VI (Names of fungi with a pleomorphic life cycle) of the International Code of Botanical Nomenclature (McNeill et al. 2006), mycologists are eligible to give asexually reproducing fungi (anamorphs) separate names from their sexual states (teleomorphs).

Primarily, fungal classification is carried out on the basis of structures associated with sexual reproduction, due to the characteristics of this phase of the life cycle which are much more stable and reliable for taxonomic purposes.
However, many fungi reproduce only asexually and therefore are difficult to classify. Fungi which lack a sexual cycle or which are not known to produce a teleomorph were historically classified into an artificial phylum, the *Deuteromycota*, also known as *Fungi Imperfecti***.**
Fungal taxonomy has undergone extensive reconstruction over the last two decades. Important works like *Ainsworth & Bisby's Dictionary of the Fungi* (9th edition, Kirk et al. 2001) and *The Mycota VII* (McLaughlin et al. 2001a, 2001b) represented major advances toward a phylogenetic classification of fungi, but they are already out-dated. Among the most important continuously revised on-line fungal taxonomies is that of GenBank on www.ncbi.nlm.nih.gov/Taxonomy/ (Wheeler et al. 2000) and Index Fungorum (www.indexfungorum.org). Due to inconsistencies and variation in the names between different resources like GenBank, Myconet (www.fieldmuseum.org/myconet), and Ainsworth & Bisby's Dictionary of the Fungi (9th edn: Kirk et al. 2001) biologists considered it necessary to summarise the state of knowledge and to restructure higher-level classifications (Hibbett et al. 2007). Fig.1.8 shows the higher-level classification of fungi according to (Hibbett et al. 2007).

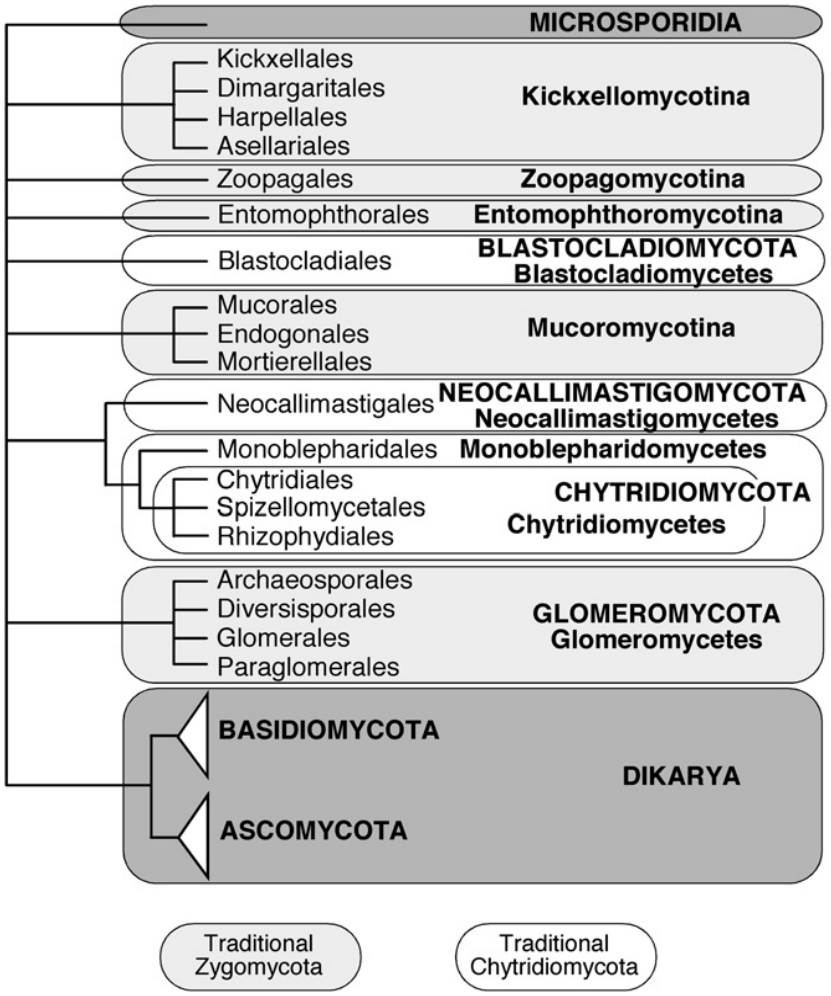


Fig.1.8. Phylogenetic affiliation of *Fungi*. a) Basal fungi and *Dikarya*. Branch lengths are not proportional to genetic distances (Hibbett et al. 2007).

Fungi are one of the most diverse organismic groups on earth. Around 100,000 fungal species are already described; however the actual species number is estimated to be as high as 1.5 million (Hawksworth 2001; Hawksworth 2004). Today, sequences representing 24,295 fungal “species names” are available in GenBank[1].

[1] Data accessed 03rd August 2008.

1.8. The rRNA Operon

Ribosomes are particles responsible for protein synthesis. They are composed of protein (40%) and a special type of RNA called ribosomal RNA (rRNA) (60%). Eukaryotic ribosomes have 80S[1] units composed of a 40S small subunit (SSU) containing the 18S rRNA and a 60S large subunit (LSU) containing three rRNA species (the 5S, 5.8S and 28S rRNAs). The synthesis of rRNAs and the assembly of ribosomal subunits in eukaryotes take place in the nucleolus, a sub-compartment within the nucleus. The genes coding for rRNAs are arranged in the rRNA operons.

1.8.1. Genomic structure of rRNA genes

In eukaryotes, the coding region for rRNA consists of 18S, 5.8S and 28S rRNA genes, which are separated by internal transcribed spacers (ITS) and flanked by an external transcribed spacer (ETS) and a non-transcribed spacer (NTS). A section from one ETS to another represents a repeat unit. The structure of such a repeat unit or rDNA array, as it is also referred to by some authors, is shown in Fig.1.9 (Hillis and Dixon 1991).

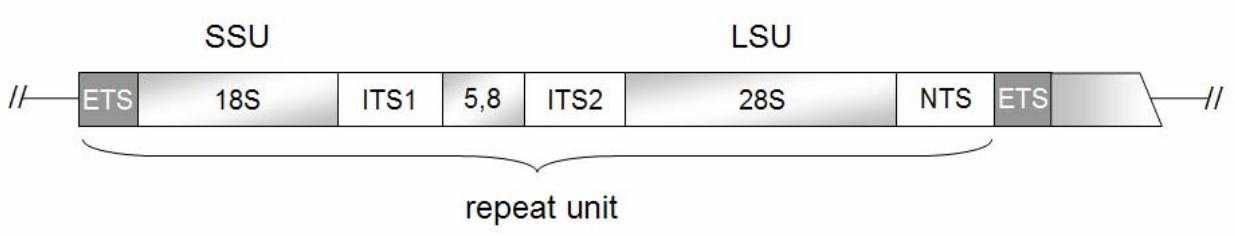


Fig.1.9. Schematic structure of the three rRNA genes and two internal transcribed spacer regions of the rRNA gene repeat unit (modified after (Hillis and Dixon 1991), not to scale). LSU, large subunit; SSU, small subunit.

The region located between the 18S and 5.8S rRNA genes is called ITS1, while the 5.8S and 28S rRNA genes are linked by ITS2 (Hillis and Dixon 1991). The whole section is transcribed without cessation, forming a single pre-rRNA molecule from which internal spacer sequences are subsequently removed by a series of endo- and exonucleolytic cleavage steps producing the mature 18S, 5.8S, and 25S rRNAs. Recent findings in the field of rRNA processing are outlined by (Granneman and Baserga 2005; Schneider et al. 2007; Sirri et al. 2008).

[1] Svedberg units refer to the sedimentation rate in an ultra-centrifuge

Eukaryotic nuclear 5S rRNA genes are relatively independent of the other rRNA genes, concerning both transcription and genomic location. In several *Ascomycota*, like *Aspergillus*, *Neurospora* and *Schizosaccharomyces*, the 5S rRNA genes are dispersed throughout the genome in a complex manner, whereas in other fungi a 5S rRNA gene is embedded into the NTS of the repeat unit (Garber et al. 1988; Belkhiri et al. 1992).

In *Bacteria*, genes encoding 5S, 16S, and 23S rRNAs ("5.8S rRNA" being part of the 23S rRNA molecule) are typically organized in a single operon (Klappenbach et al. 2000), whereby copy numbers can vary from one to 15 per bacterial genome (Rainey et al. 1996).
Bacterial and archaeal sequence-regions analogous to eukaryotic ITS are usually named intergenic spacer regions (ISR) (Gurtler 1999; Ranjard et al. 2001), although some authors (Perez-Luz et al. 2002; Jones and Thies 2007) use the term ITS as well.

1.8.2. rRNA gene cluster copy numbers in fungi

The number of tandemly repeated copies of a eukaryotic nuclear rRNA gene repeat unit (*i.e.* transcription unit and nontranscribed spacer) can vary from only one to several thousands (Hillis and Dixon 1991; Prokopowich et al. 2003). rRNA gene copy numbers can vary from 39 to 19.300 in animals, and from 150 to 26.048 in plants (Prokopowich et al. 2003). Some examples for copy numbers in fungi are listed in Tab.1.1.

Multiple copies of an rRNA unit usually evolve in concert instead of independently of each other. In other words, each copy of an rRNA gene repeat unit is usually very similar to the other copies within individuals and species, although differences among species accumulate rapidly in parts of the array.
The responsible process of homogenisation appears to be unequal crossing over and gene conversion (Hillis and Dixon 1991; Ganley and Kobayashi 2007).

Tab.1.1. Copy numbers of rRNA gene repeat units in fungi.

Fungus (taxonomy[1]: order)	Copy number of the rRNA gene cluster[2]	Reference
Pneumocystis carinii f. sp. *hominis (Pneumocystidales*)	1	(Tang et al. 1998)
Pachysolen tannophilus (Saccharomycetales)	28	(Maleszka and Clark-Walker 1993)
Aspergillus nidulans (Eurotiales)	~ 45	(Ganley and Kobayashi 2007)
Ashbya gossypii (Saccharomycetales)	~ 50	(Ganley and Kobayashi 2007)
Cryptococcus neoformans (Tremellales)	~ 55	(Ganley and Kobayashi 2007)
Kluyveromyces lactis (Saccharomycetales)	68	(Maleszka and Clark-Walker 1993)
Kluyveromyces wickerhamii (Saccharomycetales)	72	(Maleszka and Clark-Walker 1993)
Saccharomyces paradoxus (Saccharomycetales)	~ 90	(Ganley and Kobayashi 2007)
Dictyostelium discoideum (cellular slime mold[3])	~ 100	(Wostemeyer 1985)
Physarum polycephalum (cellular slime mold[1])	~ 100	(Wostemeyer 1985)
Torulaspora delbrueckii (Saccharomycetales)	115	(Maleszka and Clark-Walker 1993)
Candida glabrata (Saccharomycetales)	> 115	(Maleszka and Clark-Walker 1993)
Schizosaccharomyces pombe (Schizosaccharomycetales)	> 115	(Maleszka and Clark-Walker 1993)
Cochliobolus heterostrophus (Pleosporales)	130	(Garber et al. 1988)
Saccharomyces cerevisiae (Saccharomycetales)	~ 140	(Wostemeyer 1985)
Leptosphaeria maculans (Pleosporales)	56 to 225[4]	(Howlett et al. 1997)

[1] according to Taxonomy Browser on http://www.ncbi.nlm.nih.gov/ (Feb, 2008).
[2] per haploid genome
[3] originally (Whittaker's five-kingdom scheme of life), slime molds (*Mycetozoa*) were considered to be members of *Fungi*, but now they are placed directly among the domain *Eukaryota* (Baldauf et al. 1997).
[4] amongst only four field isolates

1.8.3. ITS function

In addition to being non-coding sequences that are physically separating the rRNA genes, ITS structures are thought to be involved in processing the rRNA transcript (Hillis and Dixon 1991; Beiggi and Piercey-Normore 2007).

ITS secondary structures like hairpins interact with protein factors building a complex which functions as a chaperone for ribosome maturation in *S. pombe* involving a complex cleavage pathway (Abeyrathne and Nazar 2005). Although the processing is not yet fully understood in biochemical terms, it is known that the folding pattern of the initial RNA transcript plays a role in guiding processing (Coleman 2007). For further details on pre-rRNA processing, which is well characterised in yeast, see Venema and Tollervey (1995) and Abeyrathne and Nazar (2005).

1.8.4. ITS as a target

A breadth of applications like culture identification, phylogenetic research, direct detection from medical specimens or the environment, and molecular typing for epidemiological investigations use the fungal ITS sequences, pointing to the great potential as target in molecular-based assays for the characterisation and identification of fungi (Iwen et al. 2002). ITS sequences are ideal for species (sometimes even subspecies) identification with sufficient reference data. However, identification of sequences from previously undescribed species is almost impossible if only the ITS sequence is available due to its high sequence variability. As a consequence, a more conserved region (*e.g.* 18S or 28S rRNA genes) is needed for classification.

1.8.5. rRNA genes as phylogenetic marker

Molecules have to fulfil some requirements to serve as a suitable phylogenetic marker: ubiquitous distribution in the living world, functional constancy, selective pressure, genetic stability, sufficient information content, and a database comprising a wide spectrum of phylogenetically diverse organisms.

rRNA genes turned out to be useful for phylogenetic studies. Due to the presence of ribosomes in all living systems (ubiquitous distribution), a high degree of functional constancy and different functional selective pressure resulting in both evolutionary conserved regions as well as highly variable regions within the same rRNA gene have made this molecule the target gene of choice for many applications.

rRNA genes are commonly used for phylogenetic classification and identification of organisms. Islands of highly conserved sequence regions within rRNA genes allow the construction of nearly “universal” primers for amplification by PCR, which in fungi is further facilitated by the usually high copy number of the rRNA repeat unit (Hillis and Dixon 1991; Miller and Huhndorf 2005).

1.8.6. ITS as phylogenetic marker

In fungi the ITS region is currently the most widely sequenced DNA locus for species identification. GenBank stored about 67,000 fungal ITS sequences in 2006, from which approximately two thirds were identified to species level, others remaining insufficiently identified (Nilsson et al. 2006).

Furthermore, the ITS region is particularly valuable for systematics, because of its level of variation, making it suitable for phylogenetic analysis at various taxonomic levels within families, depending on the respective lineage (Anderson et al. 2003).

Due to its species-specific sequence, ITS2 is the most commonly used ITS region (Fig. 1.10). It has already been applied extensively and very successfully for plants, some protistans and a few animals (Coleman 2007).

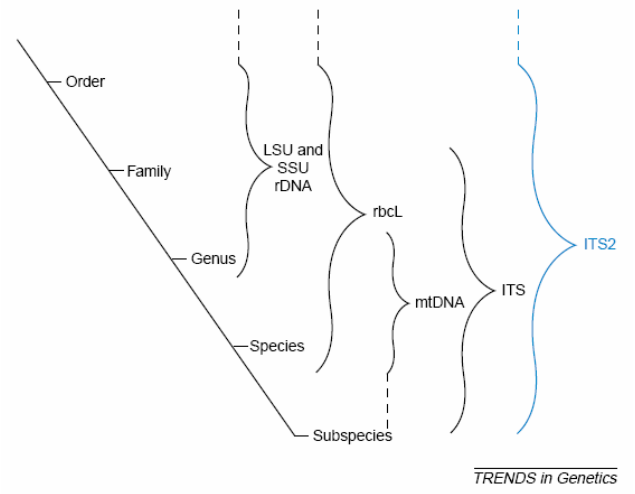


Fig.1.10. “Diagram of the rank order of taxonomic categories. Brackets indicate the approximate range for which DNA sequences commonly utilised for phylogenetic comparisons apply. mtDNA, non-coding regions of animal mitochondrial DNA; *rbcL*, the large subunit of the chloroplast gene encoding ribulose bisphosphate carboxylase” from (Coleman 2003).

1.8.7. ITS secondary structure

Already in 1991 Hillis et al. reported that the major feature of rRNA secondary structure is highly conserved throughout life. Although RNA secondary structure is dependent on its primary sequence, secondary structure maintenance occurs despite

the continued evolution of the primary sequence. This is due to compensatory mutations occurring between the paired nucleotides (Hillis and Dixon 1991).

In particular ITS2 RNA transcript secondary structures have been studied (Coleman 2007) (Fig. 1.11). She explored that, among the examined eukaryotic groups, all share the same ITS2 secondary structure model including the hallmark helices II and III. ITS2 typically has four helices: the most variable helices I and IV are specific to species and subspecies level, whereas the most conserved regions of primary structure are found in helices II and III and their adjacent single-stranded regions (Coleman 2007).

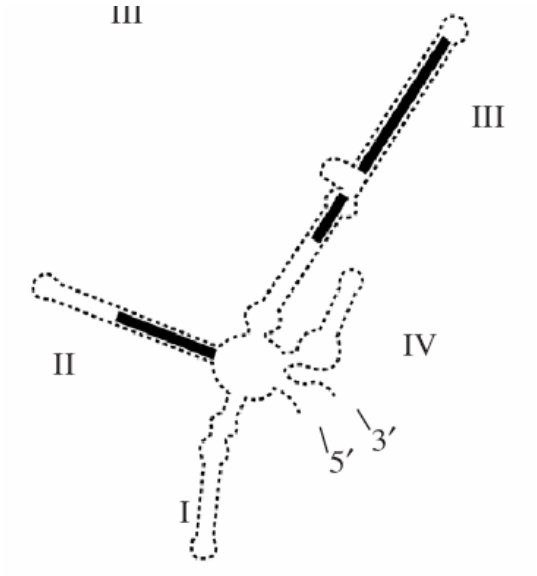


Fig.1.11. Eukaryote-universal ITS2 secondary structure. Cartoon shows with black fill the relatively conserved regions of sequence (Coleman 2007).

Coleman (2007) and Beiggi and Piercey-Normore (2007) are in agreement about ITS regions containing specific subsequences and structures that are important for ribosomal RNA processing.

1.8.8. Fungal identification using ITS (ITS1 and ITS2)

Ribosomal RNA genes have a relatively conserved nucleotide sequence among fungi, *e.g.* 18S rRNA gene sequences generally are only able to resolve taxonomic groups to the genus level (Anderson et al. 2003). However, ITS sequences show variations among species, thus serving as signature regions for molecular assays (Iwen et al. 2002). Many fungal taxonomy studies have used ITS regions for resolving relationships at the species and genus level (Lumbsch 2000; Nugent and Saville 2004).

Apart from the resolution to species level, ITS was selected, because several established universal fungal primers are based on the conserved regions of rRNA genes, making it possible to obtain PCR products from most fungi. Moreover, there is a large number of ITS sequences available in GenBank (Nilsson et al. 2006), which is advantageous for similarity searches (Wu et al. 2003).

Due to concerted evolution, ITS paralogues[1], even when present in high copy numbers, show near uniformity, so that sequencing of pooled PCR products is enabled for many species. In fungi with divergent ITS paralogues, cloning is required, offering multiple estimates of organismic relationships (Baldwin et al. 1995).

1.8.9. The 28S rRNA of the large subunit (LSU rRNA)

The LSU rRNA sequence not only provides large phylogenetic information content, but also contains highly variable regions, called divergent (D) domains or expansion segments (Hwang and Kim 1999). These regions vary in size and sequence among highly divergent eukaryotes, whereas the secondary structures of some domains have remained conserved (Chilton et al. 2003). Generally, the LSU rRNA gene shows more variation than the SSU (18S) rRNA gene (Baroin et al. 1988; Hillis and Dixon 1991; Lumbsch 2000), but less variability than the ITS region (Nugent and Saville 2004; Sonnenberg et al. 2007). Fast evolving portions (with regard to insertions/deletions and substitutions) can be used for comparing species that are expected to be closely related, while conserved regions provide information about relationships at different taxonomic levels (*e.g.* order, family, genus, etc.) (Baroin et al. 1988).

For nearly two decades, the SSU rRNA gene has been used as preferred marker to study eukaryote phylogeny. While the use of the SSU rRNA gene has undergone a magnificent boom, leading to a rich taxonomic representation, LSU rRNA sequences have been less popular for studying phylogeny (Van der Auwera and De Wachter 1998; Lumbsch 2000; Moreira et al. 2007). This is due to variable areas as well as extreme variable expansion segments present in eukaryotic LSU rRNAs, which pose a problem for studying ancient phylogenies. As a result, sequencing has often been limited to a small conserved part of the entire gene (Van der Auwera and De Wachter 1998).

[1] genes that derive from the same ancestral gene after duplication (within the same genome)

Many molecular systematic studies on fungi, *e.g.* (e.g. Hansen et al. 2005; e.g. da Silva et al. 2006; Fernandez et al. 2006), utilise the first few hundreds of nucleotides of the 5′ end of LSU gene, often referred to as partial LSU (rRNA gene/rDNA). This part contains divergent domains (D1, D2, sometimes also D3) which are among the most variable regions within the whole gene (Hopple and Vilgalys 1999; Miller and Huhndorf 2005).

1.8.10. Combination of LSU with other regions of the rRNA operon

In recent years, numerous studies on fungal phylogeny have been carried out by analysing a combination of (partial) LSU and other sequences of the rRNA operon, mainly the SSU, *e.g.* (Miller and Huhndorf 2005; Aime et al. 2006; Binder and Hibbett 2006; Schoch et al. 2006; Spatafora et al. 2006; Wang et al. 2006; White et al. 2006). Recently, some studies on phylogenetic inference (*i.e.* the estimation of history through the proposition and testing of phylogenetic hypotheses) as well as for identification purposes using the ITS and partial LSU region in particular were conducted (Dombrink-Kurtzman and Engberg 2006; Vega et al. 2006; Tsui et al. 2007). Advantages of combining these evolutionary different regions within the rRNA operon are (i) identification to species level using ITS and/or LSU, if corresponding sequences are available in public databases, (ii) higher order identification with no highly homologous reference sequences in public databases using LSU (usage of Blast tree view widget might be helpful), (iii) utilisation of two molecular markers (ITS, LSU) and their databases, and (iv) ease of alignment generation for phylogenetic studies (Vega et al. 2006). An overview of parts of the rRNA gene repeat unit and positions of primer binding sites (of primers applied in this study) are given in Fig.1.12.

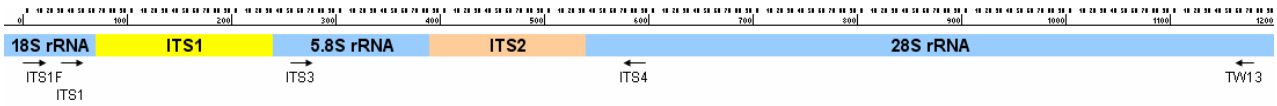


Fig.1.12. Parts of the rRNA gene repeat unit.
Sections can be amplified by PCR through the use of primers indicated by arrows.

1.9. The aims of this work

Yet little is known about the size and components of the fungal network that contributes to ecological functions. Future research is needed to link diversity and function. There is poor understanding of the specific role of fungi in general and the specific roles of individual species for most ecosystem processes in which they participate. This is due to the fact that research often does not differentiate the relative contributions of fungal and bacterial communities. Therefore it is important to identify the individual fungal species and their responses to environmental cues (Dighton et al. 2005).

Environmental conditions in respect to N supply which can be adjusted by different fertiliser applications and fungal responses to these different nitrogen sources are a main issue of the “Nitro-Genome”-project. Fungi are capable to utilise a wide range of nitrogen sources and therefore play an important role in the global N-cycle.

1.9.1. Fungal community composition in agricultural soils

Fungi play a central role in most ecosystems. They are important decomposers and take a notable place in the natural nitrogen and carbon cycles. Despite their relevancy, the vast majority of fungi have not yet been isolated and identified due to the limitations of culture-based methods. Therefore, community fingerprinting approaches are frequently used, providing simple and rapid methods with high sample throughput and the possibility of comparing different environments (Kennedy and Clipson 2003). However, a complete inventory of the fungal species in different kinds of soils is desired, resulting in high-quality data detailing fungal members of the environmental sample.

The characterisation of fungal diversity in five contrasting agricultural soils represents the first step of an extensive investigation which is aimed at exploring the role of fungi in N-transformation processes. Accordingly, description of biodiversity acts as a precondition for the development of a functional gene (involved in nitrogen cycling) microarray and subsequent studies (transcriptional analysis of short-term and long-term responses to different N-treatments). Fungal community composition in agricultural soils and its correlation to specific soil characteristics will provide useful information for environmental microbiology.

For the investigation of fungal biodiversity two different approaches should be applied. On the one hand, filamentous fungi and yeasts will be cultivated, while, on

the other hand, a culture-independent method will be applied by extracting total soil DNA. Identification of fungi present in the soils will be accomplished by DNA sequence analysis of the fungal ITS/LSU region.

1.9.2. Generation of a dataset of nitrate reductase sequences from soil fungi for the development of a *niaD*-microarray

Knowledge on the fungal nitrate reductase (NR) activity under different fertiliser conditions will improve our understanding of the role of fungi in N-transformation processes. To screen a soil sample for its fungal NR activity it is essential to determine the existing NR sequences (*niaD* gene) of fungi present in the soil. Since only a few *niaD* gene sequences are available in public databases so far, the generation of an extended set of *niaD* sequences of known isolates as well as from uncultured fungi from soil should be achieved. This set of sequences serves as a basis for the design of specific probes. Furthermore, a chip equipped with dozens of different *niaD* probes (microarray with a limited set of probes) targeting a diverse group of fungal nitrate reductases will facilitate the monitoring of functional activities of fungal populations expressing the *niaD* gene under different environmental settings.

It is already shown that *niaD* is suitable as a phylogenetic marker (Stolz and Basu 2002). Uncultured fungal species can be assigned to phylogenetic groups from sequences obtained from total soil DNA by calculation of a phylogenetic *niaD* tree. Therefore, a *niaD* array not only could be used to obtain information regarding functionality, but would also allow the determination of phylogenetic affiliation of nitrate-reducing fungi.

A better understanding of nitrate utilisation would provide initial stages for the development of strategies to improve N-fertiliser use efficiency. Increase of N-fertiliser efficiency will result in higher yields and sustainable agriculture which are targeted by farmers, researchers, politicians and society.

1.9.3. Fluorescence *in situ* hybridisation

Fingerprinting techniques as well as the analysis of rRNA genes/ITS regions from environmental samples require PCR amplification of the target gene segments from the total gene pool (Lau and Liu 2007). However, PCR associated biases and variation of rRNA operon copy number in fungi complicates the quantification of

different fungal species in a mixed DNA pool (Anderson and Cairney 2004). For quantification purposes fluorescence *in situ* hybridisation (FISH) is a beneficial technique, which enables the visualisation and phylogenetic identification of microbial cells. Furthermore, additional detection of *niaD* mRNA would allow an assertion about the physiological status of the present fungi. In the course of this the model organism *Aspergillus nidulans* will be used to establish suitable conditions for this method regarding cultivation, fixation and hybridisation parameters.

2. Materials and Methods

Applied chemicals and kits are listed in Tab.2.1.

Tab.2.1. Chemicals and Kits used.

Chemical	Company
2x ReddyMix™PCR Master Mix14	ABgene
6x Loading Dye	MBI Fermentas GmbH
Acetone	Lactan/Roth
Agar-Agar	Lactan/Roth
Agarose	Sigma
Agarose high resolution	Lactan/Roth, Art. K297.2
Ampicillin	Lactan/Roth
Benomyl	Sigma
Betaine monohydrate	Fluka
Big Dye Terminator v3.1, Cycle Sequencing Kit	Applied Biosystems, USA
Boric acid (H_3BO_4)	Pharmacia Biotech
Buffer R	Fermentas
Chloroform	Lactan/Roth
Copper II sulphate 5-hydrate	Lactan/Roth
Di-ammonium tartrate	Fluka
Di-ethyl-pyrocarbonate (DEPC)	Sigma
Di-potassiumhydrogenphosphate (K_2HPO_4)	Lactan/Roth
Ethanol	Lactan/Roth
Ethidiumbromide	Sigma
Ethylene-di-amine-tetra-acetic acid (EDTA)	Sigma
Formaldehyde 37 % solution	Roth
Formamide HIDI	Applied Biosystems, USA
Gene Ruler™ 100bp DNA Ladder	Fermentas
Gene Ruler™ 1kb DNA Ladder	Fermentas
Glass beads	B. Braun Biotech International
Glucose	Merck
Glycerol	Lactan/Roth
Hydrochloric acid (HCl)	Lactan/Roth
Hydrogen peroxide (H_2O_2) 30%	Lactan/Roth
Hygromycin B	VWR
Isoamyl alcohol	Lactan/Roth
Isopropanol (2-propanol)	Lactan/Roth
Kanamycin	Lactan/Roth
Ligation buffer (10x)	Fermentas
Magnesium chloride ($MgCl_2$)	Finnzymes
Magnesium sulphate ($MgSO_4$) heptahydrate	Lactan/Roth
Malt extract-Agar	Merck
Nickel(II) chloride ($NiCl_2$) hexahydrate	Lactan/Roth
N,N-di-methylformamide (DMF)	Sigma
p-Amino benzoic acid (paba)	Sigma

Tab.2.1. (continued)

Chemical	Company
PEG4000 (Polyethylenglycol) 50 % (w/v)	Fermentas
Peptone	Lactan/Roth
Phenol	VWR
Plasmid Mini Kit	Qiagen
plasmid vector pTZ57R/T	Fermentas
Poly-L-lysine solution	Sigma
Potassium chloride (KCl)	Lactan/Roth
Potassium-di-hydrogenphosphate (KH_2PO_4)	Lactan/Roth
Primers	VBC
QIAquick PCR Purification Kit	Qiagen
Restrictionenzymes (BsuRI, Hin6I)	Fermentas
Rose Bengal	Sigma
Sodium chloride (NaCl)	Lactan/Roth
Sodium di-hydrogenphosphate (NaH_2PO_4)	Lactan/Roth
Sodium hydroxide (NaOH)	Lactan/Roth
Sodium molybdate (Na_2MoO_4) dihydrate	Sigma
T4 DNA ligase 5u/µl	Fermentas
Tips, tubes, pipettes	Greiner
Tris Ultra Qualität	Lactan/Roth
Triton X-100	Lactan/Roth
Ultra Clean Soil DNA Isolation Kit	MoBio, USA
X-Gal	Lactan/Roth
Yeast extract	Lactan/Roth
Zinc sulphate ($ZnSO_4$) heptahydrate	Lactan/Roth

2.1. Media, Buffers and Solutions

Antibiotics:

Ampicillin in H_2O (stock: 100 mg/ml → 1,000x)
Benomyl in DMSO (stock: 10 mg/ml → 2,000x)
Kanamycin in H_2O (stock: 50 mg/ml → 1,000x)
Rose Bengal in H_2O (stock: 50 mg/ml → 1,000x)

Media:

LB (Lysogeny Broth[1])
10 g/l peptone (bacto tryptone), 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar-agar

Malt extract-Agar (MEA, Merck)
48 g/l H_2O

Aspergillus Minimal Media (AMM)
10 g/l glucose, 20 ml/l Aspi-Salts, 10 ml/l SL-4, 20 g/l agar-agar, 10 mM di-ammonium tartrate, pH 6.8

Salt Solution (Aspi-Salts)
26 g/l KCl, 26 g/l $MgSO_4$ x 7 H_2O, 76 g/l KH_2PO_4, 2 ml/l chloroform (for sterility reasons)

SL6
0.1 g/l $ZnSO_4$ x 7 H_2O, 0.03 g/l $MnCl_2$ x 4 H_2O, 0.3 g/l H_3BO_3, 0.2 g/l $CoCl_2$ x 6 H_2O, 0.01 g/l $CuCl_2$ x 2 H_2O, 0.02 g/l $NiCl_2$ x 6 H_2O, 0.03 g/l Na_2MoO_4 x 2 H_2O

SL-4
100 ml/l SL6, 0.5 g/l EDTA, 0.2 g/l $FeSO_4$ x 7 H_2O

TB (Terrific Broth[2])
11.8 g/l peptone, 23.6 g/l yeast extract, 9.49 g/l K_2HPO_4, 2.29 g/l KH_2PO_4

SOC
20 g/l peptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose

Supplements:

p-Amino Benzoic Acid (paba)
200 mg/l H_2O

[1] The acronym LB actually stands for “lysogeny broth” as Bertani himself emphasised in his commentary (Bertani, 2004), although the abbreviation is commonly used for Luria Bertani medium.
[2] acronym according to AppliChem GmbH

Buffers and Solutions:

X-Gal in DMF (stock: 40 mg/ml → 500x)

PCI
equilibrated Phenol, chloroform, and isoamyl alcohol (25:24:1, v/v)

10 % Triton X-100
100 ml/l in H_2O

1x TAE buffer
4.8 g/l Tris, 0.5 M EDTA, pH 8.0 (acetic acid)

10x PBS
15.6 g/l NaH_2PO_4, 87.66 g/l NaCl, pH 7.5 (NaOH)

1x PBS/0.1 % Triton X-100
100 ml/l 10x PBS, 10 ml/l 10 % Triton X-100

40 % Glycerol/PBS
460 ml/l 87 % glycerol, 100 ml/l 10x PBS

NTE
40 ml/l 5 M NaCl, 50 ml/l 1 M Tris, 10 ml/l 500 mM EDTA, pH 7.5

Agarose-Gel
2 % agarose in 1x TAE buffer
For improved separation of smaller bands (~ 50 – 250 bp) 3 % high resolution agarose was used.

Ethidiumbromide (10 mg/ml → 15 µl/l agarose gel)

2.2. Soil sampling

Soils were collected from four different agricultural fields and one grassland near Vienna (Austria) and subsequently stored at 4 °C. Samples from these five soils were assayed and are referred to as soils M, N, P, R and T (Tab.2.2). Prior to experiments soils were characterised according to chemical parameters (Tab.2.3).

Tab.2.2. Soil sample origins and code annotation.

Registercode	Location	Internal Code
KB177/BF19	Maissau	M
KB177/BF8	Niederschleinz	N
KB9/BF6	Purkersdorf	P
KB9/BF8	Riederberg (grassland)	R
KB28/BF7	Tulln	T

Tab.2.3. Soil chemical analysis.

			KB177/BF19 (M)	KB177/BF8 (N)	KB9/BF6 (P)	KB/9/BF8 (R)	KB28/BF7 (T)
moisture content [%]			18.57	18.69	24.27	31.23	25.86
$CaCO_3$ [%]			0.2	8.5	0.06	2.11	0.04
pH-KCl			6.99	7.15	5.67	6.63	6.21
cation exchange capacity		**mval%**[1]	8.87	15.38	11.17	33.23	37.93
base saturation	**V**	**% v. T**	92.3	98.08	81.38	82.05	70.37
	Ca%T	**% v. T**	71.44	83.75	71.16	76.87	52.85
	Mg%T	**% v. T**	11.93	8.51	6.52	4.12	16.81
	K%T	**% v. T**	6.66	4.88	2.68	0.46	0.22
AOS [%]	**(degradable organic matter)**		2.28	3.16	2.78	8.53	5.57
C_{org} [%]	**(organic carbon)**		1.33	1.84	1.62	4.96	3.24
N_{tot} [%]	**(total nitrogen)**		0.134	0.194	0.163	0.525	0.353
N_{org} [%]	**(organic nitrogen)**		0.132	0.193	0.162	0.523	0.352
N_{min} [mg%]	**(mineralised nitrogen)**		5.1	2.7	2.3	4.2	2.7
C/N	**(carbon/nitrogen ratio)**		9.9	9.5	9.9	9.4	9.2
Ca I+II [mg/100g]			132.35	262.2	161.15	520.83	405.55
Mg			13.61	16.2	9.06	17.07	78.56
K			24.63	30.47	12.04	6.03	3.51
NH_4-N			2.39	0.99	1.18	1.88	1.07
NO_3-N			2.7	1.68	1.09	2.29	1.62
Fe [mg/kg]			32.45	1.67	21.79	69.93	84.1
Mn			0.32	0.01	0.53	0.47	0.37
Cu			<0.05	0.06	<0.05	0.15	0.09
Zn			0.11	<0.06	0.23	0.31	0.15
sieving curve	**water supply**	**ml/100g TM**	47.15	56.6	33.66	94.55	81.23
	2-0.63 mm	**% TM**	9.2	0.6	2.6	2.7	1.1
	0.63-0.25	**% TM**	7.2	1.1	6.5	3.2	1.6
	0.25-0.125	**% TM**	6	1.4	10.6	4.3	2.3
	0.125-0.063	**% TM**	7.2	4.9	13.7	7	3.6
	0.063-0.02	**% TM**	63.9	67.2	56	43	32.8
	0.02-0.006	**% TM**	3.4	3.6	5.3	5.7	10.6
	0.006-0.002	**% TM**	3.1	3.4	3.6	5.1	4.9
	<0.0020 mm	**% TM**	<0.1	17.7	1.7	29.1	43.2

[1] milli-equivalents (meq/100 g)

2.3. Plate counts & culturing of fungi

Dilution series

1 g soil was weighed in a 50 ml Greiner flask (polypropylen-tube), resuspended in 10 ml PBS/0.1 % Tween and incubated at room temperature (RT) with gentle shaking (50 rpm) for 30 min. Dilution series (10^{-1} to 10^{-4}) from the soil suspension were prepared in 1x PBS.

100 µl from dilutions 10^{-1} to 10^{-4} were plated on mycological media like MEA and AMM, respectively. To prevent inhibition of fungal growth due to bacterial proliferation the antibiotic Rose Bengal (BR) was added to the media as well as Benomyl (B), a biocide, which primarily represses growth of *Ascomycota*, therefore selecting for *Basidiomycota*. The following combinations were prepared: MEA + BR, AMM + BR as well as MEA + BR + B. After 5 days of incubation at 26 °C plates were regularly checked for the growth of fungal colonies.

Morphologically distinct colonies (according to spore colour, colour of mycelium, colony morphology, colour of colony on reverse, etc.) were subcultured.

In general, DNA was extracted from spores. DNA from non sporulating fungi was obtained from the mycelium. If the mycelium was impossible to detach from the medium, colonies had to be grown on cellophane disks to avoid media remnants, which possibly could hamper DNA extraction.

DNA extraction was performed with Phenol/Chloroform/Isoamyl alcohol (PCI). Mycelium and/or spores were resuspended in 600 µl NTE and 60 µl 10 % SDS. 600 µl of the lower, clear, organic layer of PCI (4 °C) as well as glass beads of three different diameters (Ø = 1 mm, 0.4 – 0.6 mm, 0.10 – 0.11 mm) were added. Tubes were rigorously shaken in a FastPrep120 (Bio101) for 30 sec at 6.5 m/s. After centrifugation for 20 min at 10,000 rpm and 4 °C the supernatant (SN) was transferred into a new tube. One volume CI (Chloroform:Isoamyl alcohol 24:1) was added, mixed and centrifuged for 10 min. The upper phase was then precipitated with isopropanol, washed with 70 % ethanol and dissolved in 100 µl H_2O.

For the **strain collection** the spore/mycelium suspension was combined with 40 % glycerol/1x PBS to achieve a final glycerol concentration of 20 % and stored at -80°C.

2.4. ITS/LSU-PCR

Generally, for PCRs (Polymerase Chain Reaction) the 2x ReddyMix™PCR Master Mix (ABgene) and primers obtained from VBC (Tab.2.4) were used. PCR amplifications were carried out using the T3 Thermocycler (Biometra) or Mastercycler (Eppendorf); amplification steps are shown in Fig.2.1.

For identification of cultivated fungi the ITS-region was amplified with primer pair ITS1/ITS4 and sequenced. For cultivation-independent identification of soil fungi the ITS-region and partial LSU were amplified with primer pair ITS1F/TW13, elongating the extension time to 3 min due to increased fragment size. The LSU region serves for higher order identification of fungi with no highly homologous reference sequences in public databases.

Success of amplification was checked on an agarose gel. Primer binding sites are schematically depicted in Fig.2.3, primer sequences are listed in Tab.2.4.

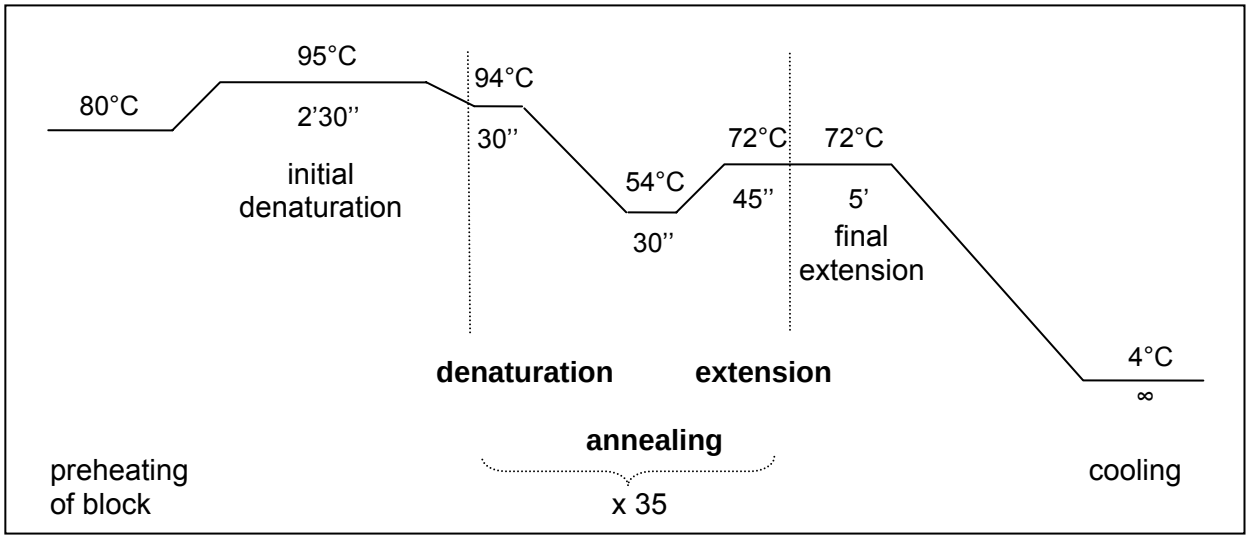


Fig.2.1. Temperature profile for ITS/LSU-PCR.

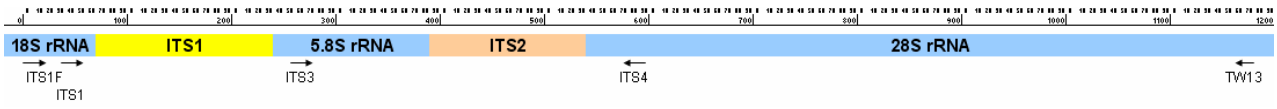


Fig.2.2. Parts of the rRNA gene repeat unit.
Sections can be amplified by PCR through the use of primers indicated by arrows.

Tab.2.4. Primers used for the amplification and sequencing of ITS/partial LSU sequence fragments.

Primer	Sequence (5'-3')	Target organisms	Gene	T_m [°C][1]	Reference
ITS1	TCCGTAGGTGAACCTGCGG	universal eukaryotic	18S	54	(White et al. 1990)
ITS1F	CTTGGTCATTTAGAGGAAGTAA	fungi	18S	54	(Gardes and Bruns 1993)
ITS3	GCATCGATGAAGAACGCAGC	fungi	5,8S	54	(White et al. 1990)
ITS4	TCCTCCGCTTATTGATATGC	fungi	28S	54	(White et al. 1990)
TW13	GGTCCGTGTTTCAAGACG	universal eukaryotic	LSU	54	(Taylor and Bruns 1999)

2.5. RFLP analysis

Restriction Fragment Length Polymorphism (RFLP) is a technique by which organisms may be differentiated by analysing patterns formed from cleavage of their DNA.

PCR products were directly subjected to RFLP analyses to estimate the sequence diversity within the clone library. The reaction was performed with the restriction endonuclease *Bsu*RI (Fermentas, isoschizomere of *Hae*III) for 2 h at 37 °C and the fragments were separated on a 3 % high resolution agarose gel. Representative clones for each pattern were selected for sequencing. Purified PCR-product served as a template for a 15 µl sequencing reaction using Big Dye Terminator v3.1, Cycle Sequencing Kit (ABI). ITS1 and ITS4 were used in a one pmol/µl dilution, respectively, corresponding to one µM final concentration. The sequencing reaction was carried out with T3 Thermocycler (Biometra) under the following conditions: initial denaturation for 1 min at 96 °C, 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min. Post-reaction clean up was performed by ethanol precipitation (96% Ethanol/NaAc/EDTA). DNA was denatured in HIDI formamide (highly deionised formamide, Applied Biosystems, USA) to be electrophoretically resolved in a DNA sequencer (ABI 3100 genetic analyzer, Pop69, BDv3.1) at the Institute of Applied Genetics und Cell Biology, University of Natural Resources and Applied Life Sciences, Vienna (Austria). Sequence files obtained in abi-format were processed as described in 2.7.

Internal lab-codes for cultivates and DNA sequences derived from them are NG_01 to 36, H01 to H18 and p01 to p53 (NG: Nitro-Genome).

[1] applied annealing temperature of the primer

2.6. Direct PCR and clone libraries produced from soil samples

Total DNA was extracted directly from soil using the Ultra Clean Soil DNA Isolation Kit (MoBio) according to the manufacturer's instructions. For each of the five soils three replicas were performed and pooled after elution. This mixture of DNA originating from different organisms was purified (QIAquick PCR Purification Kit, Qiagen) and subjected to ITS/LSU-PCR using ITS1F specific for fungi and the universal eukaryotic primer TW13 for 5' LSU.
For each soil a clone library of amplified ITS/LSU-PCR-products was constructed in plasmid pTZ57R/T (Fermentas). In the following, the cloning procedure is described:
1.5 µl of untreated PCR product was mixed with 0.165 µg plasmid vector pTZ57R/T DNA, Ligation Buffer (10x), PEG4000 (50 µg/µl final concentration), 1 µl T4 DNA Ligase (5 U/µl) and water in a final 10 µl ligation reaction. After incubation at 22 °C for at least 1 h, transformation into competent *E. coli* cells took place (heat shock for 30 sec at 42 °C in water bath, cooled on ice for 2 min, addition of 500 µl SOC medium and recovery for 1 h at 37 °C before plating on LB + X-Gal + Amp). Separated white colonies, each theoretically containing one distinct product derived from a single fungus, were picked. For each library 96 independent clones were picked and resuspended in 200 µl TB + amp in microtiter-plates. After incubation at 37 °C over night 5 µl bacterial suspension were mixed with 50 µl H_2O and boiled for 7 min at 95 °C to induce cell lysis. Inserts were amplified with primer pair ITS1F/TW13, the PCR settings are depicted in Fig.2.1. Remaining bacterial suspension was combined with 200 µl 40% glycerol/PBS and stored at -80 °C.

RFLP analyses and sequencing reactions, apart from the applied primers, were performed as described above for cultured fungi. Here the region between ITS1F and TW13 was amplified and the purified product was sequenced with ITS3 and TW13, respectively (Fig. 2.2).

In some cases it was necessary to sequence an additional part of the rRNA coding region to unambiguously identify the respective fungus. This was performed using the primer ITS1F in an additional sequencing reaction, yielding a sequence consisting of part of 18S rRNA gene (SSU) and the spacer ITS1 (Fig.2.3).

2.7. Bioinformatic analysis

For sequence analyses the commercial software Vector NTI Advance™ 10 for Windows, version 10.3.0 was used. Abi-sequence files were trimmed, contigs were created by assembling the two (or three) primer sequences (Fig.2.3), primer orientations were checked and the contig sequence was proofread.

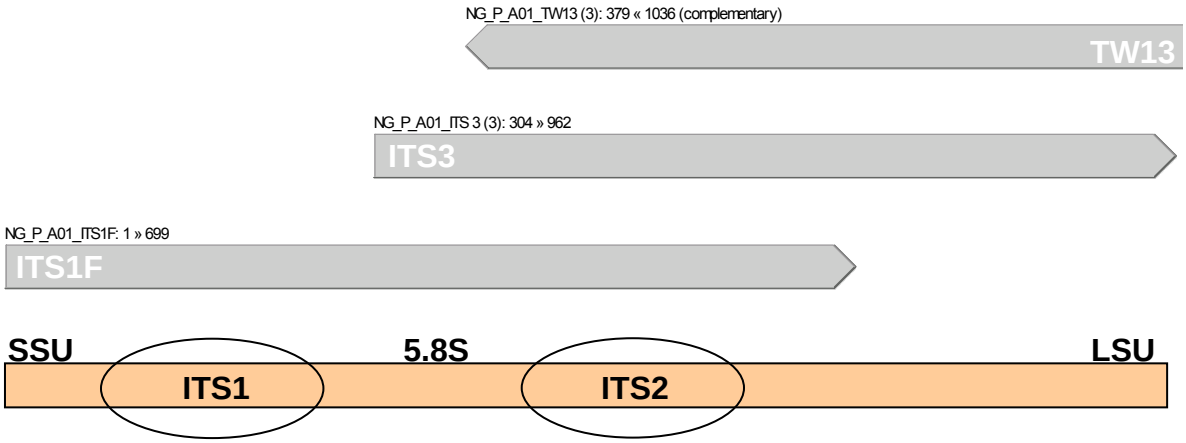


Fig.2.3. Graph pane of Vector NTI contig express showing primer orientation, lengths and contig structure (Vector NTI display modified).

Mended contig sequences were submitted to a nucleotide BLAST Search (Basic Local Alignment Search Tool) at http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al. 1990). BLAST searches were performed systematically with parts of the sequence corresponding to the ITS or partial LSU region, respectively. In order to check for chimeric sequences, the two taxonomies were compared for consistency.

Additionally to the whole sequence stretch, BLASTN searches for clone sequences were performed systematically with parts of the sequence corresponding to the ITS or partial LSU region, respectively. To differentiate between the two sequence parts, the position of the ITS4 primer was used, which is located at the 5' end of the LSU region. In order to check for chimeric sequences, the two taxonomies were compared for consistency. BLASTN search results for the whole sequence stretches were reported, but often could not be used for clear identification due to a lack of reference sequences in the public database which span the ITS as well as the LSU region. Therefore, in many cases the identification was based on the separate regions, where high % identity of ITS sequence (if available) was preferably used for species identification, whereas the partial LSU region usually served for identification at higher taxonomic levels.

For the determination of putative identities via BLASTN searches, the following threshold values were defined: For the ITS region of cultured fungi (ITS1, 5.8S and ITS2) as well as sole ITS2 region for clone sequences: species level > 98%, genus level > 97 %, class, order or family level depending on the phylogenetic classification of the reference organisms > 90 %. For partial LSU region of the clone sequences: species level > 99%, genus level > 98 %, class, order or family level depending on the phylogenetic classification of the reference organisms > 95 %, phylum level > 90 %, unidentified fungi < 90 %. Matches < 80 % identity and with a query coverage < 80 % for the ITS region and partial LSU, respectively, were regarded as below acceptable confidence levels and were not reported. Sequences lacking the ITS region were stated as not available (n/a) for the respective region and identification was based on the partial LSU sequence exclusively.

Reference hits were scrutinised concerning their reliability (e.g. strain collections were accepted as reliable references) and position in the distance tree of results.

In cases in which sequences could not reliably be identified to a certain taxonomic level, the lowest common affiliation of reliable reference sequences was taken.

Sequences with the same BLAST % identity for different fungal species were reported as different species separated by the slash-symbol.

The ITS region is currently the most widely sequenced DNA locus for species identification in fungi. There is general agreement, that high % identity of the ITS sequence is useful for species identification (see 1.8.8. Fungal identification using ITS).

In addition, many molecular systematic studies on fungi utilise the 5’ part of the LSU gene. A combination of both regions (ITS and partial LSU) was frequently used in recent years not only for fungal phylogeny, but also for identification purposes (Urban et al. 2008). The main advantage here was the higher order identification using the LSU region when no highly homologous reference sequences were available in the public database (see 1.8.10 Combination of LSU).

2.7.1. Assessment of sequences

For the analysis of the sequences on different taxonomic levels, a database was created in Microsoft Access XP (2002). For this, a bunch of tables were created. The database design in the form of an entity-relationship model is depicted in Fig.2.4. In a database, an entity type is the equivalent of a table; each individual record is

represented as a row and an attribute as a column. The descriptions of the individual tables and their attributes (the bold attributes are primary keys) are documented in the entity-relationship model. In addition, the hierarchical structure of the respective classification tables (taxonomy) is illustrated.

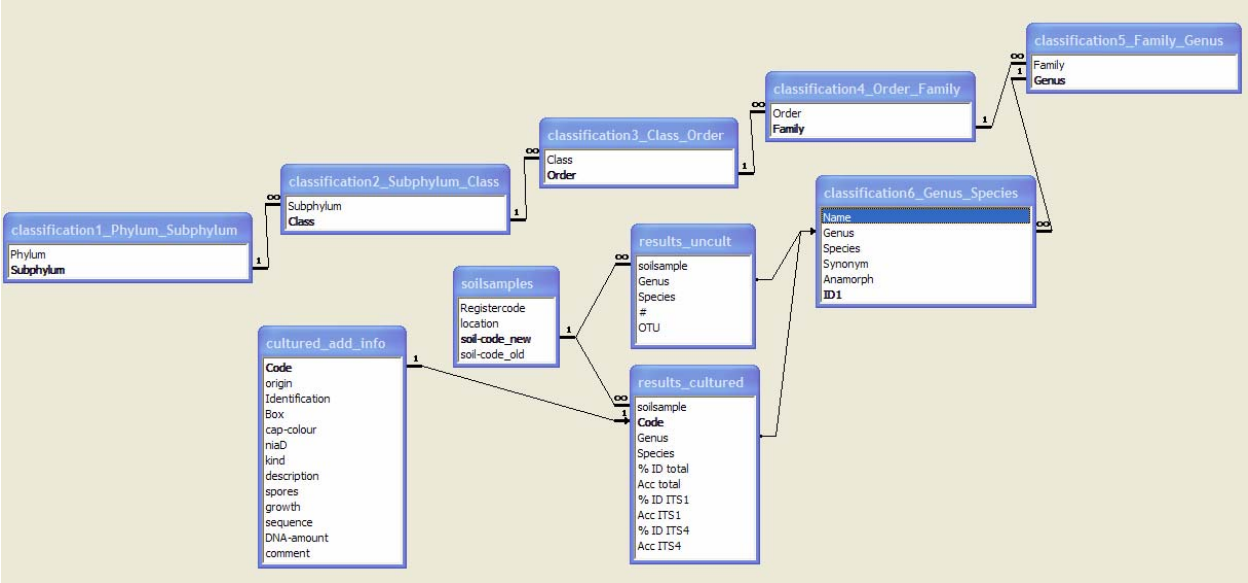


Fig.2.4. Entity-relationship model of the database.

Afterwards a query was constructed by which the classification tables were combined and connected with the data of the results tables. This resulted in a new table consisting of OTU-No, soil, abundance, identified species as well as the corresponding higher taxonomic levels for the sequences of the clone libraries. The data constructed in this way were further analysed with the software SPSS for Windows, German version 15.0.1 (2006). Therefore, cases (records) were weighted according to their abundance and sorted by soil sample. Frequency analyses were conducted and the results were figured as tables.

Furthermore, sample-based data were used for the calculation of estimators of species richness with ESTIMATES (Version 8.0.0, R. K. Colwell, http://purl.oclc.org/estimates). Five estimators were compared: Chao2 richness estimator, incidence- and abundance-based coverage estimator [incidence-based coverage estimator (ICE) and abundance-based coverage estimator (ACE)], first-order Jackknife richness estimator (Jackknife 1) and Bootstrap richness estimator.

2.8. Construction and analysis of *niaD* gene libraries

Partial fungal *niaD* genes were amplified from total DNA extracted from soil (Ultra Clean Soil DNA Isolation Kit, MoBio). 2 replicates from soils N and P from the 1st time point of the 2nd experiment were chosen. These 2 soils had been selected after the 1st experiment for following experiments and are referred to as N1, N2, P1 and P2. Extracted total DNA was purified (QIAquick PCR Purification Kit, Qiagen) and subjected to *niaD*-PCR using primers niaD01F and niaD04R (Tab.2.5) and the following optimised conditions: ReddyMix™PCR Master Mix (ABgene), 750 mM Betaine, 1 mM $MgCl_2$ or more, 2 µM primers. The cycling conditions were as follows: initial denaturation at 95 °C for 2'30'', 35 cycles of 94 °C for 45'', 52 °C for 45'' and 72 °C for 1', and a final extension at 72 °C for 5min. Subsequently, a nested PCR was performed using the primer pair niaD02F and niaD03R (Tab.2.5) in a 6 µM concentration, conditions were as stated above.

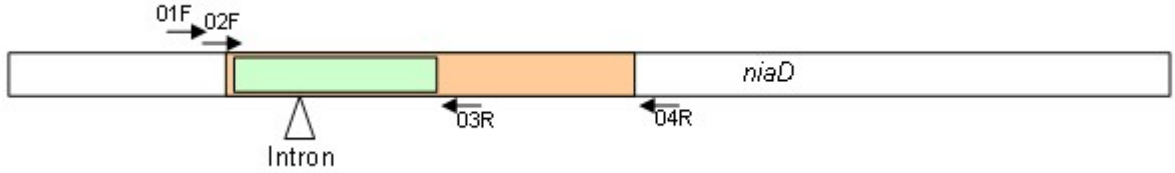


Fig.2.4. Schematic structure of the *niaD* gene including parts that can be amplified using the primer combinations niaD01F + niaD04R (orange) or niaD02F + niaD03R, which gives rise to the nested PCR product (green). Primer binding sites are indicated by arrows. The position of the intron sequence, present in some of our resulting sequences is indicated by the triangle.

Tab.2.5. Newly designed primers used for the amplification and sequencing of nitrate reductase (*niaD*) sequence fragments.

Primer	Sequence (5'-3')[1]	Target organisms	Gene	T_m [°C]
niaD01F	GTNTGYGCNGGNAA	fungi	*niaD*	52
niaD02F	MGNMGNAARGARCARAA	fungi	*niaD*	52
niaD03R	GGNARNACNCKRTTRTC	fungi	*niaD*	52
niaD04R	GTNGGRTGYTCRAA	fungi	*niaD*	52

Clone libraries of *niaD*-nested-PCR-products were constructed in plasmid pTZ57R/T (Fermentas). The cloning procedure was performed as described for ITS/LSU.

N1 and P1 clone libraries contain 96 independent clones each, while N2 and P2 contain 48 clones each. Clones were screened by *niaD*-PCR and positive candidates

[1] abbreviations according to IUPAC: R=G/A, Y=T/C, M=A/C, K=G/T, N=G/A/T/C

were compared via RFLP analyses with *Bsu*RI and *Hin*6I double digestion. Sequences were obtained by using the primers M13fwd and M13rev, respectively, for the sequencing reaction. Sequence data were edited as described for ITS/LSU sequences. Further sequence analyses were accomplished using the Vector NTI software: translation into protein sequences (exclusive flanking primers niaD02F and niaD03F), construction of a *niaD* alignment, and visualisation of a Neighbour-Joining tree.

2.9. Fluorescence *in situ* hybridisation (FISH)

Fluorescence *in situ* hybridization (FISH) uses fluorescently labelled nucleic acid probes to localise specific DNA or RNA sequences of target organisms. In the field of microbial ecology, FISH is widely used for identification of microorganisms, especially bacteria and archaea in environmental samples. In this field, the target molecule usually is rRNA, which has the advantage that probes specific for different taxonomic levels can be designed for this molecule. Moreover, visualisation of genes or chromosomes by this technique ("chromosomal painting") is used for gene mapping and for identifying chromosomal abnormalities in eukaryotic cells *e.g.* (Gerr et al. 2007). Bacterial chromosomal painting can be used for the identification of bacterial cells (Lanoil and Giovannoni 1997).

Additionally, mRNA-FISH is applied to measure and localise mRNAs within cells and tissues *e.g.* (Wagner et al. 1998; Pernthaler and Amann 2004). Generally, cells are treated with chemical substances for cell fixation, immobilised to a slide and hybridised under stringent conditions. After a washing step, the hybridisation can be analysed by epifluorescence microscopy (Daims et al. 2005).

2.9.1. Cultivation of *Aspergillus nidulans* for mRNA-FISH

A. nidulans wt paba was grown as liquid culture in AMM excl. agar, 2 mg/l paba, N-source (either 10 mM ammonium tartrate for non-induced or 10 mM $NaNO_3$ for induced conditions) at 37°C for 7 h or overnight. To super-induce *niaD*-mRNA expression, culture was grown with ammonium tartrate overnight, followed by N-starvation for 30 min and induction with $NaNO_3$ for another 30 min.

Samples were once washed with PBS, centrifuged, the resulting pellet resuspended in PBS and kept on ice.

2.9.2. Cell fixation with paraformaldehyde (PFA)

4% PFA
108.1 ml/l 37% formaldehyde solution (Roth) in 1 x PBS

3 Vol 4% PFA were added to 1 Vol of sample and incubated for 2 h at 4°C. After incubation, the sample was centrifuged (15,000 rpm, 15 min) and the pellet was washed once with 1 x PBS. Finally, the pellet was resuspended in 1 Vol 1 x PBS and 1 Vol ethanol$_{abs}$. After fixation samples were stored at -20°C.

2.9.3. Cell fixation with EtOH

The sample was centrifuged to harvest the cells, washed once with 1 x PBS and the pellet resuspended in 1 x PBS. Afterwards, 1 Vol ethanol$_{abs}$ was added and samples were stored at -20°C.

2.9.4. Oligonucleotide probes

To select appropriate probes the online database probeBase (Loy et al. 2003) on http://www.microbial-ecology.net/probebase/ was used. The characteristics of oligonucleotide probes used are listed in Tab.2.6.

Tab.2.6. Characteristics of oligonucleotide probes used for FISH analyses.

Probe	Sequence (5'-3')	Target molecule	Specificity	Label	Reference
EUK516	ACCAGACTTGCCCTCC	18S rRNA	Eukarya	5' Fluos	(Amann et al. 1990)
NonEUB	ACTCCTACGGGAGGCAGC	-	control	5' Fluos	(Wallner et al. 1993)
niaD_2527R	CAGGCCCGCAAACCAAAACCA	*niaD* mRNA	*A. nidulans*	5' Cy3	unpublished

High Performance Liquid Chromatography (HPLC)-purified and lyophilized oligonucleotides were obtained from Thermo Electron GmbH or VBC, respectively. Working solutions with a final concentration of 50 ng/µl for Fluos-probes, and of 30 ng/µl for Cy3-probes were prepared. Both stock and working solutions were stored at -20°C in the dark.

2.9.5. Preparation of poly-L-lysine coated slides

To increase cell immobilization for samples, slides were coated with poly-L-lysine, which is known not to exhibit auto-fluorescence under laser irradiation.

10 well slides (Paul Marienfeld) were washed in 1% HCl in 70% EtOH for 5 min, prior to coating with 0.01% poly-L-lysine solution for 5 min. Coated slides were dried for 1.5 h at 60°C in a Heraeus T20 drying oven (Kendro Lab. Products) and stored in a dry and dustless box at RT.

In order to minimise mRNA degradation by RNases DEPC treated MQ water, PBS and SSC was used, slides were incubated for 20 min in 0.3 % H_2O_2 and immobilized and dehydrated samples were subjected to DEPC-treatment prior to hybridisation.

2.9.6. H_2O_2-treatment of coated slides

Slides were treated with 0.3 % H_2O_2 in H_2O for 20 min at RT, washed twice with MQ-H_2O_{DEPC} for 1 min each and let dry.

2.9.7. Immobilisation and dehydration of the sample

10 µl sample were immobilised on wells by drying at 46°C in a hybridisation oven for ~10 min. After liquid had dried, an increasing ethanol-series was undertaken. For this, slides were in succession put into 50%, 80% and 96% ethanol for 3 min each.

2.9.8. Carbethoxylation and inactivation of endogenous RNases (DEPC treatment)

DEPC-treatment was performed with 0.1% (v/v) freshly prepared DEPC in PBS for 12 min at RT. Slides were washed once with PBS_{DEPC} for 1 min and with MQ-H_2O_{DEPC} for 1 min.

2.9.9. Probe hybridisation

1 M Tris/HCl, pH 8.0
10 % (w/v) SDS

20 x SSC (Saline-Sodium Citrate)
0.3 M Na3-Citrat, 3 M NaCl

Hybridisation buffer1
0.9 M NaCl, 20 mM Tris/HCl, 10 % FA, 0.01 % SDS

Hybridisation buffer2
SSC (0.9 M Na), 10 % FA, 0.01 % SDS

Washbuffer1
0.45 M NaCl, 20 mM Tris/HCl, 0.01 % SDS

Washbuffer2
SSC (0.45 M Na), 0.01 % SDS

DEPC reacts with primary amines and can therefore not be used to treat Tris buffers, since Tris contains an amino group. For this reason, SSC buffer, which can be treated with DEPC, was tested and compared with the untreated Tris buffer.

Hybridisations were performed at a temperature of 46°C. Stringency was achieved by addition of formamide (FA) to the hybridisation buffer (HB) and the concentration of salts in the washing buffer (WB). Corresponding volumes are listed in Tab.2.7.

Tab.2.7. Volumes of FA, NaCl and EDTA used in hybridisation and washing buffers.

Hybridisation buffer			Washing buffer		
FA [%]	FA [µl]	H_20 [µl]	NaCl [mM]	5 M NaCl [µl]	0.5 M ETDA [µl]
0	0	800	900	9,000	-
10	100	700	450	4,500	-
20	200	600	225	2,150	500
25	250	550	159	1,490	500
30	300	500	112	1,020	500
35	350	450	80	700	500
40	400	400	56	460	500
45	450	350	40	300	500
55	550	250	20	100	500
70	700	100	0	0	500

10 µl HB and 1 µl of the respective probe were pipetted on the well and mixed gently. The slide was put into a 50 ml tube (Greiner Bio-One GmbH), in which a tissue, soaked in remaining HB had been placed. The closed tube was incubated at 46°C in a hybridisation oven UE500 (Memmert GmbH) for 3 hours. After hybridisation, the slide was washed in pre-warmed WB at 48°C for 10 min. After dipping the slide into ice-cold H_2O_{DEPC} it was immediately dried in an air stream. Slides were stored at -20°C in the dark until microscopic analysis.

2.9.10. Staining with 4'-6'-di-amidino-2-phenylindole (DAPI)

Before microscopic analysis, samples were stained with 4'-6'-di-amidino-2-phenylindole (DAPI), which preferentially stains double stranded DNA (emission maximum: 461 nm) to visualise all cells for microscopic analyses.

DAPI (Lactan)
1:1,000 dilution in H_2O

15 µl DAPI dilution were used and incubated for 7 min in the dark. DAPI was removed and the sample was washed with 25 µl of H_2O to remove free DAPI and slides were dried for ~20 min (RT) in the dark. Due to the high mutagenicity of DAPI, particular pipettes were used.

2.9.11. Detection of fluorescently labelled cells

Samples were embedded in Citifluor AF1 (Agar Scientific Limited) to decrease bleaching during microscopic analysis and covered with a coverslip. Evaluation of hybridisations was done using a Confocal Laser Scanning Microscope LSM 510 Meta (Zeiss) and the software included. The microscope was equipped with an Ar-laser (430-514 nm; for excitation of the Fluos-fluorophore) and two He-Ne-lasers (543 nm; for excitation of Cy3). Excitation of DAPI was done via exposure to UV radiation (~350-365 nm) by a UV lamp. Plan-Neoflar objectives with 40x, 63x and 100x magnification were used in combination with an 10x ocular. Documentation was done using the software delivered with the CLSM.

3. Results

3.1. Plate counts & cultured fungi

To establish a collection of fungi from agricultural soil for future laboratory studies with selected strains, soil suspensions were plated on different media:

- malt extract agar (MEA) + rose bengal (BR), a nutrient rich complex growth medium with BR added to suppress growth of bacteria. Under illumination with visible light, RB produces singlet oxygen which is known to inactivate bacterial cells efficiently (Schäfer et al. 2000).
- MEA + BR + Benomyl, as above but with the addition of Benomyl, which inhibits growth of Ascomycetes more strongly than growth of Basidiomycetes (Summerbell 1993) by depolymerisation of microtubules (Mini and Raudaskoski 1993).
- *Aspergillus* minimal medium (AMM) + BR, a defined minimal medium with glucose as carbon source and di-ammonium tartrate as nitrogen source

100 µl from dilutions 10^{-1} to 10^{-4} of the soil suspension (1 g soil resuspended in 10 ml PBS/0.1 % Tween) were plated on the aforementioned mycological media. After 5 days of incubation at 26 °C plates were regularly checked for the growth of fungal colonies. Colony forming units (CFU) of filamentous fungi and yeasts on plates with different media composition were counted (Tab.3.1). Plates with rapidly growing and heavily sporulating *Mucorales* were discarded, since colony counting and subculturing from such plates was hardly possible.

Tab.3.1. Plate counts: numbers signify colonies of total filamentous fungi and yeasts counted. n.c., not countable.

a) MEA + BR plates

	dilutions			
soils	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**
M	14	5	0	1
N	102	17	3	0
P	55	4	0	0
R	83	13	0	0
T	34	16	0	0

Tab.3.1. (continued)

b) MEA + BR + Benomyl plates

	dilutions			
soils	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**
M	67	0	0	0
N	3	5	0	0
P	2	0	0	0
R	0	0	0	0
T	4	0	0	0

c) AMM + BR plates

	dilutions			
soils	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**
M	n.c.	93	9	0
N	n.c.	100	6	0
P	n.c.	255	1	0
R	542	57	44	0
T	n.c.	102	1	0

Morphologically distinct colonies were subcultured and identified using comparative sequence analysis (BLAST) of the ITS region. For this, a DNA extraction of pure cultures was performed with Phenol/Chloroform/Isoamyl alcohol. Purified DNA isolated from two selected pure cultures is shown in Fig.3.1 on the right, indicated by 1 and 2. For identification of cultivated fungi the ITS region was amplified with primer pair ITS1/ITS4 and sequenced using ITS1 and ITS4, respectively. ITS-PCR products of selected fungi, indicated by their internal lab codes (p09 – p31), are shown in Fig.3.1 on the left. Length variabilities of ITS regions in different organisms are displayed in Fig.3.2. Identification of pure cultures was carried out by sequence analysis of the ITS region as described in 2.7. Identified fungal cultures are listed in Tab.3.2 and an overview of the taxonomy is depicted in Fig.3.3.

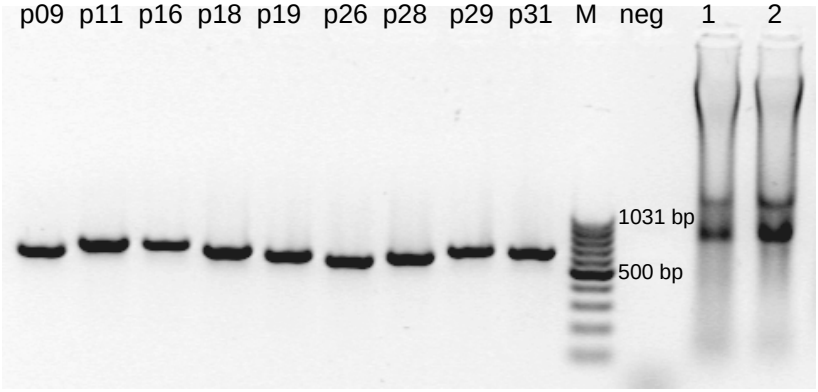


Fig. 3.1. Agarose-Gel of ITS-PCR-products. Numbers indicate colony number (internal lab code). 1 and 2, purified DNA isolated from subcultures NG_34 and NG_35, respectively; M, marker; neg, negative control.

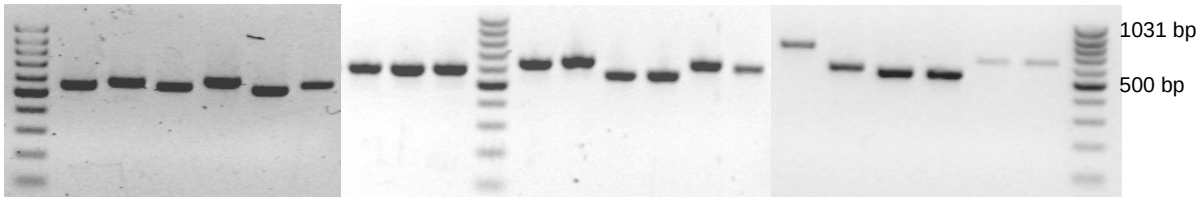


Fig. 3.2. Agarose-Gel illustrating the length variability of ITS regions exemplified by ITS-PCR-products obtained from cultured fungi.

Tab.3.2. Cultured fungi. Acc. No, Accession number of closest hit in NCBI database; ID, identity to closest match in NCBI database; Putative Identification, classification based on ITS resp. LSU sequence; phylum: A, *Ascomycota*; B, *Basidiomycota*; sp., no specific species identified.

Soil	Code	Putative Identification	M*	Phylum	% ID	Acc. No
M	**p28**	*Beauveria tenella*	1	A	100%	AJ345087.1|BTE345087
M	**p52**	*Capnodiales sp.*	1	A	94%	AJ972856.1
M	**NG_H03**	*Chaetomium sp.*	3	A	98%	DQ093661.1
M	**p44**	*Chaetomiaceae sp.*	1	A	92%	EU326205.1|
M	**p37**	*Cordyceps bassiana*	1	A	100%	AJ345089.1
M	**NG_28**	*Cryptococcus terricola*	3	B	99%	AF444377.1
M	**NG_H16**	*Fusarium oxysporum*	3	A	100%	AY188919.1
M	**p46**	*Sordariomycetes sp.*	1	A	95%	AB231012.1
M	**p47**	*Massarina rubi*	1	A	99%	AF383963.1
M	**p39**	*Oidiodendron cerealis*	1	A	100%	AF062788.1
M	**p10**	*Penicillium glandicola*	1	A	99%	AY373916.1
M	**NG_17**	*Penicillium sp.*	1	A	98%	AF033440.1
M	**NG_08**	*Phoma cf. eupyrena*	1	A	100%	AJ890436.1
M	**p12**	*Talaromyces flavus*	1	A	99%	AF455513.1
M	**NG_H18**	*Trichocladium/Humicola sp.*	1	A	99%	AM292050.1
M	**NG_05**	*Trichoderma koningiopsis*	1	A	100%	DQ379015.1
M	**p45**	*Verticillium nigrescens*	1	A	100%	AJ292440.1|VNI292440
N	**p38**	*Cryptococcus aerius*	1	B	100%	AF145324.1|AF145324
N	**p40**	*Eurotium chevalieri*	1	A	100%	AY373886.1
N	**NG_H04**	*Fusarium sp.*	1	A	99%	DQ452447.1
N	**p31**	*Hypocrea lixii*	1	A	100%	EF442080.1
N	**NG_H06**	*Hypocreales sp.*	3	A	94%	AF130140
N	**NG_H14**	*Nectriaceae sp.*	2	A	94%	AY677294.1
N	**p41**	*Onygenales sp.*	1	A	97%	DQ317338.1
N	**NG_26**	*Penicillium sp.*	3	A	100%	DQ339570.1
N	**p49**	*Pleosporales sp.*	1	A	90%	AY943061.1
N	**p15**	*Talaromyces flavus*	1	A	99%	AF455513.1
N	**NG_02**	*Trichoderma sp.*	1	A	98%	DQ345813.1
N	**NG_01**	*Trichoderma velutinum*	1	A	99%	EF417479.1
P	**NG_H02**	*Aspergillus flavipes*	3	A	99%	AY214443.1
P	**NG_H01**	*Bionectria ochroleuca*	3	A	100%	AJ608977.1
P	**p06**	*Chloridium sp.*	1	A	100%	AM262403.1
P	**p07**	*Microascales sp.*	1	A	97%	EF029213.1
P	**NG_H15**	*Fusarium oxysporum*	1	A	99%	DQ452451.1
P	**p20**	*Hypocreales sp.*	1	A	94%	AB114223.1
P	**NG_33**	*Penicillium chrysogenum*	3	A	100%	AY373903.1
P	**NG_23**	*Penicillium janthinellum*	3	A	99%	AJ608945.1
P	**NG_H17**	*Phaeosphaeriaceae sp.*	2	A	90%	AF181710.1|AF181710

Tab.3.2. (continued)

Soil	Code	Identification	M*	Phylum	% ID	Acc. No
P	**p50**	*Pseudogymnoascus roseus*	1	A	100%	AJ608972.1
P	**NG_15**	*Sordariales sp.*	1	A	92%	EF017204.1
P	**p08**	*Talaromyces flavus*	1	A	99%	AF455513.1
P	**NG_22**	*Trichoderma rossicum*	3	A	99%	DQ083024.1
P	**NG_13**	*Trichoderma viridescens*	1	A	100%	EU280135.1
R	**p03**	*Aspergillus flavipes*	1	A	99%	AY214443.1
R	**NG_34**	*Phlebia bresadolae* (only ITS2)	3	B	99%	AF141617.1
R	**NG_H11**	*Bionectria ochroleuca*	1	A	99%	AJ608977.1
R	**p11**	*Debaryomyces castellii*	1	A	100%	AB054102.1
R	**p51**	*Microascales sp.*	1	A	97%	EF029213.1
R	**NG_H10**	*Fusarium oxysporum*	1	A	99%	DQ459007.1
R	**p01**	*Acremonium sp.*	1	A	100%	EF577238.1
R	**NG_35**	*Helotiales sp.*	1	A	95%	AY188359.1
R	**NG_H13**	*Nectria haematococca mpVI*	3	A	99%	AY633746.1
R	**NG_H08**	*Penicillium atramentosum*	3	A	99%	AF033483
R	**p02**	*Penicillium canescens*	1	A	100%	AY373901.1
R	**p43**	*Penicillium sp.*	1	A	98%	AF455543.1
R	**NG_25**	*Penicillium ochrochloron*	3	A	99%	DQ093695.1
R	**NG_18**	*Penicillium sp.*	1	A	99%	AB274312.1
R	**p53**	*Preussia sp.*	1	A	99%	AY943061.1
R	**NG_16**	*Trichoderma atroviride*	1	A	100%	DQ841734.1
T	**NG_36C**	*Bionectriaceae sp.*	3	A	98%	EU552110.1
T	**NG_32**	*Candida sp.*	2	A	100%	AM160629.1
T	**NG_21B**	*Cercophora/Apodus sp.*	3	A	99%	AF064642.1|AF064642
T	**NG_09**	*Davidiella tassiana*	1	A	99%	AF297231.1|AF297231
T	**NG_21A**	*Helicodendron sp.*	3	A	98%	EF029238.1
T	**NG_36B**	*Helotiales sp.*	3	A	94%	AY188359.1
T	**NG_27**	*Hypocrea lixii*	3	A	99%	EF191309.1
T	**NG_06**	*Penicillium canescens*	1	A	99%	AY373901.1
T	**NG_20**	*Penicillium sp.*	3	A	98%	AF178522.1|AF178522
T	**NG_11**	*Penicillium sp.*	1	A	99%	EF070712.1
T	**NG_10**	*Phoma cf. eupyrena*	1	A	100%	AJ890436.1
T	**NG_21C**	*Sordariomycetes sp.*	3	A	90%	EF027383.1
T	**NG_12**	*Hypocreaceae sp.*	1	A	98%	EF417482.1
T	**NG_04**	*Trichoderma koningiopsis*	1	A	100%	DQ379015.1

*) M = medium, 1: MEA + BR, 2: MEA + BR + B, 3: AMM + BR

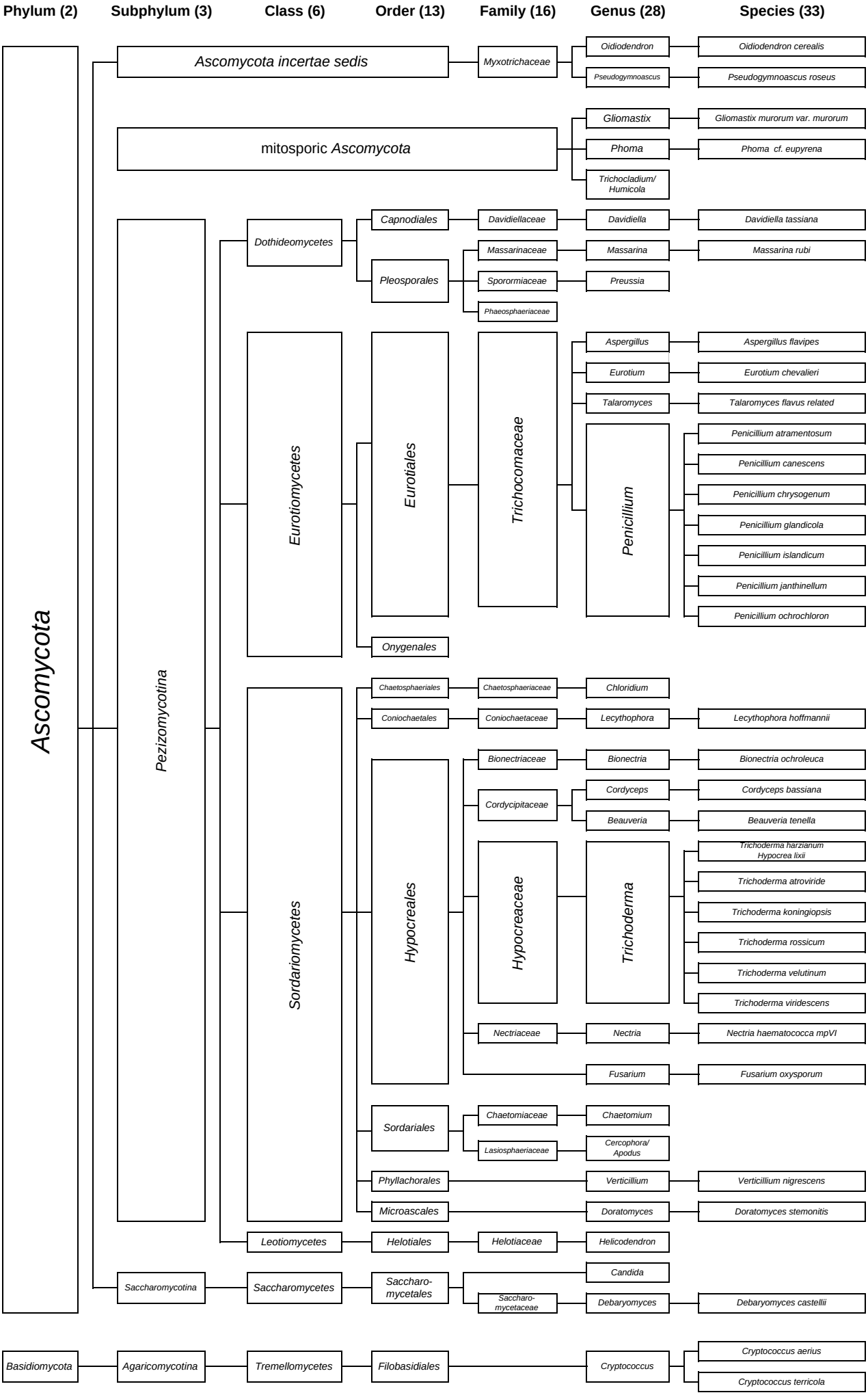


Fig.3.3. Taxonomy of cultured fungi which could be identified to the species level. Numbers in parentheses indicate counts in the respective taxonomic level.

The fungal community, illustrated here by cultivable (under described conditions) fungi, of all soil samples taken together consisted of 61 different species, 4 species belonging to the phylum *Basidiomycota* and 57 species belonging to the phylum *Ascomycota*. Cultivable fungi from the *Mucorales* (subphylum *Mucoromycotina*) were found on plates but not further investigated (see above). Altogether, species belonging to 16 different families, 13 different orders, 6 different classes as well as 3 different subphyla were found (exclusive uncertain taxonomic groups indicated as *incertae sedis*, Fig.3.3). Most of the species belong to the *Pezizomycotina*, which cover the classes *Sordariomycetes* (25 found species), *Eurotiomycetes* (15) and *Dothideomycetes* (7), and which contribute the most to the species variety. The class *Eurotiomycetes* almost exclusively consists of species, which belong to the *Trichocomaceae* (14) containing *Penicillium*, *Talaromyces*, *Aspergillus* and *Eurotium* species, whereas the class *Sordariomycetes* is mainly represented by members of the *Hypocreales* (16). The most abundant order of *Dothideomycetes* is *Pleosporales* (5) including *Massarina* and *Preussia*, among others.
In total, 28 different genera were detected. The genera *Penicillium* and *Trichoderma* showed the highest diversity, comprising 11 different *Penicillium* species and 8 different *Trichoderma* species (teleomorph *Hypocrea*), including species designated as *Penicillium* sp. or *Trichoderma* sp., respectively, for which the identification to the species level was not possible.

3.2. Diversity analysis of fungal ITS/LSU sequences (clone libraries)

To assess the fungal community of the soil samples, which were amenable to DNA analysis, a direct PCR approach was undertaken. For this, DNA was extracted directly from soil and subjected to ITS/LSU-PCR using the primer ITS1F specific for fungi and the universal eukaryotic primer TW13 for the 5’ LSU rRNA gene region. Clone libraries containing 96 clones for each soil sample were constructed in plasmid pTZ57R/T. Inserts were amplified with primer pair ITS1F/TW13. PCR products were directly subjected to RFLP analyses to estimate the sequence diversity within the clone libraries. The reaction was performed with the restriction endonuclease *Bsu*RI (isoschizomere of *Hae*III) and the fragments were separated on a 3 % high resolution agarose gel. Representative clones for each pattern were selected randomly for sequencing.

RFLP-patterns for each library are shown in Fig.3.4 – Fig.3.8. RFLP results are depicted in a subjacent table for each soil (Tab.3.3 – Tab.3.7): clones of distinct restriction patterns are listed in the left column. Clones which gave rise to a similar pattern are mentioned beside and the frequency at which a pattern occurs is shown in the right column. Codes of the clones refer to the position of the 96-well microtiter plate in which they are stored. Letters and numbers denote lines and columns of the microtiter plate, respectively.

Clone library M (soil Maissau)

The scheme for restriction products applied on the gels is as follows.

A	01	02	03	04	05	06	B	01	02	03	04	05	06	C	01	02	03	04	05	06	D	01	02	03	04	05	06
E	01	02	03	04	05	06	F	01	02	03	04	05	06	G	01	02	03	04	05	06	H	01	02	03	04	05	06
A	07	08	09	10	11	12	B	07	08	09	10	11	12	C	07	08	09	10	11	12	D	07	08	09	10	11	12
E	07	08	09	10	11	12	F	07	08	09	10	11	12	G	07	08	09	10	11	12	H	07	08	09	10	11	12

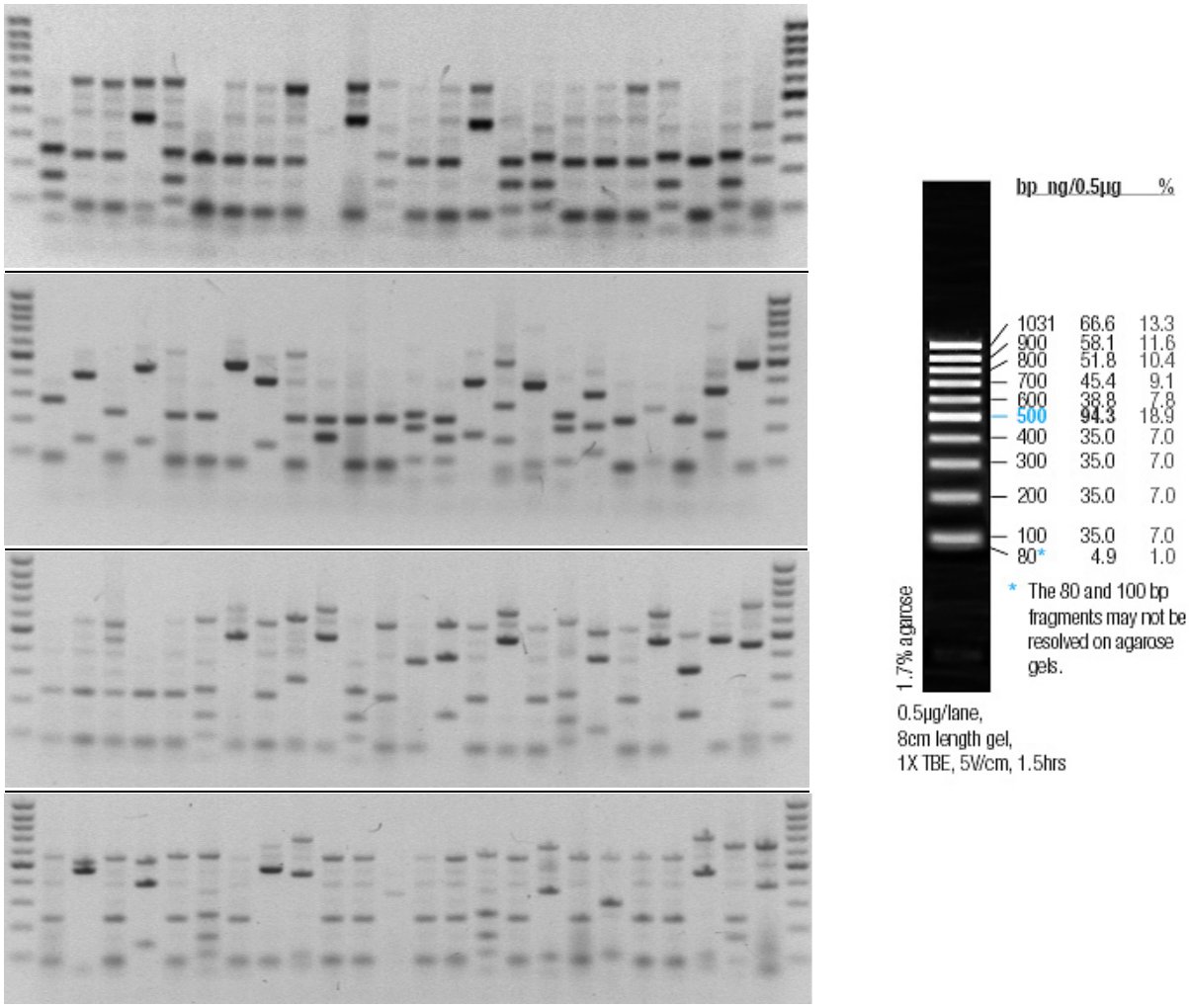


Fig.3.4. RFLP-analysis of 96 clones obtained from soil sample M (left) and GeneRuler™ 100bp DNA Ladder (right).

Tab.3.3. RFLP-analysis of 96 clones obtained from soil sample M.

Clone	Identical patterns	Frequency
A01	D05	2
A02	A03 B01 B02 B03 C01 C11 C02 C06 C09 D01 D02 D08 E05 F10 F11 F03 G10 G07 G08 H08 H09	22
A04	B05 C03	3
A05	B06 B12 C05 C12 D03 E12 G12 G09 H12	10
A06	A07 A10 A11 D04 E01 E03 E06 F05 F06 F07 H02 H04	13
A08	A09 B08 B09 E07 E09	6
B04	F12 H03	3
B10	C10 D09 D11	4
B11		1
C04	G02 F04	3
C08		1
D06	H01	2
D07	E10	2
D10		1
D12	F09 H10	3
E02		1
E04		1
E08		1
F01	H06 B07 F08	4
F02		1
G01	G06	2
G03	H05	2
G04		1
G05	C07	2
G11	H12	2
H07		1
H11		1

Clone library N (soil Niederschleinz)

A 01 02 03 04 05 06 B 01 02 03 04 05 06 C 01 02 03 04 05 06 D 01 02 03 04 05 06
E 01 02 03 04 05 06 F 01 02 03 04 05 06 G 01 02 03 04 05 06 H 01 02 03 04 05 06
A 07 08 09 10 11 12 B 07 08 09 10 11 12 C 07 08 09 10 11 12 D 07 08 09 10 11 12
E 07 08 09 10 11 12 F 07 08 09 10 11 12 G 07 08 09 10 11 12 H 07 08 09 10 11 12

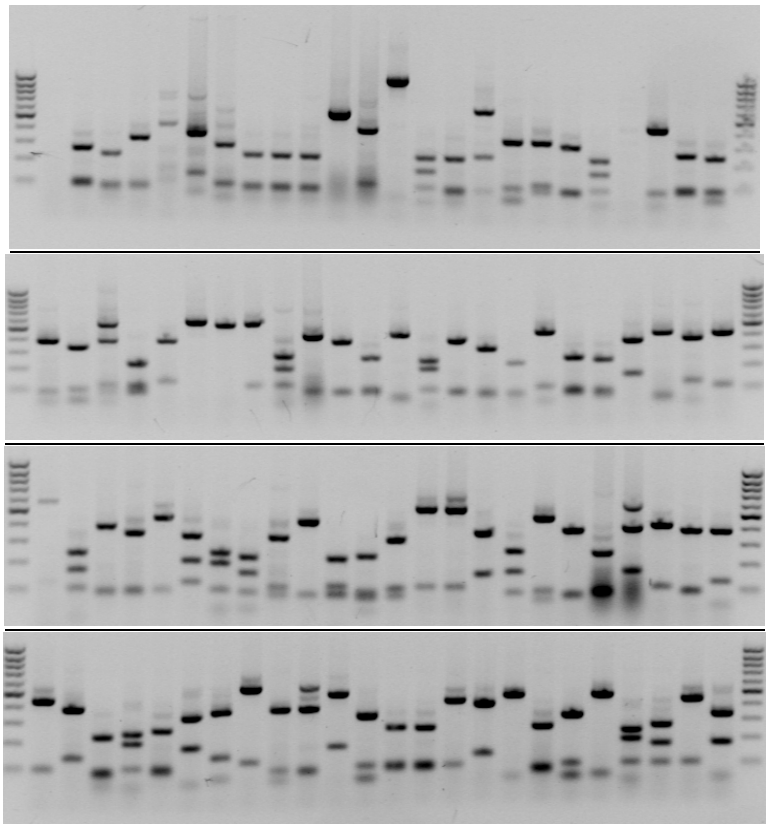


Fig.3.5. RFLP-analysis of 96 clones obtained from soil sample N.

Tab.3.4. RFLP-analysis of 96 clones obtained from soil sample N.

Clone	Identical patterns										Frequency
A02	D05	F06	G05	H01	H02	E11	G12	G07	G08	B12	11
A03	B02	B03	B04	C03	D06						6
A04	C06	B10	D11	F09							5
A05											1
A06	C10	D12	E08	F07	H05	H03					7
A08	C11	H10									3
A11	C12	C08	C09	G11							5
A12	E12	H12									3
B01	D01	G04									3
B05	F01	F02									3
B06	D04	G01	H04	A09	D10	G06	H06				8
C01											1
C02	D02										2
C04											1
C05	C07	B09	F12	H07							5
D09											1
E01	D07	F04									3

E02	F05 G03 A10 E05	5
E03		1
E04	B11	2
E06	A07	2
E07	G09 H11	3
E09		1
E10	H09	2
F08	A08	2
H09	E10 B07 G02	4

Clone library P (soil Purkersdorf)

A 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 B
C 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 D
E 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 F
G 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 H

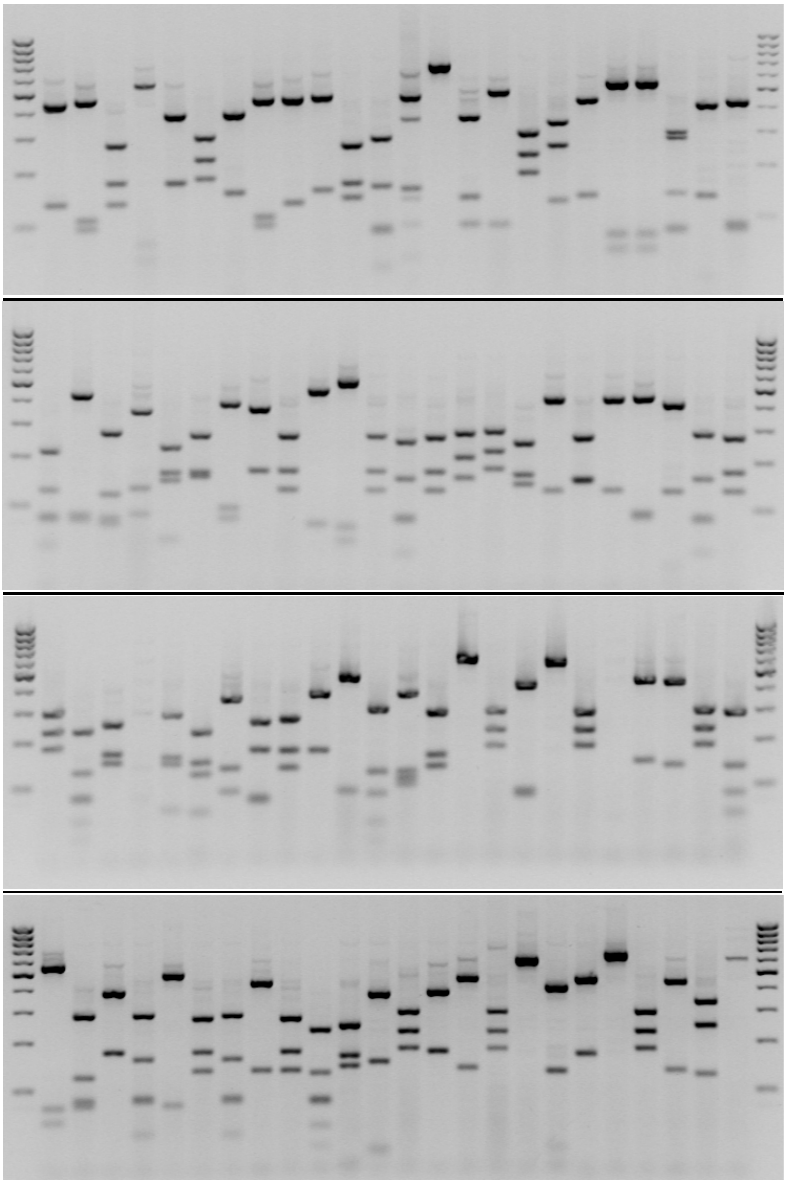


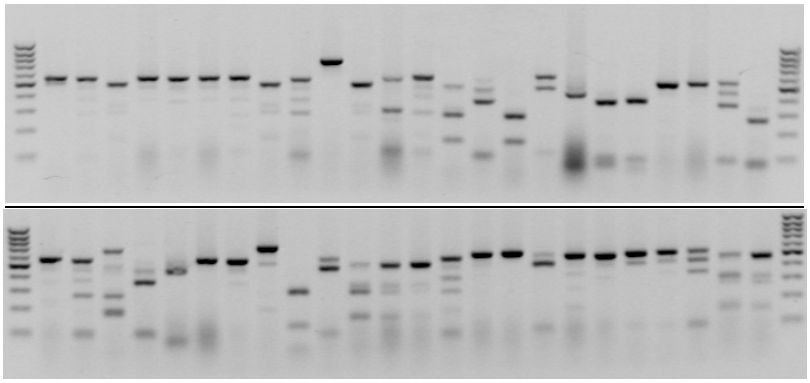
Fig.3.6. RFLP-analysis of 96 clones obtained from soil sample P.

Tab.3.5. RFLP-analysis of 96 clones obtained from soil sample P.

Clone	Identical patterns									Frequency
A01	B11	D06	D08	F10	H06	B07	F09	A09		9
A02	A08	B12								3
A03	C12	D02	D12	E09	G06	G09	C09			8
A04	B08	B09	C11	G01						5
A05	C08	E10	G03	H02						5
A06	B05	D04	E01	F04	F07	F11	H01	H04	H09	10
A07	D10	F01	G12	C07						5
A10	H03	H10								3
A11	D05	C05	E03	G11						5
A12	D11	E12	F12	G02						5
B01										1
B02	F03	F06	H05	H08	H12					6
B03	C04	E07								3
B04	E11	G05	C10							4
B06	H11									2
B10										1
C01										1
C02	D09	F05								3
C03										1
D01	G10									2
D03										1
D07										1
E02										1
E03										1
E05	F02									2
E06										1
E08										1
G04	G07									2
G08	H03	H10								3
H02										1
H07										1

Clone library R (soil Riederberg)

A 01 02 03 04 05 06 B 01 02 03 04 05 06 C 01 02 03 04 05 06 D 01 02 03 04 05 06
E 01 02 03 04 05 06 F 01 02 03 04 05 06 G 01 02 03 04 05 06 H 01 02 03 04 05 06
A 07 08 09 10 11 12 B 07 08 09 10 11 12 C 07 08 09 10 11 12 D 07 08 09 10 11 12
E 07 08 09 10 11 12 F 07 08 09 10 11 12 G 07 08 09 10 11 12 H 07 08 09 10 11 12



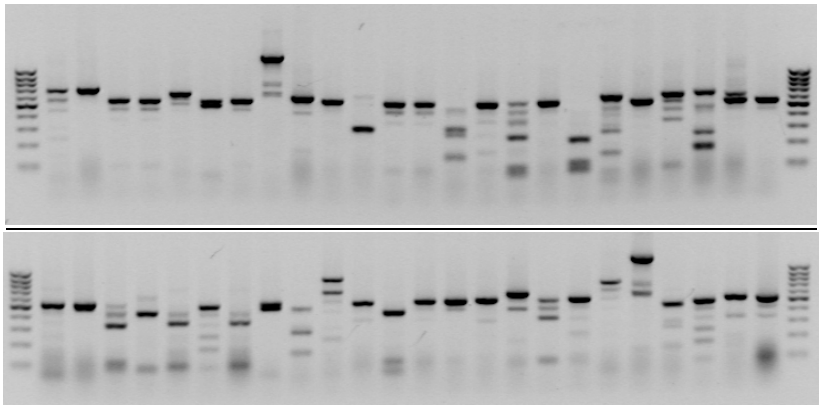


Fig.3.7. RFLP-analysis of 96 clones obtained from soil sample R.

Tab.3.6. RFLP-analysis of 96 clones obtained from soil sample R.

Clone	Identical patterns	Frequency
B01	A06 A05 A04 A02 G09 E01	7
B04		1
B06		1
B08	H08	2
B12	A01 B07 A10 A09 H02 H03 G08 D12 C07 G07 G09 H12 F01 G03	15
C04		1
C05		1
C06	E05	2
C12		1
D01		1
D02		1
D03	D04 E06 G04	4
D06		1
D10	E03	2
E09	D05 G11 H04 C03	5
E11	F07 E04	3
F02	H07	2
F03		1
F04	G05	2
F10		1
G01	A03 B02 B05 F06 H09	6
G02	B03 G06 C09 H10 C01 E02	7
H06	H05 C02 C08 F05 F09	6
H11	G09	2

Clone library T (soil Tulln)

A 01 02 03 04 05 06 B 01 02 03 04 05 06 C 01 02 03 04 05 06 D 01 02 03 04 05 06
E 01 02 03 04 05 06 F 01 02 03 04 05 06 G 01 02 03 04 05 06 H 01 02 03 04 05 06
A 07 08 09 10 11 12 B 07 08 09 10 11 12 C 07 08 09 10 11 12 D 07 08 09 10 11 12
E 07 08 09 10 11 12 F 07 08 09 10 11 12 G 07 08 09 10 11 12 H 07 08 09 10 11 12

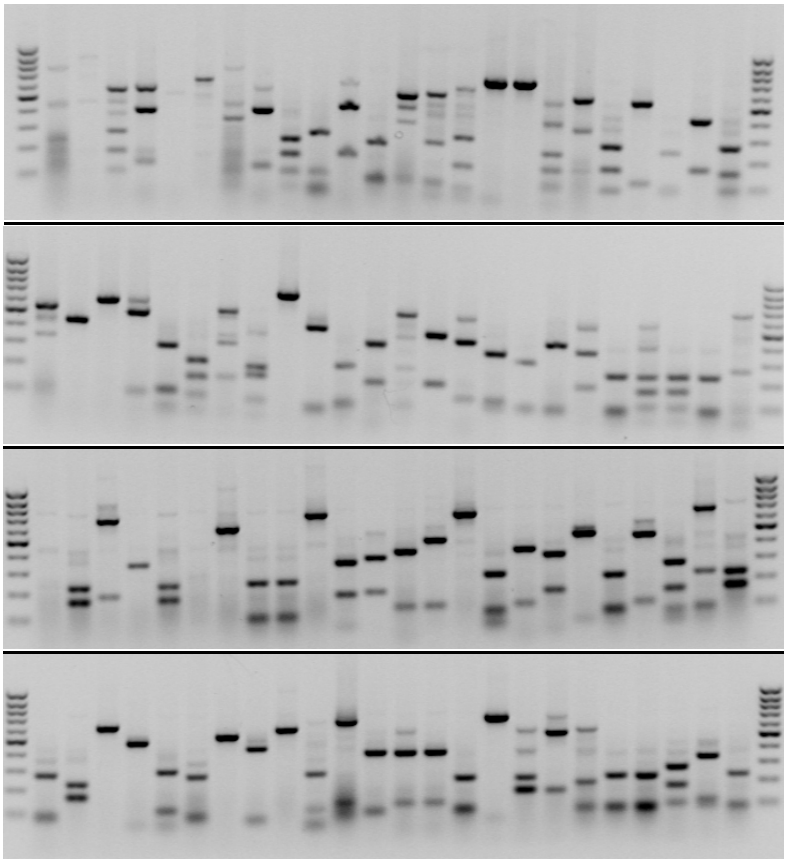


Fig.3.8. RFLP-analysis of 96 clones obtained from soil sample T.

Tab.3.7. RFLP-analysis of 96 clones obtained from soil sample T.

Clone	Identical patterns	Frequency
A01		1
A03		1
A04	B02 G12 G3	4
A10		1
B11	B12 F06 C12	4
C01	E01	2
C02		1
C04	C05 F03 B10 C09 G10 A09	7
C06	H03 H04 E06	4
C07	G02 D05 C11 F12	5
C10	F10 B04 E05	4
D01		1
D03	F07	2
D07		1
D12	A08 A11 E08 F02 B03	6
E04	D09 C08	3
E09	F09 B07 E03	4

E10	E02 F04	3
F01		1
F08	G06	2
F11		1
G01		1
G11		1
H01		1
H06		1
H07		1
H09	H08 G09 D08 B08 B09 H05 H02 F05 E07 H12 E11 D04 B06	14
H10	D02 D06 D10	4
H11	G07 G08 G04 G05 E12	6

3.2.1. Diversity screening via RFLP

RFLP screening was done for 96 clones per each soil sample using enzyme *Bsu*RI. The number of obtained patterns for cloned ITS/LSU fragments was 27 for M, 26 for N, 31 for P, 24 for R and 29 for T (Tab.3.8). At least one representative of each pattern was sequenced. The number of clones which were sequenced for each soil sample is given in Tab.3.8. Taken together, 201 clones were sequenced.

Tab.3.8. Overview of RFLP-analyses

soils:	**M**	**N**	**P**	**R**	**T**
different patterns:	27	26	31	24	29
drop-outs (no PCR-product obtained):	0	2	2	0	4
maximal frequency of identical patterns:	22	11	10	15	14
number of clones sequenced:	30	48	40	48	35

The whole set of identified fungal sequences from the cloning approach is given in Tab.3.9.

After sequences have been obtained for each RFLP-pattern, RFLP-types were defined (Tab.3.10). This was performed manually by comparing the RFLP-pattern-analysis with the sequencing results as well as by sequence analysis of individual sequences.

Tab.3.9. Identified fungi from soil sample clone libraries.

Clone	soil	Identification	% ID (ITS + LSU)	Acc.No. (ITS + LSU)	% ID (ITS)	Acc.No. (ITS)	% ID (LSU)	Acc.No. (LSU)	#
A02	M	*Chaetomiaceae*	99	AY346305.1	98	EF192179.1	99	AY346305.1	22*
A03	M	*Trichocladium asperum*	94	EU040239.1	98	AM292050.1	98	AM292051.1	
A06	M	*Lasiosphaeriaceae*	95	EU040239.1	94	AY219880.1	99	AY780059.1	13
A05	M	*Myrothecium sp.*	97	AJ302002.1|MAT302002	94	AJ302005.1	99	AJ302000.1	11
A08	M	*Trichocladium asperum*	95	EU040239.1	98	AM292050.1	98	AM292051.1	6
B07	M	*Scleroderma bovista*	90	EU046005.1	98	AB211267.1	99	AF336264.1	4*
H06	M	*Scleroderma bovista*	90	EU046005.1	98	AB211267.1	99	AF336264.1	
B10	M	*Scleroderma bovista*	90	EU046005.1	98	AB099901.1	99	AF336264.1	4
A04	M	*Hapsidospora/Acremonium sp.*	93	AM421065.1	98	U57672.1|ACU57672	99	AF096192.1|AF096192	3
C04	M	*Cercophora sp.*	93	EU040239.1	99	AY999136.1	99	AY780067.1	3
D12	M	*Hebeloma sp.*	97	AF430291.1	99	AY311526.1	99	EF561632.1	3
H03	M	*Nectria sp.*	90	AJ301967.1|VCI301967	100	DQ317342.1	95	AB027379.1	3
A01	M	*Myrothecium sp.*[1]	-	n/a	-	n/a	97	AJ301999.1|MVE301999	2
D07	M	*Lasiosphaeriaceae*	90	EU040239.1	98	DQ166963.1	97	AF064642.1|AF064642	2
G01	M	*Cyphellophora laciniata*	99	EU035416.1	98	EU035416.1	99	EU035416.1	2*
G06	M	*Cyphellophora laciniata*	98	EU035416.1	98	EU035416.1	99	EU035416.1	
G03	M	*Soradariales*	96	EF027383.1	94	AF443851.1|AF443851	97	AY999099.1	2
G05	M	*Hapsidospora/Acremonium sp.*	94	AM421065.1	98	U57672.1|ACU57672	99	AF096192.1|AF096192	2
G11	M	*Paecilomyces carneus*	92	L07138.1|EPIRGITSH	100	AB258369.1	100	EF468843.1	2
H01	M	*Minimedusa polyspora*	99	DQ915476.1	98	DQ915476.1	99	DQ915476.1	2
B11	M	*Myrothecium sp.*	97	AJ302002.1|MAT302002	94	AJ302005.1|MIN302005	99	AJ302000.1|MLE302000	1
C08	M	*Lasiosphaeriaceae*	96	EF027383.1	95	AF443851.1|AF443851	97	AY999099.1	1
D10	M	*Lasiosphaeriaceae*	91	EU040239.1	96	AY999128.1	98	AY999106.1	1
E02	M	*Lasiosphaeriaceae*	90	EU040239.1	98	DQ166963.1	97	AF064642.1|AF064642	1
E04	M	*Hypocreales*	89	AF335453.1	98	AJ890439.1	95	AF210671.1	1
E08	M	*Trichosporon dulcitum*	97	AB180200.1	99	AF444428.1	100	AF075517.1|AF075517	1
F02	M	*Lasiosphaeriaceae*	97	AF064642.1|AF064642	97	DQ166963.1	97	AF064642.1|AF064642	1
H07	M	*Pleosporales*	90	DQ885897.1	99	AJ246158.1|PMA246158	99	DQ810223.1	1

[1] Identification was based on the position in the distance tree of results

Tab.3.9. (continued)

Clone	soil	Identification	% ID (ITS + LSU)	Acc.No. (ITS + LSU)	% ID (ITS)	Acc.No. (ITS)	% ID (LSU)	Acc.No. (LSU)	#
H11	M	*Trichocladium asperum*	94	EU040239.1	99	AM292050.1	98	AM292051.1	1
A02	N	*Hypocreales*	92	AJ301967.1|VCI301967	99	DQ779785.1	96	AY138481.1	12*
D08	N	*Hypocreales*	91	AJ301967.1|VCI301967	99	DQ317342.1	95	AY138481.1	
B06	N	*Botryotinia sp.*	88	AY394901.1	99	EU563125.1	99	AY544651.1	8*
A09	N	*Stachybotrys chartarum*			99	AY095980.1	99	AF081468.2	
A06	N	*Pyrenophora tritici-repentis*	95	AY154681.1	100	AY739861.1	99	DQ384097.1	7
A03	N	*Sordariales*	95	EU040239.1	95	DQ854987.1	97	EU040239.1	6
A04	N	*Ascomycota*	85	AF081480.2|AF081480	-	MUC	92	AF081478.2|AF081478	5
A11	N	*Tetracladium sp.*	99	EU883430.1	99	EU883430.1	99	EU883430.1	5*
C08	N	*Thanatephorus cucumeris*	97	AF354078.1	100	AF472512.1	99	AF354111.2	
C09	N	*Thanatephorus cucumeris*	97	AF354078.1	99	AY684922.1	98	AF354111.2	
C12	N	*Mortierella alpina*	90	EU428773.1	99	EU076962.1	94	DQ273794.1	
G11	N	*Davidiella sp.*	100	EF679369.2	100	EU622923.1	100	EF679390.2	
C05	N	*Verticillium nigrescens*	90	AJ301962.1|VCO301962	100	AJ292440.1|VNI292440	99	EF543841.1	5*
B09	N	*Fusarium incarnatum*	99	AY633745.1	99	AY147368.1	99	AY213706.1	
C07	N	*Fusarium incarnatum*	99	AY633745.1	100	AY147362.1	99	EU214561.1	
E02	N	*Sordariomycetes*	97	AF222497	98	AM922222.1	98	EF543841.1	5
C02	N	*Sordariales*	96	DQ900985.1	95	AY999129.1	97	AY346271.1	4*
B08	N	*Sordariales*	96	DQ900985.1	95	AY999129.1	97	AY346271.1	
D02	N	*Lasiosphaeriaceae*	96	DQ900985.1	94	AY999129.1	97	DQ900985.1	
F03	N	*Basidiomycota*	95	DQ915476.1	98	DQ915476.1	98	DQ457641.1	
E10	N	*Cyphellophora laciniata*	98	EU035416.1	98	EU035416.1	99	EU035416.1	4*
H09	N	*Cyphellophora laciniata*	98	EU035416.1	98	EU035416.1	98	EU035416.1	
A08	N	*Acremonium strictum*	96	AJ558115.1|NMA558115	99	AY138848.1	100	AY138485.1	3
A12	N	*Minimedusa polyspora*	98	DQ915476.1	98	DQ915476.1	99	DQ915476.1	3*
E12	N	*Ascomycota*	94	AY188359.1	100	DQ420923.1	98	AY188359.1	
B01	N	*Pleosporales*	93	AY293790.1	89	EF120414.1	96	AY510387.1	3
B05	N	*Stachybotrys chartarum*	100	AF081468.2	100	AF081468.2	100	AF081468.2	3*
F01	N	*Xylariales*	95	DQ384572.1	99	EF187912.1	99	AF452030.1	

Tab.3.9. (continued)

Clone	soil	Identification	% ID (ITS + LSU)	Acc.No. (ITS + LSU)	% ID (ITS)	Acc.No. (ITS)	% ID (LSU)	Acc.No. (LSU)	#
E01	N	*Lasiosphaeriaceae*	87	EU040239.1	98	AY999135.1	99	AY999110.1	3*
D07	N	*Nectria mauritiicola*	99	AJ558115.1|NMA558115	99	AY138846.1	100	AJ558115.1|NMA558115	
E07	N	*Tetracladium sp.*	99	EU883430.1	99	EU883430.1	99	EU883430.1	3*
H11	N	*Tetracladium sp.*	97	EU883430.1	99	DQ068996.1	94	AY204612.1	
E04	N	*Nectria haematococca*	99	AY633746.1	99	AB305107.1	100	AY188918.1	2
E06	N	*Pleosporales*	98	EF590319.2	98	AM901685.1	98	DQ885894.1	2
F08	N	*Thanatephorus praticola*	98	AF354078.1	100	DQ355140.1	99	AF354111.2	2*
H08	N	*Cystofilobasidiales*	87	AM922290.1	99	AF444418.1	100	EF551318.1	
A05	N	*Botryotinia sp.*	88	AY394901.1	100	EU563125.1	99	AY544651.1	1
C01	N	unidentified fungus	-	MUC	-	MUC	89	DQ273789.1	1
C04	N	unidentified fungus	84	EF413029.1	86	EF434096.1	84	EF413029.1	1
D09	N	*Hypocrea sp.*	98	AB027384.1	98	AY857210.1	99	AF127148.1|AF127148	1
E03	N	*Lasiosphaeriaceae*	91	EF027383.1	98	AY999135.1	99	AY999110.1	1
E09	N	*C. globosum related*	97	AJ620951.1	98	AY533556.1	97	AJ746203.1	1
F10	N	*Nectria mauritiicola*	99	AJ558115.1|NMA558115	99	AY138846.1	99	AJ558115.1|NMA558115	1
F11	N	*Chytridiomycota*	92	AY546692.1	95	AF216770.1	92	AY546692.1	1
G10	N	*Alternaria sp.*	99	AY154712.1	100	EU326181.1	99	DQ678068.1	1
A06	P	*Basidiomycota*	86	AY542864.1	84	AM113462.1	95	AF506709.1	10*
B05	P	*Basidiomycota*	95	AF506709.1	86	AM113462.1	95	AF506709.1	
A01	P	*Tetracladium sp.*	96	EU883430.1	94	EU754979.1	100	EU883432.1	9*
B11	P	*Bionectriaceae*	98	AF210677.1	97	EU567315.1	99	AF210690.1	
D08	P	*Tetracladium sp.*	98	EU883432.1	96	FJ000374.1	99	EU883432.1	
A03	P	*Tetracladium maxilliforme*	99	EU883430.1	100	DQ068996.1	99	EU883430.1	8*
C09	P	*Tetracladium sp.*	99	EU883432.1	99	FJ000375.1	99	EU883432.1	
B02	P	*Mortierellaceae*	93	EF027378.1	88	EF027378.1	95	AF157197.1|AF157197	6*
F03	P	*Pyronemataceae*	88	DQ206862.1	99	AF072091.1|AF072091	96	AY544654.1	
A04	P	*Cryptococcus terricola*	99	AM922287.1	99	EU252550.1	100	EF068204.1	5
A05	P	*Helotiales*	93	AF284122.1	97	EF029238.1	97	AF222454.1	5*
H02	P	*Helotiales*	93	AY394907.1	97	EF029238.1	96	EU040231.1	
C08	P	*Helotiales*	93	EU040232.1	98	AF128439S2	96	EU040231.1	

Tab.3.9. (continued)

Clone	soil	Identification	% ID (ITS + LSU)	Acc.No. (ITS + LSU)	% ID (ITS)	Acc.No. (ITS)	% ID (LSU)	Acc.No. (LSU)	#
A07	P	*Schizothecium sp.*	89	EU040239.1	97	AY999118.1	99	AY780076.1	5
A12	P	*Herpotrichiellaceae*	99	AF050276.1	99	EU046043.1	100	EU046043.1	5
A11	P	*Lecythophora sp.*	97	AY219880.1	96	AY219880.1	98	AY219880.1|	4
B04	P	*Neonectria radicicola*	93	AJ301967.1|VCI301967	99	AJ279490.1|CSP279490	100	AY677313.1	4*
G05	P	*Mycosphaerellaceae*	97	EU019284.1	96	EU019284.1	98	EU019257.2	
A02	P	*Pucciniomycotina*	90	AM410637.1	82	AF444507.2	97	AF189896.1	3
A10	P	*Pyronemataceae*	87	DQ206862.1	90	DQ491500.1	99	AY500531.1	3
B03	P	*Tetracladium maxilliforme*	99	EU883430.1	100	DQ068996.1	99	EU883432.1	3
C02	P	*Nectria mauritiicola*	99	AJ558115.1	100	AJ558115.1|NMA558115	99	AJ558115.1|NMA558115	3
E07	P	*Tetracladium maxilliforme*	99	EU883430.1	100	DQ068996.1	100	EU883432.1	3
B06	P	*Minimedusa polyspora*	99	DQ915476.1	98	DQ915476.1	100	DQ915476.1	2
D01	P	*Herpotrichiellaceae*	99	AF050276.1|AF050276	98	EU046043.1	100	AB100625.1	2
D07	P	*Leotiomycetes*	98	EU035444.1	94	DQ317329.1	99	EU035444.1	2
E05	P	*Leptodontidium orchidicola*	96	EU035444.1	99	AF486133.1	98	EU035444.1	2
G04	P	*Herpotrichiellaceae*	99	EU046043.1	99	EU046043.1	100	EU046043.1	2
B01	P	*Pyronemataceae*	98	AY500531.1	87	DQ491500.1	98	AY500531.1	1
B10	P	*Helotiales*	94	EF596821.1	94	U57494.1|CPU57494	97	AF222478.1	1
C01	P	*Sordariales*	94	EU040239.1	95	EU543258.1	96	AJ620951.1	1
C03	P	*Sordariomycetes*	89	EU040239.1	92	EF197068.1	94	AY346300.1	1
D03	P	*Leotiomycetes*	97	EU035444.1	97	DQ679495.1	99	AB100621.1	1
E02	P	*Chaetomiaceae*	97	EU040239.1	98	AY533556.1	99	AF096186.1|AF096186	1
E03	P	*Fusarium sp.*	96	EF453189.1	95	AY230193.1	98	EF453189.1	1
E06	P	*Boliniales*	90	EU040239.1	86	DQ185070.1	96	EF154451.1	1
E08	P	*Helotiales*	97	AY188359.1	95	AY188359.1	98	AY188359.1	1
G08	P	*Tetracladium sp.*	97	EU883430.1	93	FJ000371.1	99	EU883432.1	1
H07	P	*Hypocrea sp.*	95	AB027384.1	100	EF601618.1	98	AB027384.1	1
E09	P	*Tetracladium sp.*	98	EU883432.1	97	J000374.1	99	EU883432.1	1

Tab.3.9. (continued)

Clone	soil	Identification	% ID (ITS + LSU)	Acc.No. (ITS + LSU)	% ID (ITS)	Acc.No. (ITS)	% ID (LSU)	Acc.No. (LSU)	#
B12	R	*Tetracladium sp.*	99	EU883430.1	97	DQ068996.1	100	EU883432.1	14*
G03	R	*Sordariomycetes*	87	AB278173.1	96	AM922199.1	97	U46889.1|LCU46889	
A09	R	*Tetracladium sp.*	99	EU883430.1	99	DQ068996.1	100	EU883432.1	
C07	R	*Tetracladium sp.*	99	EU883430.1	100	DQ068996.1	100	EU883432.1	
H12	R	*Hypocreales*	92	AF310976.1|AF310976	100	DQ247775.1	99	AB067706.1	
A03	R	*Fungi SCGI*	86	EU046087.1	91	DQ182440.1	89	EU292556.1	7
G02	R	*Ascomycota*	88	AJ558115.1|NMA558115	85	U57671.1|ASU57671	91	AY281098.1	7*
C09	R	*Tetracladium furcatum*	100	EU883432.1	100	FJ000375.1	100	EU883432.1	
B01	R	*Tetracladium sp.*	98	EU883430.1	100	DQ068996.1	98	EU883432.1	6
H06	R	*Fungi SCGI*	88	EU292507.1	91	DQ182440.1	91	EU292556.1	6*
C02	R	*Fungi SCGI*	87	EU292507.1	91	DQ182440.1	95	EU179598.1	
E09	R	*Fusarium oxysporum*	99	EU214564.1	99	EU214564.1	99	EU214568.1	5*
D05	R	*Tetracladium sp.*	99	EU883430.1	99	DQ068996.1	99	EU883432.1	
D03	R	*Lasiosphaeriaceae*	99	AY780061.1	97	AY999128.1	99	AY780061.1	4*
D04	R	*Lasiosphaeriaceae*	92	DQ900985.1	91	DQ166962.1	96	AY780064.1	
G01	R	*Fungi SCGI*	86	EU292507.1	90	DQ182440.1	89	EU179995.1	4*
E12	R	*Lasiosphaeriaceae*	91	EU040239.1	91	AY999128.1	98	AY780061.1	
F06	R	*Fungi SCGI*	87	EU179598.1	91	DQ182440.1	95	EU179598.1	
C06	R	*Cryptococcus aerius*	94	AM922287.1	99	AF145324.1|AF145324	99	AF181527.1|AF181527	3*
E05	R	*Cryptococcus aerius*	92	AM922287.1	100	AF145324.1|AF145324	99	AF181544.1|AF181544	
E11	R	*Tetracladium sp.*	99	EU883430.1	100	DQ068996.1	99	EU883432.1	3
H11	R	*Phyllachoraceae*	99	DQ286218.1	98	DQ779781.1	99	DQ286218.1	3*
G12	R	*Fusarium sp.*	99	EU563588.1	98	AY633561.1	99	AB363765.1	
B08	R	*Pyronemataceae*	93	EF417800.1	83	EF417800.1	97	EF417800.1	2
B09	R	*Pyronemataceae*	89	DQ206862.1|	97	AF072091.1|AF072091	97	DQ220322.1	2
D06	R	*Ascomycota*	89	AJ558115.1|NMA558115	87	U57671.1|ASU57671	93	AY281098.1	2
D10	R	unidentified fungus	84	AY239018.1	85	AY035644.1	85	DQ273788.1	2*
E03	R	unidentified fungus	-	MUC	92	EU076962.1	85	DQ273788.1	
E08	R	*Davidiella/Cladosporium*	99	EF679369.2	100	EU622923.1	99	EF679390.2	2
F02	R	unidentified fungus	-	MUC	-	MUC	82	AF506409.1	2

Tab.3.9. (continued)

Clone	soil	Identification	% ID (ITS + LSU)	Acc.No. (ITS + LSU)	% ID (ITS)	Acc.No. (ITS)	% ID (LSU)	Acc.No. (LSU)	#
F04	R	*Neonectria radicicola*	93	EU214559.1	100	AJ279490.1|CSP279490	100	AY677313.1|	2
F08	R	*Davidiella/Cladosporium*	100	EF679369.2	100	EU622923.1	100	EF679390.2	2
A11	R	*Cryptococcus aerius*	97	EU046047.1	100	AF145324.1|AF145324	99	AF181544.1|AF181544	1
B04	R	unidentified fungus	82	AF115333.1	85	DQ421136.1	89	DQ341997.1	1
B06	R	*Ascomycota*	-	n/a	-	n/a	96	AY293785.1	1
C04	R	*Fungi SCGI*	-	n/a	-	n/a	95	EU179598.1	1
C05	R	*Hypocreales*	97	AY129552.1	92	AY677294.1	97	AY281098.1	1
C10	R	*Fusarium sp.*	95	AF310976.1|AF310976	98	AY729072.1	97	AY097324.1	1
C12	R	*Fusarium solani*	99	AY633746.1	99	AY633746.1	100	AY097318.1	1
D01	R	*Basidiomycota*	-	MUC		MUC	95	DQ341916.1	1
D02	R	*Cryptococcus sp.*	99	DQ000318.1	100	AM160648.1	99	AM160648.1	1
D07	R	*Herpotrichiellaceae*	97	AF050274.1|AF050274	98	DQ008140.1	99	EU046043.1	1
D09	R	*Blastocladiomycota*	-	MUC	92	AY997034.1	84	X90411.1|BEDNA28RR	1
D11	R	*Tetracladium sp.*	99	EU883430.1	99	DQ068996.1	99	EU883432.1	1
F03	R	*Fungi SCGI*	89	EU292507.1	93	DQ182440.1	91	EU292556.1	1
F11	R	*Hypocreales*	86	AJ301967.1|VCI301967	85	EF060655.1	91	AY281098.1	1
H09	T	*Trichocladium related*	94	EU040239.1	99	AM292050.1	99	AM292051.1	14*
B06	T	*Sordariales*	96	EU040239.1	97	AJ390388.1|CSY390388	99	AY346305.1	
G09	T	*Chaetomiaceae*	96	EU040239.1	97	AJ271583.1|THY271583	99	AF286412.1|AF286412	
A06	T	*Herpotrichiellaceae*	97	AF050274	95	AF050274	100	EU046043.1	8*
C05	T	*Coprinellus sp.*	91	AY228352.1	98	AY521250.1	99	AY663837.1	
D12	T	*Sordariales*	97	AY587938.1	96	AF177155	97	DQ376251.1	6
H11	T	*Fusarium sp.*	99	AY188919.1	100	EU214567.1	99	EU214568.1	6
C07	T	*Schizothecium vesticola*	99	AY780076.1	97	AY999118.1	99	AY780076.1	5
B11	T	*Pleosporales*	95	DQ384105.1	90	AJ972795.1	95	DQ384105.1	4
C06	T	*Sordariales*	96	EU040239.1	97	AJ390388.1|CSY390388	99	AY346305.1	4
C10	T	*Pezizomycotina*	91	DQ227261.1	88	DQ227261.1	93	EF608074.1	4
E09	T	*Mucoromycotina*	92	DQ273794.1	86	EF126343.1	92	DQ273794.1	4
H10	T	*Tetracladium sp.*	98	EU883432.1	97	FJ000374.1	99	EU883432.1	4*
D06	T	*Phialophora sp.*	94	EU035444.1	99	AY805586.1	97	EU040232.1	

Tab.3.9. (continued)

Clone	soil	Identification	% ID (ITS + LSU)	Acc.No. (ITS + LSU)	% ID (ITS)	Acc.No. (ITS)	% ID (LSU)	Acc.No. (LSU)	#
A04	T	*Lasiosphaeriaceae*	99	AY780076.1	98	EF197082.1	99	AY780076.1	3
E04	T	*Pezizales*	97	AY500531.1	94	DQ491500.1	97	AY500531.1	3
E10	T	*Davidiella sp.*	100	EF679390.2	100	EF679390.2	100	EF679390.2	3
C01	T	*Neonectria ramulariae*	99	AY677333.1	99	AJ608955.1|	99	AB067706.1	2
H06	T	*Sordariomycetes*	83	AB031196.1	80	DQ528789.1	82	DQ470951.1	1
D03	T	*Psathyrella sp.*	93	AY228352.1	89	AY461837.1	98	DQ986271.1	2
F08	T	*Cryptococcus aerius*	94	AM922287.1	100	AF145324.1|AF145324	99	EF644448.1	2
A01	T	*Lasiosphaeriaceae*	96	AY587936.1	97	AY587911.1	96	AY587936.1	1
A03	T	*Lasiosphaeriaceae*	-	n/a	-	n/a	99	AF064642.1|AF064642	1
B05	T	*Herpotrichiellaceae*	97	AF050274	95	AF050274	100	EU046043.1	1
C02	T	*Helotiales*	91	DQ227263.1	88	DQ227260.1	94	EF608074.1	1
C03	T	*Agaricomycetes*	94	EU118620.1	90	EU118620.1	96	EU118620.1	1
D01	T	*Hypocreales*	93	AJ558115.1|NMA558115	97	AM262391.1	97	AJ558115.1|NMA558115	1
D07	T	*Davidiella sp.*	100	EF679390.2	100	EF679390.2	100	EF679390.2	1
F01	T	*Pleosporales*	96	DQ384098.1	88	AJ972795.1	96	DQ384105.1	1
F11	T	*Chytridiomycota*	-	n/a	-	n/a	93	DQ273776.1	1
G01	T	*Chaetomiaceae*	96	EU040239.1	97	EF192179.1	99	AY346305.1	1
G03	T	*Lasiosphaeriaceae*	88	EU040239.1	96	EF197082.1	99	AY780076.1	1
G11	T	*Sordariales*	97	AY587936.1	97	EF197073.1	97	AY587936.1	1
H01	T	*Ascomycota*	93	EU292653.1	98	AF128440.1|AF128439S2	96	EU040231.1	1
H07	T	*Chaetomiaceae*	96	EU040239.1	97	EF192179.1	99	AY346305.1	1

Identification: classification based on ITS, LSU or both sequences
Acc. No.: accession number of closest hit in NCBI database
ID: identity to closest match in NCBI database
#: frequency
n/a: not available
MUC: match under cutoff
*: additional clone of an RFLP-pattern
SCGI: Soil Clone Group I, unclassified fungal sequences, which have been postulated as a novel sub-phylum of *Ascomycota* by phylogenetic analyses by Porter and co-workers (2008).

Tab.3.10. Classification of sequences in OTUs. Sorting after frequency within each soil.

soil	OTU	Name	#
M	3	*Trichocladium asperum*	**18**
M	6	*Lasiosphaeriaceae sp.7*	**13**
M	5	*Myrothecium sp.2*	**12**
M	2	*Chaetomiaceae sp.1*	**11**
M	7	*Scleroderma bovista*	**8**
M	4	*Hapsidospora/Acremonium sp.*	**5**
M	10	*Lasiosphaeriaceae sp.9*	**4**
M	20	*Nectria sp.*	**3**
M	12	*Hebeloma sp.1*	**3**
M	8	*Cercophora sp.*	**3**
M	19	*Minimedusa polyspora*	**2**
M	18	*Paecilomyces carneus*	**2**
M	17	*Sordariales sp.1*	**2**
M	16	*Cyphellophora laciniata*	**2**
M	1	*Myrothecium sp.1*	**2**
M	21	*Pleosporales sp.1*	**1**
M	14	*Trichosporon dulcitum*	**1**
M	13	*Hypocreales sp.1*	**1**
M	11	*Lasiosphaeriaceae sp.10*	**1**
M	9	*Lasiosphaeriaceae sp.8*	**1**
N	44	*Hypocreales sp.2*	**12**
N	105	*Stachybotrys chartarum*	**7**
N	91	*Pyrenophora tritici-repentis*	**7**
N	99	*Sordariales sp.2*	**6**
N	121	*Ascomycota sp.1*	**5**
N	116	*Tetracladium related*	**5**
N	96	*Botryotinia sp.*	**5**
N	16	*Cyphellophora laciniata*	**4**
N	126	*Acremonium strictum*	**3**
N	106	*Thanatephorus cucumeris*	**3**
N	100	*Sordariales sp.3*	**3**
N	86	*Pleosporales sp.2*	**3**
N	41	*Fusarium incarnatum*	**3**
N	107	*Verticillium nigrescens*	**2**
N	102	*Sordariomycetes sp.1*	**2**
N	87	*Pleosporales sp.3*	**2**
N	78	*Nectria mauritiicola*	**2**
N	67	*Lasiosphaeriaceae sp.1*	**2**
N	43	*Nectria haematococca*	**2**
N	130	*Basidiomycota sp.1*	**1**
N	122	*Ascomycota sp.2*	**1**
N	120	*Alternaria sp.*	**1**
N	108	*Xylariales sp.*	**1**
N	73	*Mortierella alpina*	**1**
N	72	*Minimedusa polyspora*	**1**
N	54	*Hypocrea sp.1*	**1**
N	38	*Fungi sp.1*	**1**
N	34	*Davidiella sp.1*	**1**
N	33	*Cystofilobasidiales sp.*	**1**
N	27	*Chytridiomycota sp.2*	**1**
N	26	*Chytridiomycota sp.1*	**1**
N	25	*Chaetomium globosum related*	**1**

soil	OTU	Name	#
P	127	*Basidiomycota sp.2*	**10**
P	111	*Tetracladium related*	**9**
P	53	*Herpotrichiellaceae sp.2*	**9**
P	112	*Tetracladium related*	**8**
P	95	*Schizothecium vesticola*	**5**
P	52	*Helotiales sp.2*	**5**
P	32	*Cryptococcus terricola*	**5**
P	92	*Pyronemataceae sp.1*	**4**
P	68	*Lecythophora sp.*	**4**
P	116	*Tetracladium related*	**3**
P	94	*Pyronemataceae sp.2*	**3**
P	90	*Pucciniomycotina sp.*	**3**
P	79	*Nectria mauritiicola*	**3**
P	74	*Mortierellaceae sp.*	**3**
P	69	*Leotiomycetes sp.*	**3**
P	81	*Neonectria radicicola*	**2**
P	76	*Mycosphaerellaceae sp.*	**2**
P	72	*Minimedusa polyspora*	**2**
P	70	*Leptodontidium orchidicola*	**2**
P	118	*Boliniales sp.*	**1**
P	103	*Sordariomycetes sp.2*	**1**
P	98	*Sordariales sp.4*	**1**
P	77	*Bionectriaceae sp.*	**1**
P	55	*Hypocrea sp.2*	**1**
P	51	*Helotiales sp.3*	**1**
P	50	*Helotiales sp.4*	**1**
P	45	*Fusarium sp.1*	**1**
P	23	*Chaetomiaceae sp.2*	**1**

Tab.3.10. (continued)

soil	OTU	Name	#
R	116	*Tetracladium related*	**14**
R	36	*Fungi SCGI sp.*	**11**
R	115	*Tetracladium related*	**6**
R	125	*Ascomycota sp.4*	**5**
R	65	*Lasiosphaeriaceae sp.2*	**4**
R	34	*Davidiella sp.2*	**4**
R	30	*Cryptococcus aerius*	**4**
R	114	*Tetracladium related*	**3**
R	104	*Sordariomycetes sp.3*	**3**
R	60	*Hypocreales sp.5*	**3**
R	94	*Pyronemataceae sp.4*	**2**
R	93	*Pyronemataceae sp.3*	**2**
R	81	*Neonectria radicicola*	**2**
R	42	*Fusarium oxysporum*	**2**
R	39	*Fungi sp.2*	**2**
R	129	*Basidiomycota sp.4*	**1**
R	128	*Basidiomycota sp.3*	**1**
R	124	*Ascomycota sp.3*	**1**
R	117	*Blastocladiomycota sp.*	**1**
R	85	*Phyllachoraceae sp.*	**1**
R	59	*Hypocreales sp.3*	**1**
R	58	*Hypocreales sp.4*	**1**
R	53	*Herpotrichiellaceae sp.3*	**1**
R	47	*Fusarium sp.3*	**1**
R	46	*Fusarium sp.2*	**1**
R	43	*Nectria haematococca*	**1**
R	40	*Fungi sp.3*	**1**
R	35	*Eukaryote sp.*	**1**
R	31	*Cryptococcus sp.*	**1**
R	11	*Lasiosphaeriaceae sp.3*	**1**

soil	OTU	Name	#
T	101	*Sordariales sp.5*	**8**
T	95	*Schizothecium vesticola*	**8**
T	97	*Sordariales sp.6*	**7**
T	48	*Fusarium sp.4*	**6**
T	88	*Pleosporales sp.4*	**5**
T	53	*Herpotrichiellaceae sp.1*	**5**
T	83	*Pezizomycotina sp.*	**4**
T	75	*Mucoromycotina sp.*	**4**
T	34	*Davidiella sp.3*	**4**
T	29	*Coprinellus sp.*	**4**
T	24	*Chaetomiaceae sp.4*	**4**
T	3	*Trichocladium sp. related*	**4**
T	82	*Pezizales sp.*	**3**
T	112	*Tetracladium related*	**2**
T	89	*Psathyrella sp.*	**2**
T	84	*Phialophora sp.*	**2**
T	80	*Neonectria ramulariae*	**2**
T	30	*Cryptococcus aerius*	**2**
T	22	*Chaetomiaceae sp.3*	**2**
T	123	*Ascomycota sp.5*	**1**
T	119	*Agaricomycetes sp.*	**1**
T	71	*Leptodontidium sp.*	**1**
T	66	*Lasiosphaeriaceae sp.5*	**1**
T	64	*Lasiosphaeriaceae sp.6*	**1**
T	62	*Lasiosphaeriaceae sp.4*	**1**
T	57	*Hypocreales sp.6*	**1**
T	49	*Helotiales sp.5*	**1**
T	28	*Chytridiomycota sp.3*	**1**

OTU: operational taxonomic unit
#: frequency
SCGI: Soil Clone Group I; unclassified fungal sequences, which have been postulated as a novel sub-phylum of *Ascomycota* by phylogenetic analyses by Porter and co-workers (2008).

3.2.2. Diversity analysis of RFLP-types

For the analysis of the sequences on different taxonomic levels, a database was created (see 2.7.1). This database contains RFLP-type data (i.e. RFLP-type-No, soil sample and abundance) as well as the identification result and the corresponding taxonomic description on each level (species, genus, family, order, class, subphylum and phylum) for each RFLP-type. The data constructed in this way were further analysed with the software SPSS by applying frequency analyses. Outputs of frequency analyses of cases (=RFLP-type) weighted according to their abundance within the respective soil for the different taxonomic levels are depicted in Tab.3.11a – e. Taxa of uncertain position are listed as *incertae sedis*. These taxa have been placed as species of the least inclusive level in the hierarchy to which they can be assigned with confidence. The term 'basal fungal lineages' represents early-diverging groups, traditionally placed within the *Zygomycota* and *Chytridiomycota*.

Tab.3.11. Composition of clone libraries from different soils in %.

a) Phylum	M	N	P	R	T
Ascomycota	88.4	89.0	75.5	85.4	83.9
Basidiomycota	11.6	6.6	21.3	8.5	10.3
Chytridiomycota		2.2			1.1
Basal fungal lineages		1.1	3.2		4.6
Fungi unidentified		1.1		3.7	
Blastocladiomycota				1.2	
Eukaryota unidentified				1.2	
Total	100.0	100.0	100.0	100.0	100.0

b) Phylum	Subphylum	M	N	P	R	T
Eukaryota	*Eukaryota i. s.*				1.2	
Fungi	*Fungi i. s.*		1.1		3.7	
Ascomycota	*Ascomycota i. s.*		6.6		7.3	1.1
	mitosporic *Ascomycota*	18.9	13.2	23.4	28.0	8.0
	Pezizomycotina	69.5	69.2	52.1	36.6	74.7
	SCGI				13.4	
Basidiomycota	*Basidiomycota i. s.*		1.1	10.6	2.4	
	Agaricomycotina	11.6	5.5	7.4	6.1	10.3
	Pucciniomycotina			3.2		
Chytridiomycota	*Chytridiomycota i. s.*		2.2			1.1
Blastocladiomycota	*Blastocladiomycota i. s.*				1.2	
Basal fungal lineage	*Mucoromycotina*		1.1	3.2		4.6
	Total	100	100	100	100	100

Basal fungal lineages: early-diverging groups, which traditionally have been placed within the *Zygomycota* and *Chytridiomycota*. SCGI: Soil Clone Group I; unclassified fungal sequences, which have been postulated as a novel sub-phylum of *Ascomycota* by phylogenetic analyses by Porter and co-workers (2008).
i. s.: incertae sedis; taxa of uncertain position, which have been placed as species of the least inclusive level in the hierarchy to which they can be assigned with confidence.

Tab.3.11. (continued)

c) Phylum	Subphylum	Class	M	N	P	R	T
Eukaryota	*Eukaryota incertae sedis*					1.2	
Fungi	*Fungi incertae sedis*	*Fungi incertae sedis*		1.1		3.7	
Ascomycota	*Ascomycota incertae sedis*	*Ascomycota incertae sedis*		6.6		7.3	1.1
	Pezizomycotina	*Sordariomycetes*	66.3	44.0	22.3	25.6	49.4
		Eurotiomycetes	2.1	4.4	9.6	1.2	5.7
		Dothideomycetes	1.1	15.4	2.1	4.9	10.3
		Leotiomycetes		5.5	10.6		1.1
		Pezizomycetes			7.4	4.9	3.4
		Pezizomycotina incertae sedis					4.6
	mitosporic *Ascomycota*	mitosporic *Ascomycota*	18.9	13.2	23.4	28.0	8.0
	SCGI	SCGI not specified				13.4	
Basidiomycota	*Basidiomycota incertae sedis*	*Basidiomycota incertae sedis*		1.1	10.6	2.4	
	Agaricomycotina	*Agaricomycetes*	10.5	4.4	2.1		8.0
		Tremellomycetes	1.1	1.1	5.3	6.1	2.3
	Pucciniomycotina	*Pucciniomycotina incertae sedis*			3.2		
Chytridiomycota	*Chytridiomycota incertae sedis*	*Chydridiomycota incertae sedis*		2.2			1.1
Blastocladiomycota	*Blastocladiomycota incertae sedis*	*Blastocladiomycota incertae sedis*				1.2	
Basal fungal lineage	*Mucoromycotina*	*Mucoromycotina incertae sedis*		1.1	3.2		4.6
		Total					

Tab.3.11. (continued)

d) Phylum	Subphylum	Class	Order	M	N	P	R	T
Eukaryota	*Eukaryota i. s.*	*Eukaryota i. s.*	*Eukaryota i. s.*				1.2	
Fungi	*Fungi i. s.*	*Fungi i. s.*	*Fungi i. s.*		1.1		3.7	
Ascomycota	*Ascomycota i. s.*	*Ascomycota i. s.*	*Ascomycota i. s.*		6.6		7.3	1.1
	Pezizomycotina	*Sordariomycetes*	*Boliniales*			1.1		
			Coniochaetales	3.2		4.3		
			Hypocreales	26.3	25.3	8.5	14.6	10.3
			Phyllachorales		2.2		1.2	
			Sordariales	36.8	13.2	7.4	6.1	36.8
			Sordariomycetes i. s.		2.2	1.1	3.7	2.3
			Xylariales		1.1			
		Eurotiomycetes	*Chaetothyriales*	2.1	4.4	9.6	1.2	5.7
		Dothideomycetes	*Pleosporales*	1.1	14.3			5.7
			Capnodiales		1.1	2.1	4.9	4.6
		Leotiomycetes	*Leotiomycetes i. s.*			3.2		
			Helotiales		5.5	7.4		1.1
		Pezizomycetes	*Pezizales*			7.4	4.9	3.4
		Pezizomycotina i. s.	*Pezizomycotina i. s.*					4.6
	mitosporic *Ascomycota*	mitosporic *Ascomycota*	mitosporic *Ascomycota*	18.9	13.2	23.4	28.0	8.0
	SCGI	SCGI not specified	SCGI not specified				13.4	
Basidiomycota	*Basidiomycota i. s.*	*Basidiomycota i. s.*	*Basidiomycota i. s.*		1.1	10.6	2.4	
	Agaricomycotina	*Agaricomycetes*	*Agaricales*					6.9
			Agaricomycetes i. s.					1.1
			Boletales	8.4				
			Cantharellales	2.1	4.4	2.1		
		Tremellomycetes	*Cystofilobasidiales*		1.1			
			Filobasidiales			5.3	6.1	2.3
			Tremellales	1.1				
	Pucciniomycotina	*Pucciniomycotina i. s.*	*Pucciniomycotina i. s.*			3.2		
Chytridiomycota	*Chytridiomycota i. s.*	*Chydridiomycota i. s.*	*Chydridiomycota i. s.*		2.2			1.1
Blastocladiomycota	*Blastocladiomycota i. s.*	*Blastocladiomycota i. s.*	*Blastocladiomycota i. s.*				1.2	
Basal fungal lineage	*Mucoromycotina*	*Mucoromycotina i. s.*	*Mortierellales*		1.1	3.2		
			Mucoromycotina i. s.					4.6

Tab.3.11. (continued) e)

Phylum	Subphylum	Class	Order	Family	M	N	P	R	T
Eukaryota	*Eukaryota i. s.*	*Eukaryota i. s.*	*Eukaryota i. s.*	*Eukaryota i. s.*				1.2	
Fungi	*Fungi i. s.*	*Fungi i. s.*	*Fungi i. s.*	*Fungi i. s.*		1.1		3.7	
Ascomycota	*Ascomycota i. s.*	*Ascomycota i. s.*	*Ascomycota i. s.*	*Ascomycota i. s.*		6.6		7.3	1.1
	Pezizomycotina	*Sordariomycetes*	*Boliniales*	*Boliniales i. s.*			1.1		
			Coniochaetales	*Cortinariaceae*	3.2				
				Coniochaetaceae			4.3		
			Hypocreales	*Nectriaceae*	3.2	4.4	5.3	3.7	2.3
				mitosporic Hypocreales	14.7	6.6	1.1	4.9	6.9
				Hypocreales i. s.	6.3	13.2		6.1	1.1
				Hypocreaceae		1.1	1.1		
				Clavicipitaceae	2.1				
				Bionectriaceae			1.1		
			Phyllachorales	*Phyllachoraceae*				1.2	
				mitosporic *Phyllachorales*		2.2			
			Sordariales	*Lasiosphaeriaceae*	23.2	2.2	5.3	6.1	12.6
				Chaetomiaceae	11.6	1.1	1.1		6.9
			Sordariomycetes i. s.	*Sordariomycetes i. s.*		2.2	1.1	3.7	
				Sordariales i. s.	2.1	9.9	1.1		17.2
				Magnaporthaceae					2.3
			Xylariales	*Xylariales i. s.*		1.1			
		Eurotiomycetes	*Chaetothyriales*	*Herpotrichiellaceae*	2.1	4.4	9.6	1.2	5.7
		Dothideomycetes	*Pleosporales*	*Pleosporales i. s.*	1.1	5.5			5.7
				Pleosporaceae		8.8			
			Capnodiales	*Mycosphaerellaceae*			2.1		
				Davidiellaceae		1.1		4.9	4.6
		Leotiomycetes	*Leotiomycetes i. s.*	*Leotiomycetes i. s.*			3.2		
			Helotiales	*Sclerotiniaceae*		5.5			
				Helotiales i. s.			7.4		1.1
		Pezizomycetes	*Pezizales*	*Pyronemataceae*			7.4	4.9	
		Pezizomycetes	*Pezizales*	*Pezizales i. s.*					3.4
		Pezizomycotina i. s.	*Pezizomycotina i. s.*	*Pezizomycotina i. s.*					4.6
	mit. Ascomycota	mitosporic *Ascomycota*	mitosporic *Ascomycota*	mitosporic *Ascomycota i. s.*	18.9	13.2	23.4	28.0	8.0
	SCGI	SCGI not specified	SCGI not specified	SCGI not specified				13.4	

Tab.3.11. (continued) e)

Phylum	Subphylum	Class	Order	Family	M	N	P	R	T
Basidiomycota	*Basidiomycota i. s.*	*Basidiomycota i. s.*	*Basidiomycota i. s.*	*Basidiomycota i. s.*		1.1	10.6	2.4	
	Agaricomycotina	*Agaricomycetes*	*Agaricales*	*Psathyrellaceae*					6.9
			Agaricomycetes i. s.	*Agaricomycetes i. s.*					1.1
			Boletales	*Sclerodermataceae*	8.4				
			Cantharellales	mitosporic *Cantharellales*	2.1	1.1	2.1		
			Cantharellales	*Ceratobasidiaceae*		3.3			
		Tremellomycetes	*Cystofilobasidiales*	*Cystofilobasidiales i. s.*		1.1			
			Filobasidiales	mitosporic *Filobasidiales*			5.3	6.1	2.3
			Tremellales	*Tremellales i. s.*	1.1				
	Pucciniomycotina	*Pucciniomycotina i. s.*	*Pucciniomycotina i. s.*	*Pucciniomycotina i. s.*			3.2		
Chytridiomycota	*Chytridiomycota i. s.*	*Chydridiomycota i. s.*	*Chydridiomycota i. s.*	*Chytridiomycota i. s.*		2.2			1.1
Blastocladiomycota	*Blastocladiomycota i. s.*	*Blastocladiomycota i. s.*	*Blastocladiomycota i. s.*	*Blastocladiomycota i. s.*				1.2	
Basal fungal lineage	*Mucoromycotina*	*Mucoromycotina i. s.*	*Mortierellales*	*Mortierellaceae*		1.1	3.2		
			Mucoromycotina i. s.	*Mucoromycotina i. s.*					4.6

Basal fungal lineages: early-diverging groups, which traditionally have been placed within the *Zygomycota* and *Chytridiomycota*.
SCGI: Soil Clone Group I; unclassified fungal sequences, which have been postulated as a novel subphylum of *Ascomycota* by phylogenetic analyses by Porter and co-workers (2008).
i. s.: incertae sedis; Taxa of uncertain position, which have been placed as species of the least inclusive level in the hierarchy to which they can be assigned with confidence.

The number of observed RFLP-types were 122 for all data. For the respective clone library we found 20 RFLP-types in M, 32 in N, 28 in P, 30 in R and 28 in T. The numbers of each taxonomic rank are listed in Tab.3.12.

Tab.3.12. Composition of clone libraries.

	M	N	P	R	T	total*
Genus	16	27	23	18	24	61
Family	14	24	21	17	20	46
Order	9	17	16	15	16	31
Class	6	12	11	12	12	17
Subphylum	3	8	6	9	6	11
Phylum	2	5	3	5	4	6

*) The total number refers to the number of different members of the respective taxonomic level and is not the sum of the numbers of each clone library.

3.2.3. Species richness estimation

Furthermore, the data obtained from the clone libraries (i.e. RFLP-types, sequence identification and abundance) were used for the calculation of estimators of species richness with ESTIMATES (see 2.7). Five estimators were compared: Chao2 richness estimator, incidence- and abundance-based coverage estimators [incidence-based coverage estimator (ICE) and abundance-based coverage estimator (ACE)], first-order Jackknife richness estimator (Jackknife 1) and Bootstrap richness estimator.
The estimated species richness of the clone libraries is shown in Fig.3.9 to Fig.3.14.

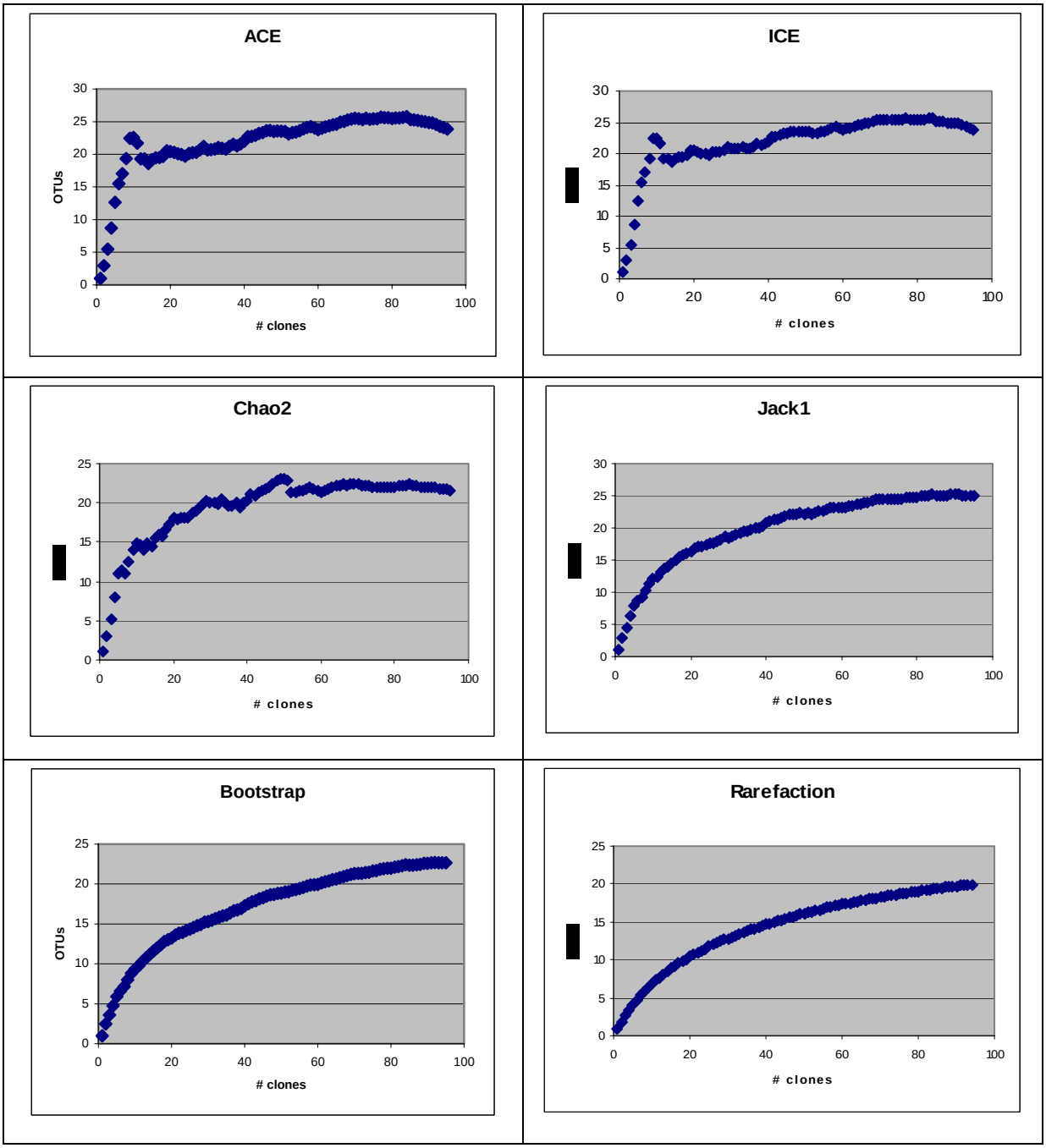


Fig.3.9. Estimators of species richness for clone library M.

ACE, Abundance-based coverage estimator; ICE, Incidence-based coverage estimator; Jack 1, first-order Jackknife richness estimator; OTU, operational taxonomic unit, corresponding to RFLP-type; # clones, number of analysed clones of the clone library.

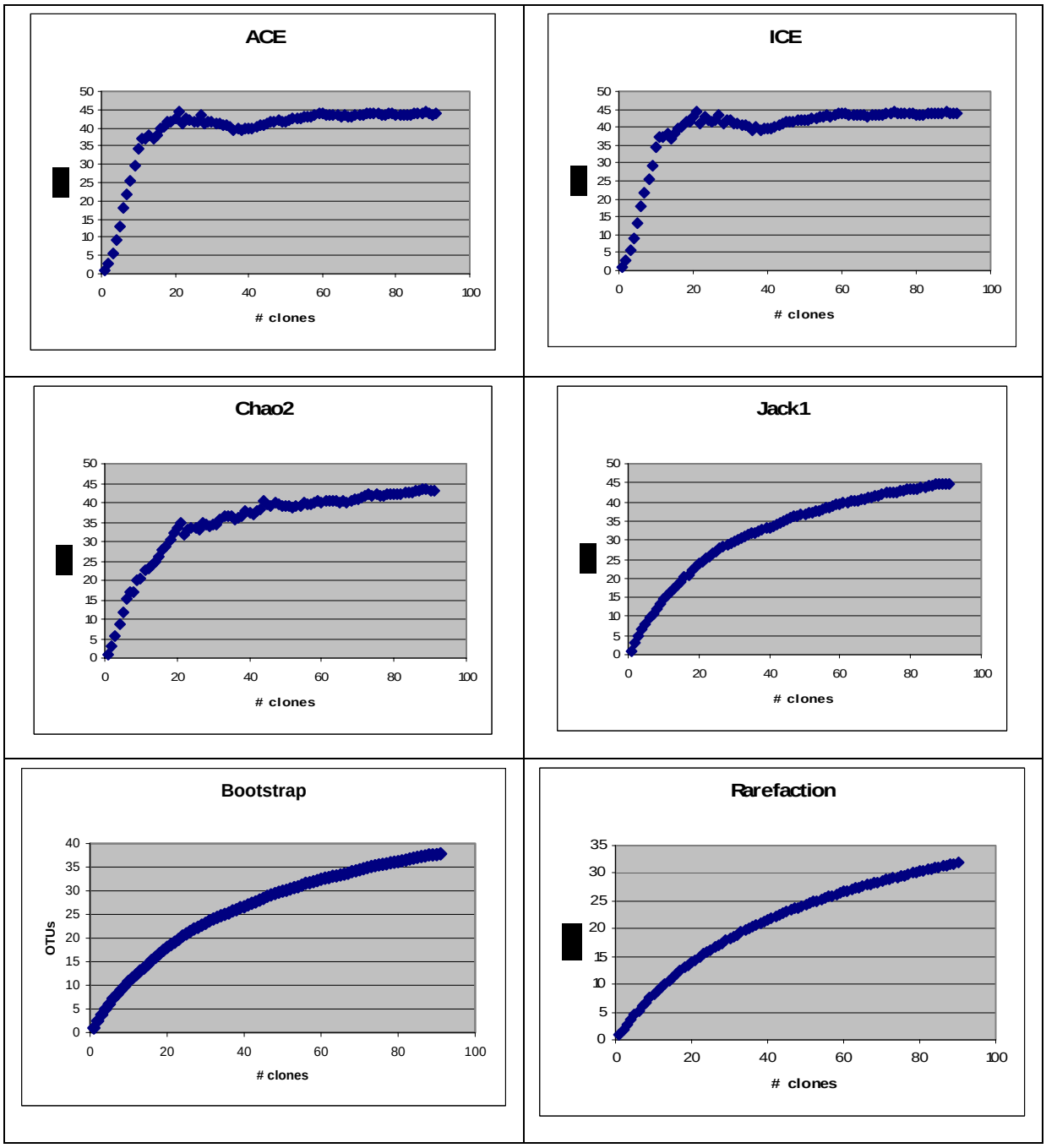


Fig.3.10. Estimators of species richness for clone library N.

ACE, Abundance-based coverage estimator; ICE, Incidence-based coverage estimator; Jack 1, first-order Jackknife richness estimator; OTU, operational taxonomic unit, corresponding to RFLP-type; # clones, number of analysed clones of the clone library.

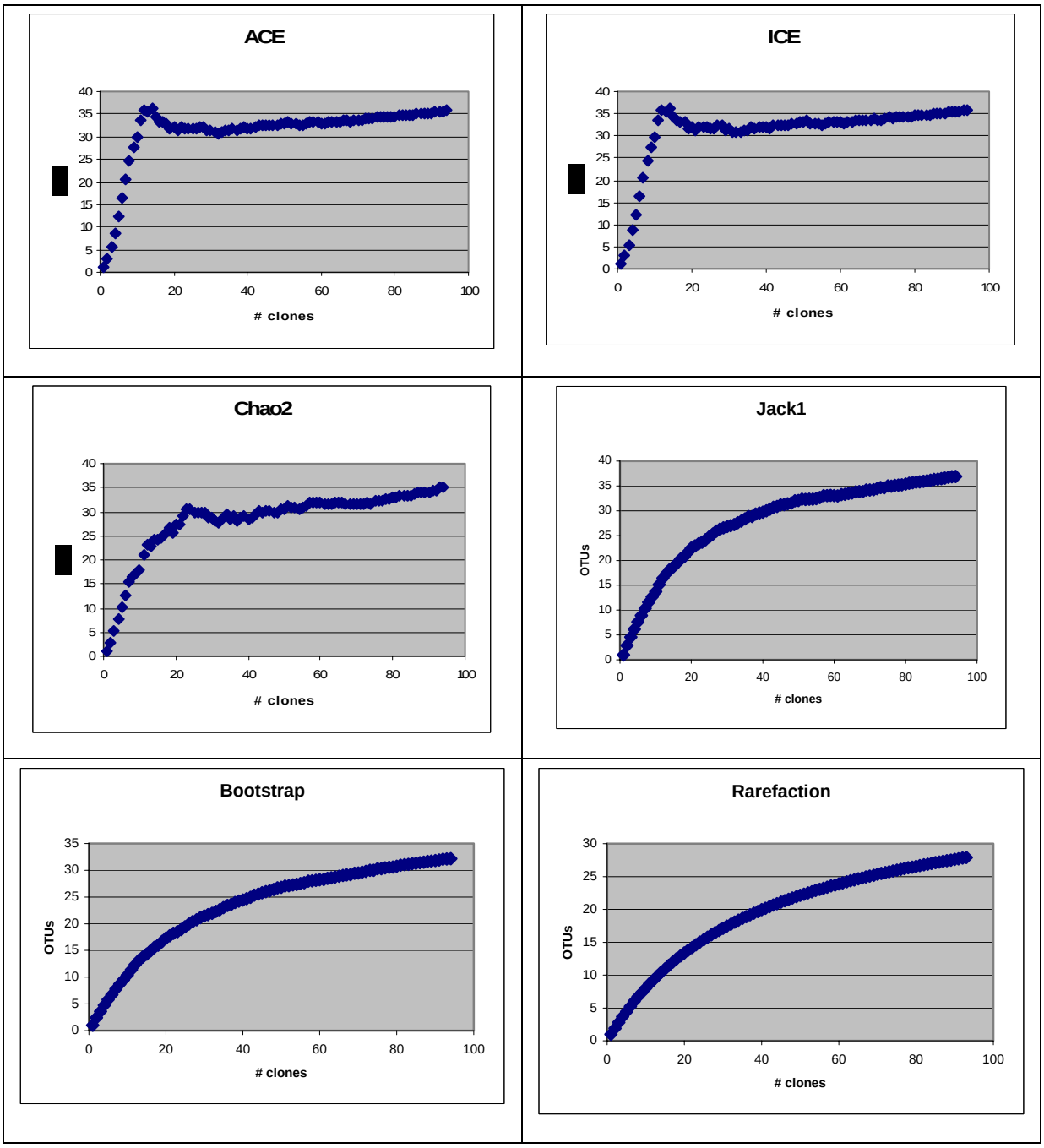


Fig.3.11. Estimators of species richness for clone library P.

ACE, Abundance-based coverage estimator; ICE, Incidence-based coverage estimator; Jack 1, first-order Jackknife richness estimator; OTU, operational taxonomic unit, corresponding to RFLP-type; # clones, number of analysed clones of the clone library.

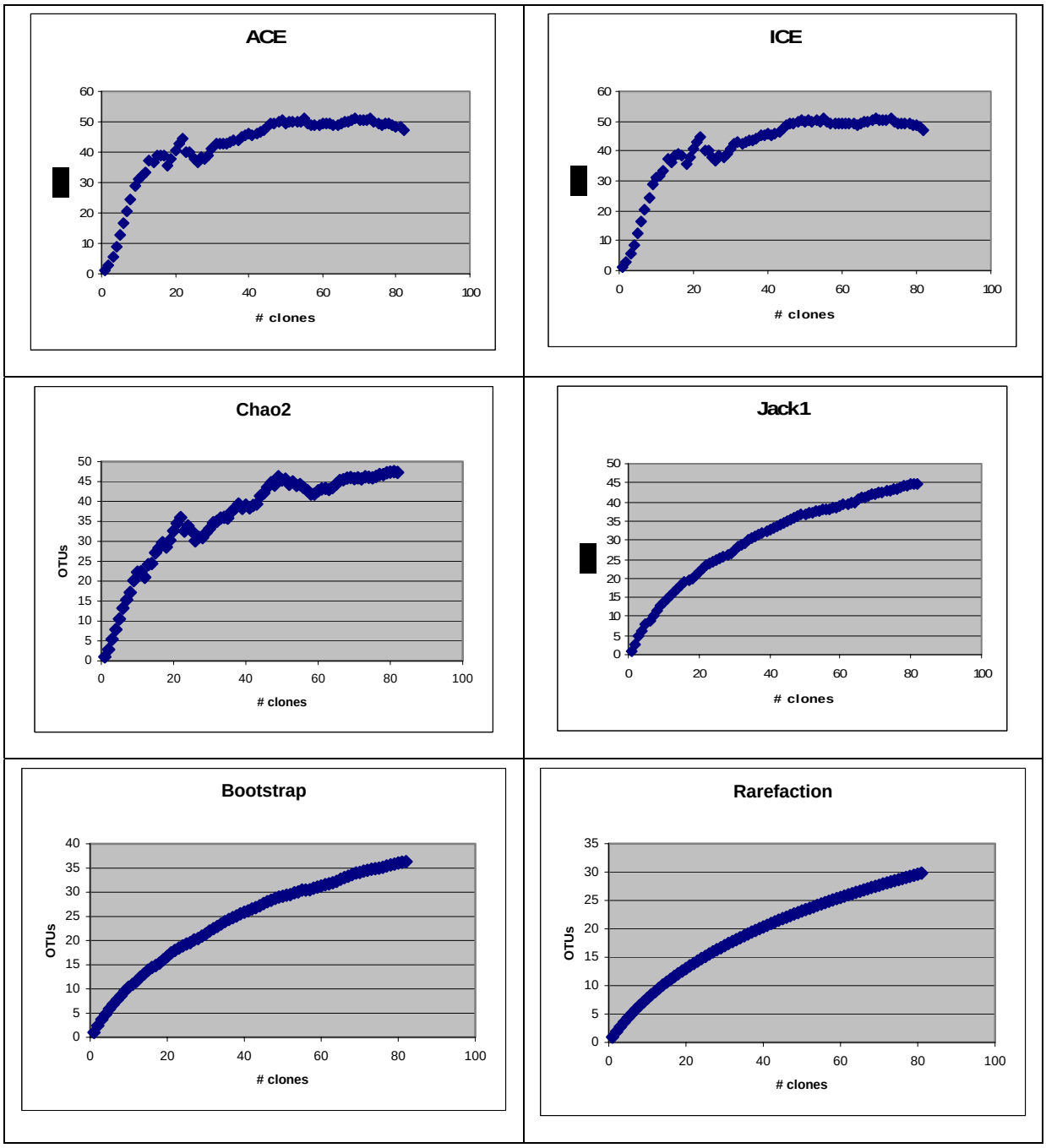


Fig.3.12. Estimators of species richness for clone library R.

ACE, Abundance-based coverage estimator; ICE, Incidence-based coverage estimator; Jack 1, first-order Jackknife richness estimator; OTU, operational taxonomic unit, corresponding to RFLP-type; # clones, number of analysed clones of the clone library.

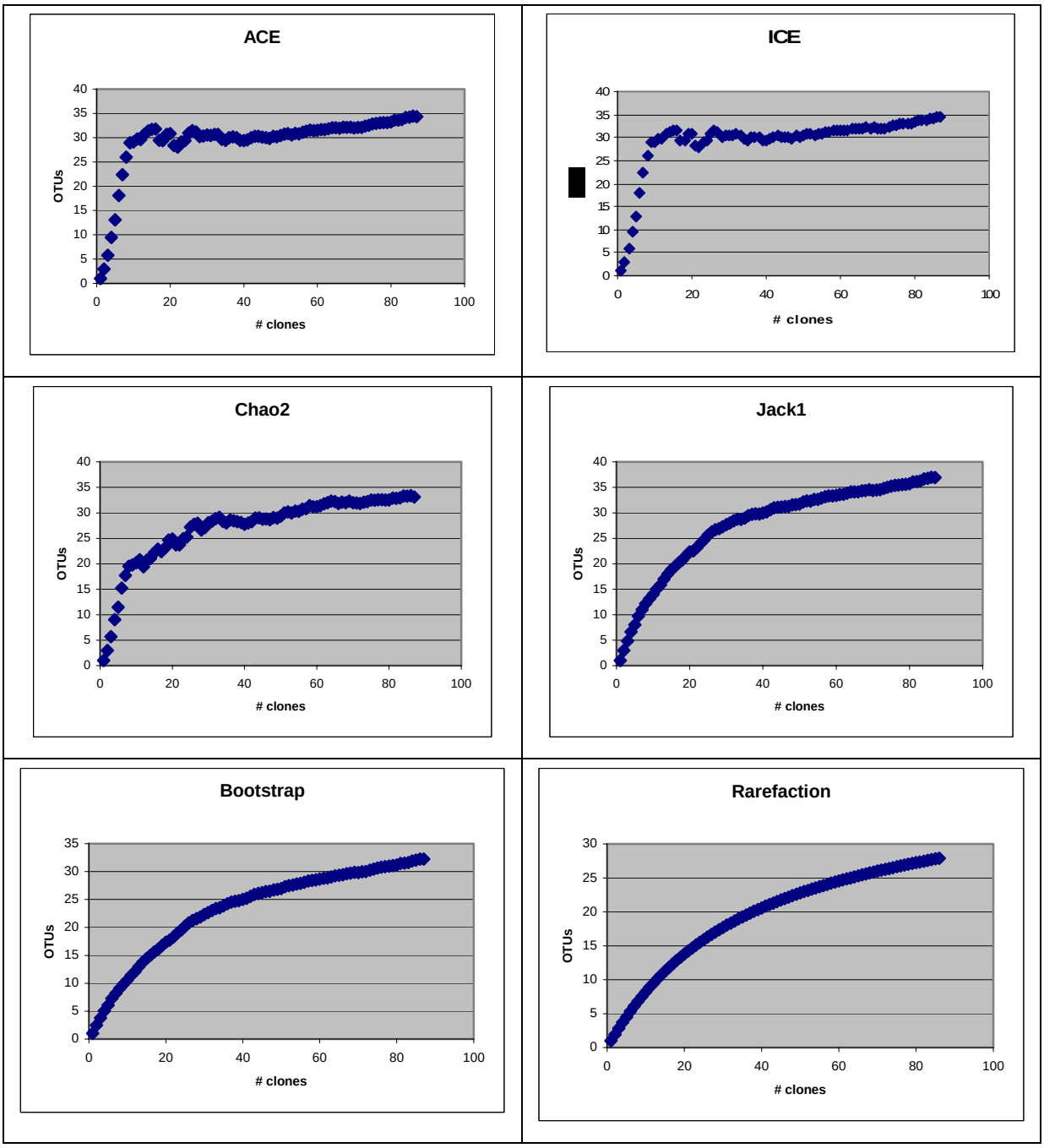


Fig.3.13. Estimators of species richness for clone library T.

ACE, Abundance-based coverage estimator; ICE, Incidence-based coverage estimator; Jack 1, first-order Jackknife richness estimator; OTU, operational taxonomic unit, corresponding to RFLP-type; # clones, number of analysed clones of the clone library.

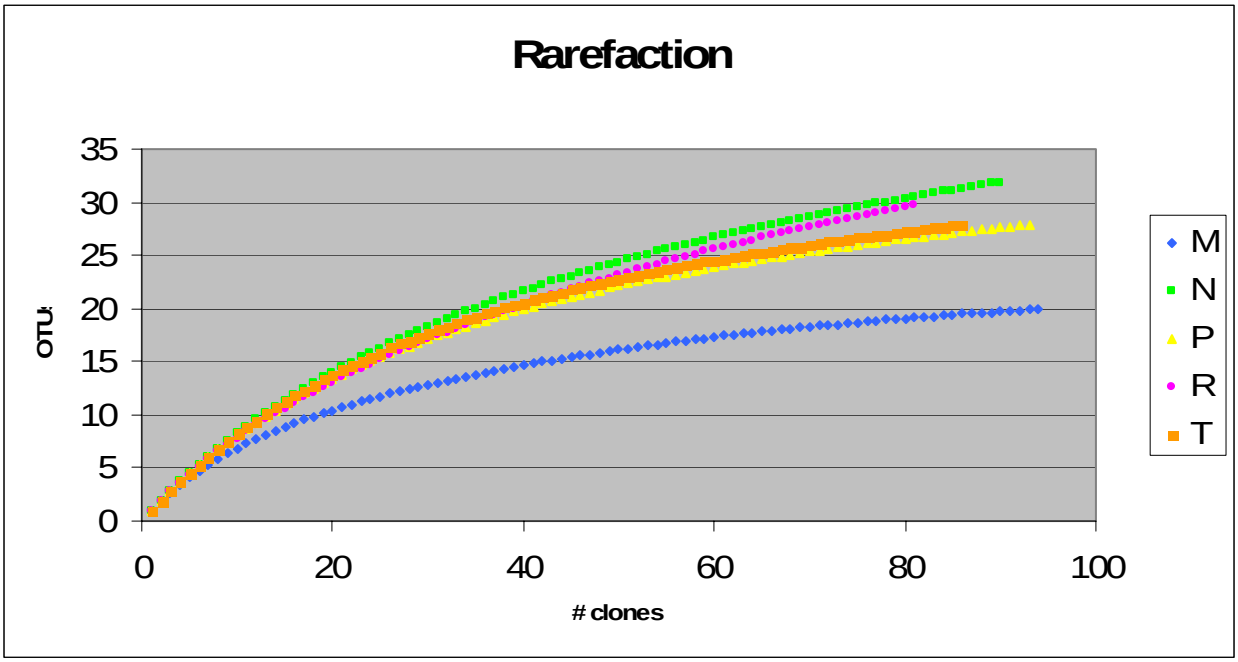


Fig.3.14. Rarefaction-species-accumulation curve of all clone libraries.

OTU, operational taxonomic unit, corresponding to RFLP-type; # clones, number of analysed clones of the clone library.

3.3. Overlap of both methods

The results of both approaches, the culturing as well as the culture-independent approach, were compared. The amplified sequence regions for identification varied between the culture-dependent and –independent approaches: For identification of cultivated fungi the ITS region between the primer binding sites ITS1 and ITS4 was sequenced (Fig.3.15). This approach therefore covered both, ITS1 and ITS2. The complete ITS region is ideal for species (sometimes even subspecies) identification with sufficient reference data. Since well characterised reference sequences were expected for cultured fungi in the sequence database, the complete ITS region was chosen. Conversely, from the culture-independent approach less well characterised reference sequences were expected. In this case the LSU served as a good marker with improved resolution at higher taxonomic levels. For the cultivation-independent identification of soil fungi the ITS region and partial LSU were amplified with primer pair ITS1F/TW13 (Fig.3.15).

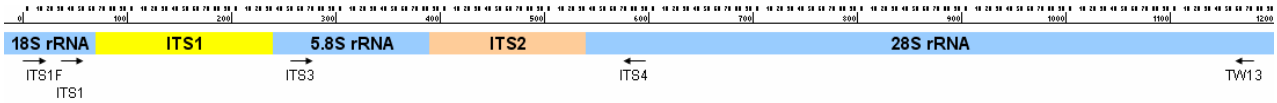


Fig.3.15. Parts of the rRNA gene repeat unit.
Primer binding sites are indicated by arrows.

The overlapping sequence from the two different approaches mainly covers the ITS2 region (~ 300 bp), which is enough to find identical species from both methods by sequence analysis. Fungal species identified by both methods are listed in Tab.3.13. The 14 overlapping species make up for 8.6 % of all identified species by both methods taken together. The majority of species were detected by the culture-independent procedure (62.6 %), while 28.8 % of all species were found by the culturing-approach exclusively (Fig.3.16).

For many species identified by both methods, the soil origin was different between the two methods. In the case of 5 species, however, the sequences identified by the culturing and the culture-independent approach, respectively, derived from the same soil sample. These species include *Bionectria ochroleuca, Cercophora/Apodus sp, Davidiella tassiana, Fusarium oxysporum and Helicodendron sp.* (Tab.3.13).

Tab.3.13.Sequences identified by both approaches.

Cultured fungi		Clone libraries			
Code	**Soil origin**	**Code**	**Soil origin**	**Identification**	**Identity [%]**
NG_p38	N	R_A11	R	*Cryptococcus aerius*	99 - 100
NG_p38	N	R_E05	R		
NG_p38	N	T_F08	T		
NG_p38	N	R_C06	R		
NG_28	M	P_A04	P	*Cryptococcus terricola*	99
NG_H01	P	P_B11	P	*Bionectria ochroleuca/ Bionectriaceae sp.*	98 - 99
NG_36C	T	P_B11	P		
NG_H11	R	P_B11	P		
NG_21B	T	T_A03	T	*Cercophora/Apodus sp./ Lasiosphaeriaceae*	100
NG_09	T	T_E10	T	*Davidiella tassiana/Davidiella sp.*	99
NG_H10	R	R_E09	R	*Fusarium oxysporum*	98 - 99
NG_H15	P	R_E09	R		
NG_H15	P	T_H11	T		
NG_H10	R	T_H11	T		
NG_H16	M	T_H11	T		
NG_21A	T	T_H01	T	*Helicodendron sp./Helotiales*	99 - 100
NG_21A	T	P_C08	P		
NG_21A	T	P_H02	P		
NG_21A	T	P_A05	P		
NG_35	R	P_E08	P	*Helotiales sp.*	100
NG_36B	T	P_E08	P		
NG_p31	N	P_H07	P	*Hypocrea lixii/Hypocrea sp.*	98
NG_H13	R	N_E04	N	*Nectria haematococca mpVI/ Nectria haematococca*	98
NG_p49	N	R_B06	R	*Pleosporales sp./Ascomycota*	96
NG_21C	T	P_E06	P	*Sordariomycetes sp./Boliniales*	100
NG_01	N	P_H07	P	*Trichoderma velutinum/Hypocrea sp.*	98
NG_p45	M	N_C05	N	*Verticillium nigrescens*	99

In cases of different identification names of the two methods, the identification of the molecular approach is stated right behind the slash-symbol.
Sequences with the same soil origin are highlighted in blue.

Fig.3.16. Species found by both approaches. Identified sequences of the culturing approach are in the left circle. A selection of species identified with the culture-independent approach is in the right circle. The intersection represents the overlap.

3.4. Construction and analysis of *niaD* gene libraries from soil fungi

It was known from previous studies, that fungi such as *Aspergillus nidulans*, *Cadophora finlandia* and *Phialocephala fortinii*, which are frequently used in our laboratory, are able to utilise nitrate as a sole nitrogen source and as a consequence possess a gene encoding nitrate reductase.

For the construction of primers amplifying the nitrate reductase gene *niaD*, a multiple protein sequence alignment was generated containing fungal full length *niaD* sequences from Genbank. Since basidiomycetous *niaD* sequences deviate highly from ascomycetous *niaD* sequences, it was not possible to develop primers allowing amplification of both, ascomycetous and basidiomycetous *niaD* genes. Since the investigated soils mainly contained *Ascomycota*, the focus was laid on the amplification of ascomycetous *niaD* sequences. Based on the protein alignment of 22 ascomycetous *niaD* sequences degenerated *niaD* primers were designed for those sections of the alignment with the most conserved regions. The part of the protein alignment of the *niaD* region, which contains the binding sites of the *niaD* primers niaD01F, niaD02F, niaD03R and niaD04R is depicted in Fig.3.17.

In order to optimise PCR-conditions for the amplification of the *niaD* gene section (~400 bp) using the newly designed *niaD* primers niaD01F/niaD04R genomic DNA of *Cadophora finlandia* and *Phialocephala fortinii* was used.

For the amplification of the *niaD* gene fragment from soil DNA, the primers niaD01F/niaD04R were used. No visible amplification products were obtained. Therefore, a nested PCR approach was undertaken using the primer pair niaD02F/niaD03R. PCR-products of the nested PCR are shown in Fig.3.18.

The soil samples N and P had been selected after the 1st time course experiment for following experiments. Two replicates from each soil DNA extracted from N and P soil samples were taken as DNA templates for the *niaD* PCR.

In silico analysis revealed that in a portion of sequences derived from P1 and P2, the amplified coding region is interrupted by a single intron (Fig.3.18). Therefore, sequences of mainly two lengths were obtained: 367 bp without an intron and 419 or 442 bp, respectively, containing a single intron; all sequences coding for 122 amino acids. Apparently, there are differences between fungal species concerning the presence and length of this intron.

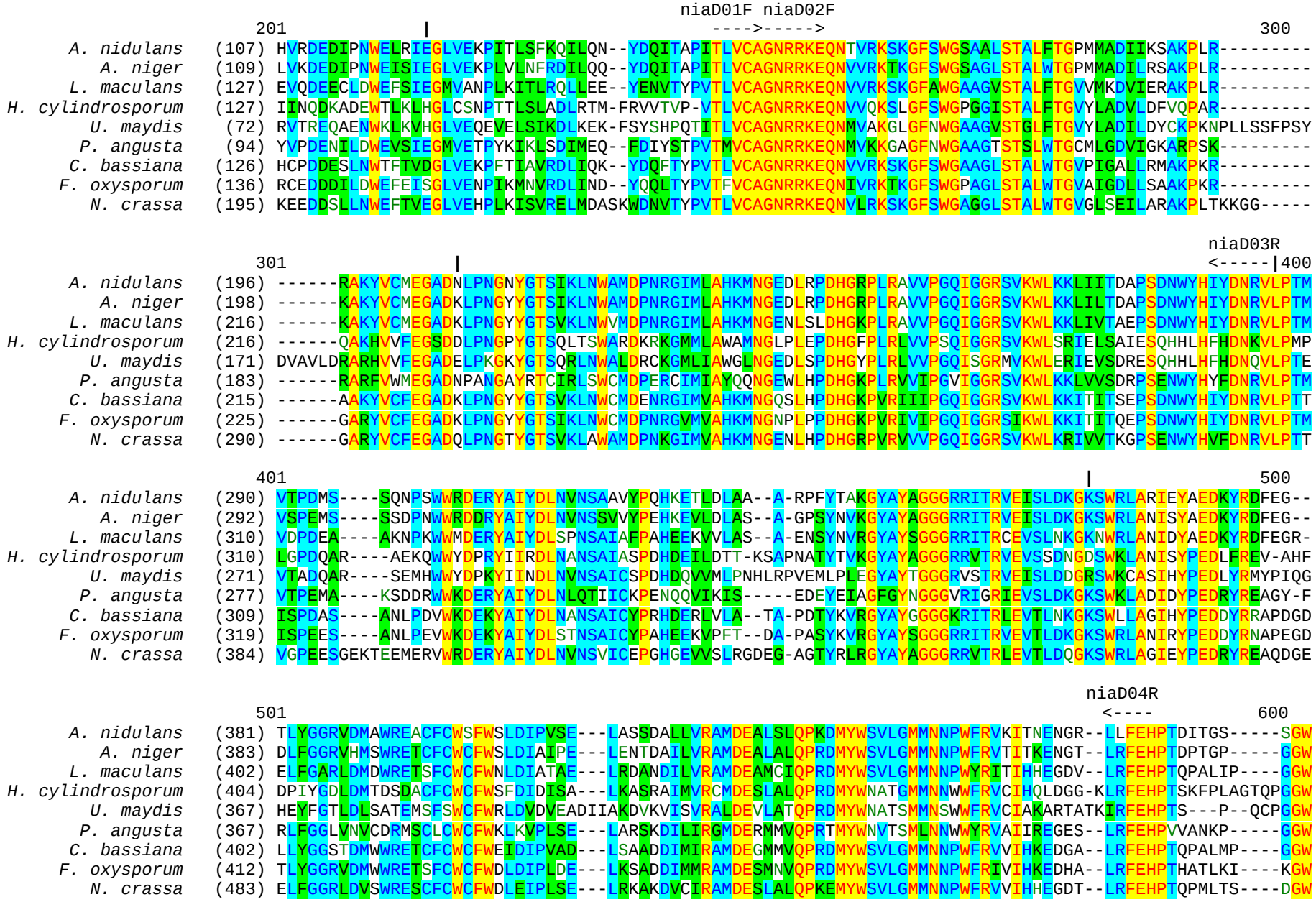


Fig.3.17. Multiple protein sequence alignment of the part of the *niaD* gene where the primer binding sites are located.

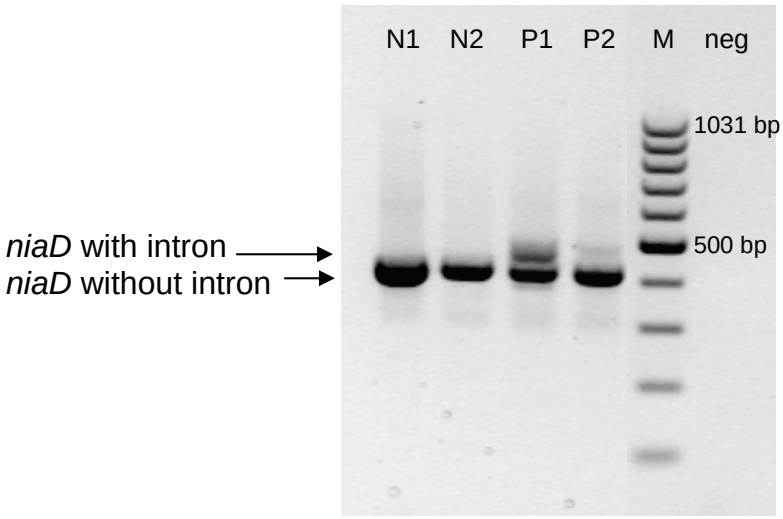


Fig.3.18. PCR products of a nested *niaD*-PCR on an agarose-gel. M, marker; neg, negative control.

PCR products of the nested *niaD*-PCR were cloned in plasmid pTZ57R/T. N1 and P1 clone libraries contain 96 independent clones each, while N2 and P2 contain 48 clones each. Clones were screened by *niaD*-PCR and positive candidates were compared via RFLP analyses with *Bsu*RI and *Hin*6I double digestion (see 2.8).
Restriction patterns of each *niaD*-gene-library are depicted in Fig.3.19. - 3.23. Codes of the clones refer to the position of the 96-well microtiter plate in which they are stored.

A 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 B
C 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 D
E 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 F
G 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 H

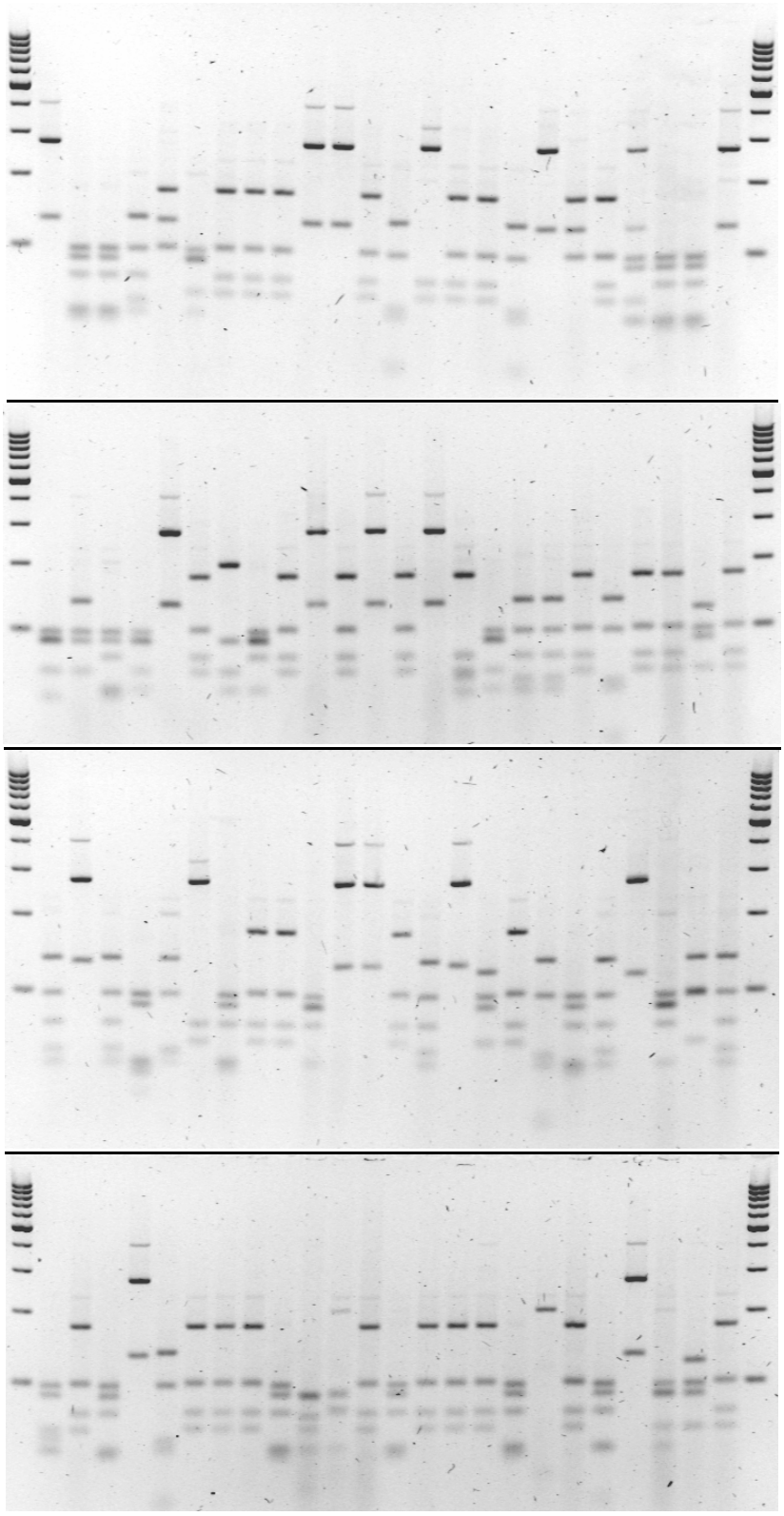


Fig.3.19. RFLP-analysis using *Bsu*RI & *Hin*6I of 96 clones obtained from sample N1.

A 01 02 03 04 05 06 B 01 02 03 04 05 06 C 01 02 03 04 05 06 D 01 02 03 04 05 06
E 01 02 03 04 05 06 F 01 02 03 04 05 06 G 01 02 03 04 05 06 H 01 02 03 04 05 06

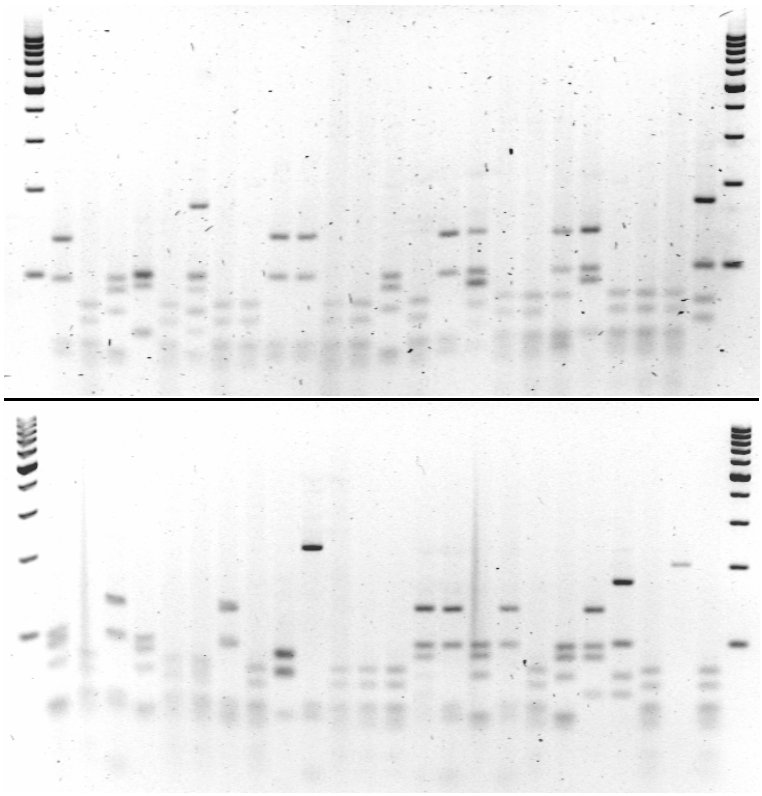


Fig.3.20. RFLP-analysis using *Bsu*RI & *Hin*6I of 48 clones obtained from sample N2.

E 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 F
G 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 H

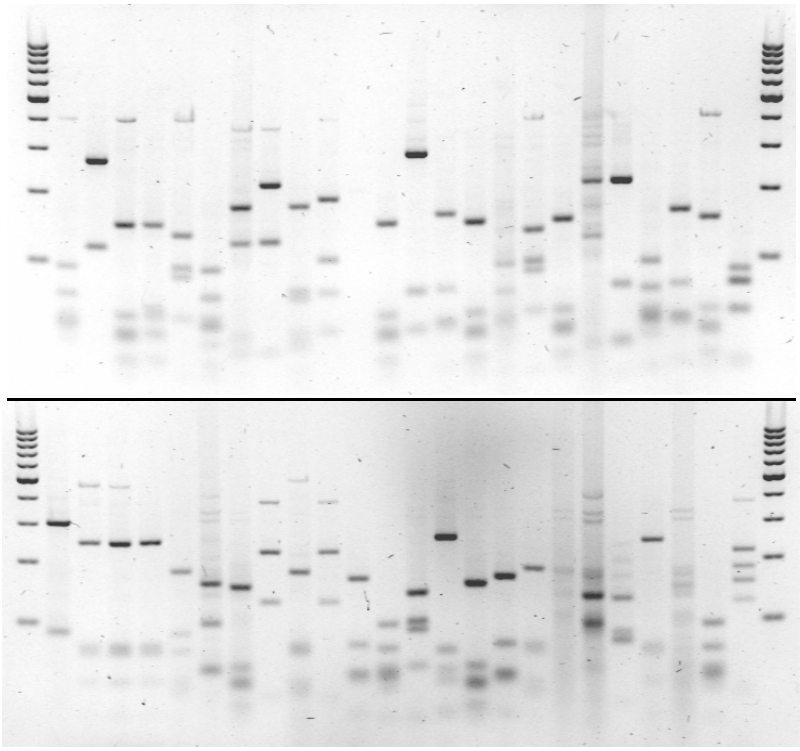


Fig.3.21. RFLP-analysis using *Bsu*RI & *Hin*6I of 48 clones obtained from sample P1.

A 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 B
C 01 02 03 04 05 06 07 08 09 10 11 12 01 02 03 04 05 06 07 08 09 10 11 12 D

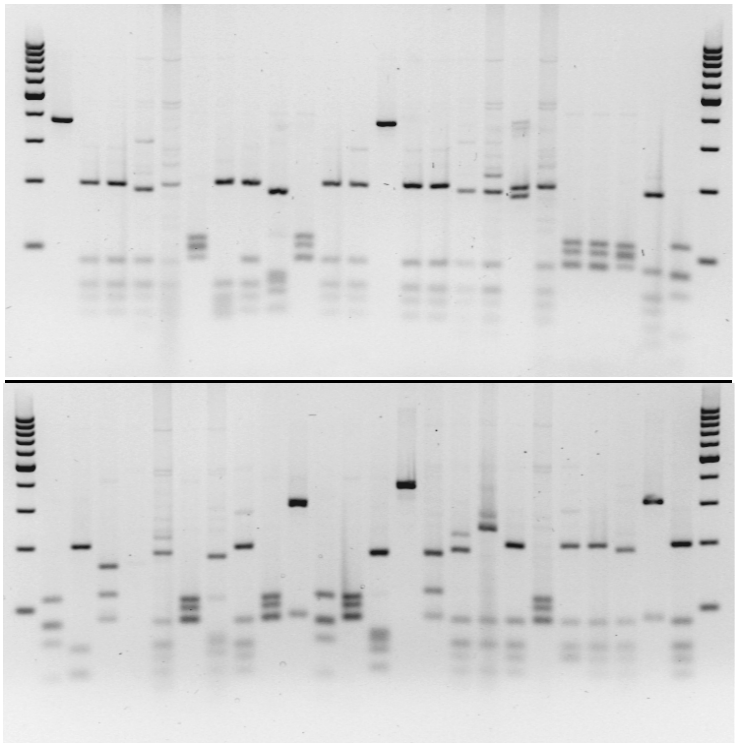


Fig.3.22. RFLP-analysis using *Bsu*RI & *Mbo*I of 48 clones obtained from sample P1.

A 01 02 03 04 05 06 B 01 02 03 04 05 06 C 01 02 03 04 05 06 D 01 02 03 04 05 06
E 01 02 03 04 05 06 F 01 02 03 04 05 06 G 01 02 03 04 05 06 H 01 02 03 04 05 06

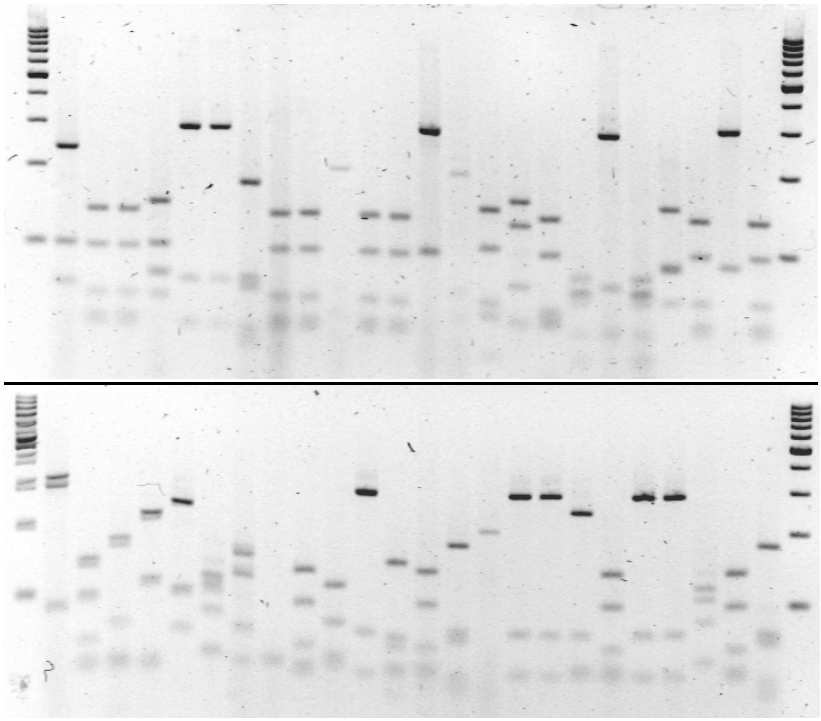


Fig.3.23. RFLP-analysis using *Bsu*RI & *Hin*6I of 48 clones obtained from sample P2.

One representative of each pattern was sequenced. Sequences were assembled and edited using the Vector NTI software (check for orientation, remove flanking niaD primers). Furthermore, the sequences were screened for introns carrying the conserved splice sites GT and YAG and marked by adding the respective sequence feature for exons and introns. The nucleotide sequences were subsequently translated into protein sequences using splicing, whereby reference sequences served as templates to find the correct open reading frame.
The set of nitrate reductase sequences (deriving from known isolates as well as direct PCR approaches) is continuously increasing and protein sequences will be used for phylogenetic analyses.

3.5. Fluorescence *in situ* hybridisation (FISH)

Fingerprinting techniques as well as the analysis of rRNA genes/ITS regions from environmental samples require PCR amplification of the target gene segments from the total gene pool. However, PCR associated biases complicate the quantification of different fungal species in a mixed DNA pool. As a result, an additional method, independent from PCR, was required.
For quantification purposes fluorescence *in situ* hybridisation (FISH) is a beneficial technique, which enables the visualisation and phylogenetic identification of microbial cells. Furthermore, additional detection of *niaD* mRNA would allow an assertion about the physiological status of the identified fungi.

3.5.1. rRNA-FISH

The FISH experiment was performed to test the method on filamentous fungi. In this case a pure culture of the model organism *Aspergillus nidulans* was used.
To establish suitable conditions for this method, parameters like culture conditions, the sample fixation method, treatment of slides as well as hybridisation and washing buffer compositions were tested initially using the 18S rRNA-targeted probes EUK516-Fluos (rRNA-FISH). Overnight cultures of the filamentous fungus *A. nidulans* scraped off from an AMM-plate were subjected to rRNA-FISH (see 2.9).
The formaledhyde-fixed sample hybridised with the EUK516-Fluos probe is depicted in Fig.3.24. A bright fluorescence signal was detected. Ribosomes are evenly distributed throughout the hyphae, whereas nuclei are clearly delimited (Fig.3.24).

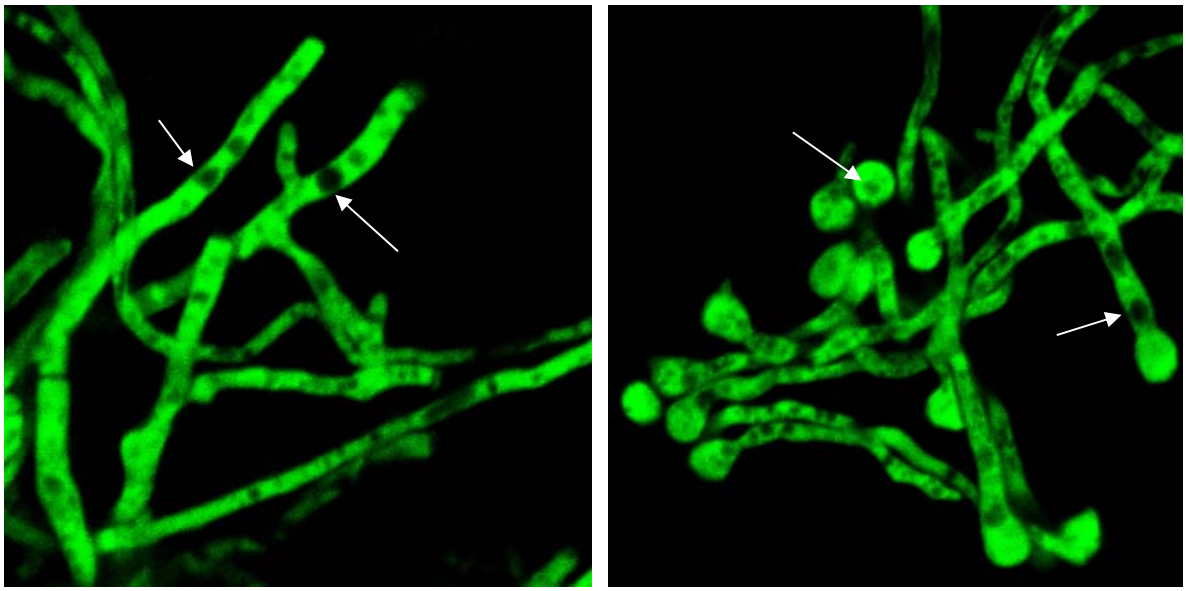


Fig.3.24. FISH analysis of *A. nidulans* with EUK516-Fluos probe at 10 % formamide. The ribosome-rich cytoplasma can be easily distinguished from nuclei (arrows).

In the course of the pre-experiment (rRNA-FISH) it turned out that uncoated, H_2O_2-treated slides can be used. formaledhyde-fixed samples appeared to work better than ethanol-fixed ones, and a 10 % formamide concentration seemed to be suitable for hybridisation. Hybridisation worked with both buffers, Tris- and SSC-buffer.
No enzymatic treatment prior to hybridisation was needed for *Aspergillus nidulans* in order to yield a strong, clear and specific signal.

3.5.2. *niaD* mRNA-FISH

Suitable conditions from the pre-experiment (usage of H_2O_2-treated slides, formaldehyde as fixative, 10 % formamide and SSC-buffer for hybridisation) were then selected for the actual mRNA FISH using the probe for *niaD* mRNA. In order to avoid mRNA degradation by endogenous and exogenous RNases several precautions were taken (*e.g.* DEPC treatment, see 2.9.8).

It is known, that *niaD* gene expression is repressed in the presence of ammonium. Induction of *niaD* transcription requires the presence of nitrate itself, and the de-repression by the absence of a primary nitrogen source such as ammonium.
Transcription of *niaD* can be even more induced by a nitrate induction after a period of N-starvation (super induction). For a super-induced *niaD*-mRNA expression, the culture was grown overnight with ammonium tartrate, followed by N-starvation for 30 min and induction with $NaNO_3$ for another 30 min.

A. nidulans cultures at different growth stages grown on ammonium tartrate (uninduced) are depicted in Fig.3.25–28. Generally, the EUK516-Fluos signal is displayed in green and the signal of the niaD-Cy3 probe in red. The co-localisation of both signals appears yellow.

A. nidulans cultures, which have been induced by nitrate are shown in Fig.3.25. The super-induced culture (nitrate induction after N-starvation) is depicted in Fig.3.26. The two induced conditions appeared to give higher fluorescence intensity for the *niaD*-targeted probe as compared with the uninduced condition, using the same microscopic settings. Interestingly, the induced sample appeared brighter than the super-induced sample. However, the intensity of the signal was not quantified.

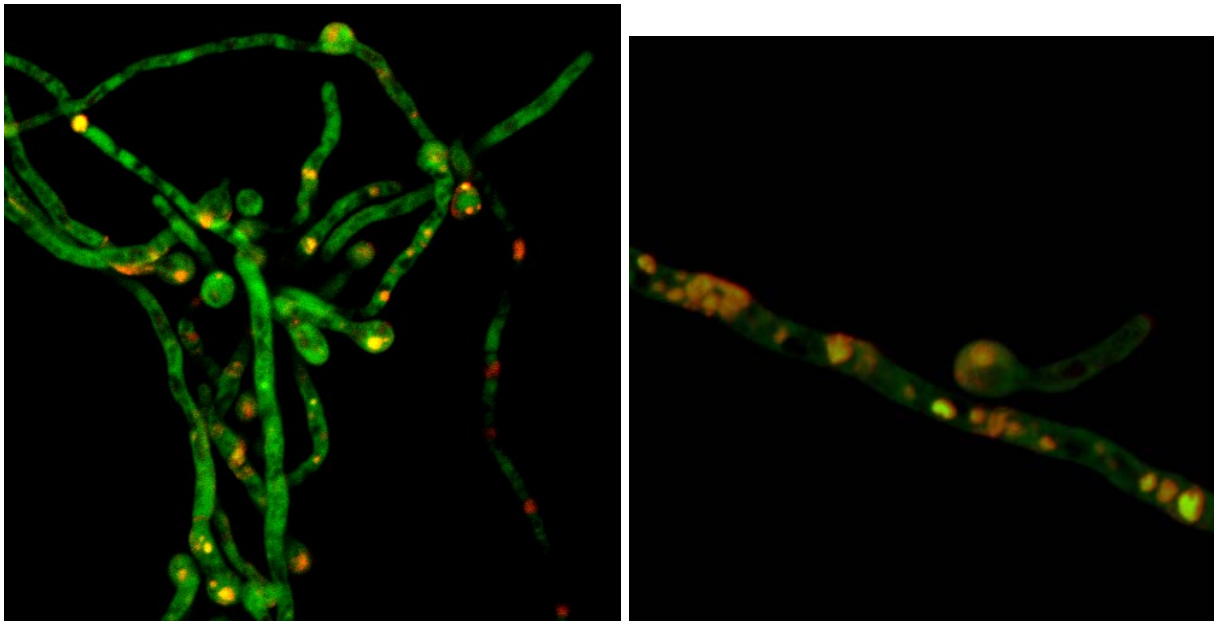


Fig.3.25. *A. nidulans* overnight culture grown on ammonia (uninduced). Overlay of EUK516-Fluos (green) and *niaD*-Cy3 (red) signals. Colocalisation of both signals appear yellow.

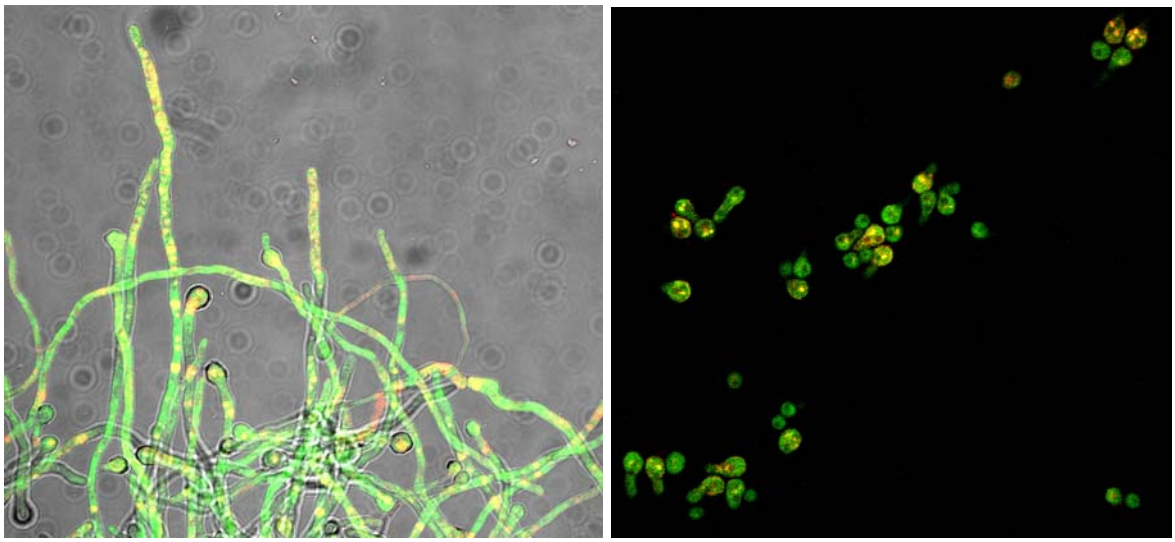


Fig.3.26. *A. nidulans* overnight culture (left) and 7 h culture (right) grown on ammonia (uninduced). Overlay of EUK516-Fluos (green) and *niaD*-Cy3 (red) signals. Left picture together with transmitted light.

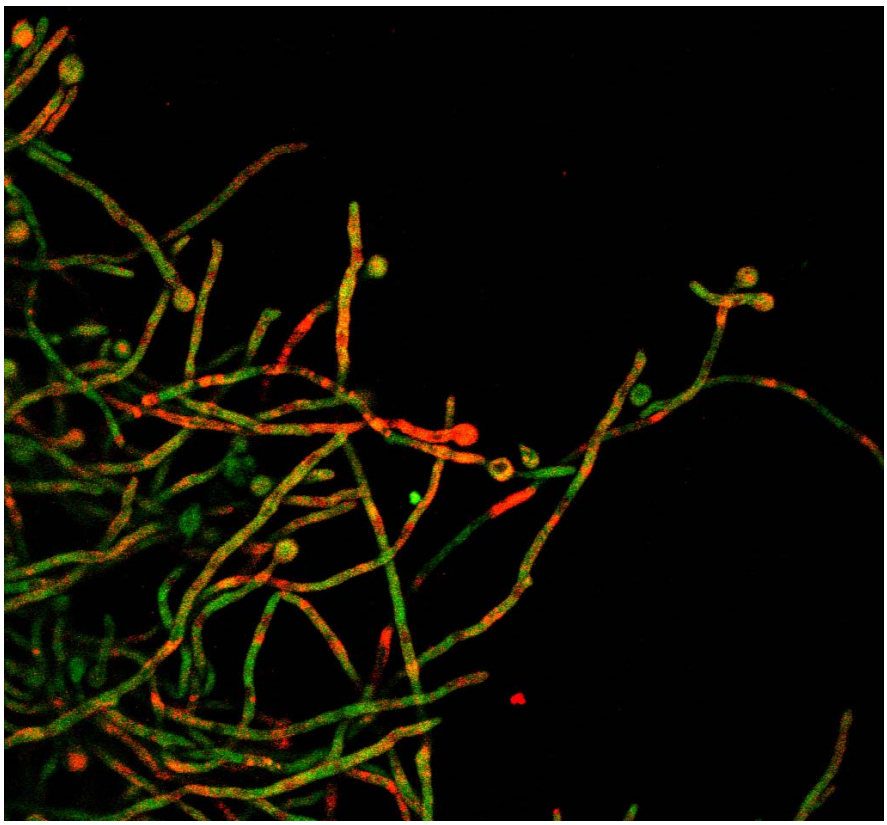


Fig.3.27. *A. nidulans* overnight culture grown on nitrate (induced). Overlay of EUK516-Fluos (green) and *niaD*-Cy3 (red) signals.

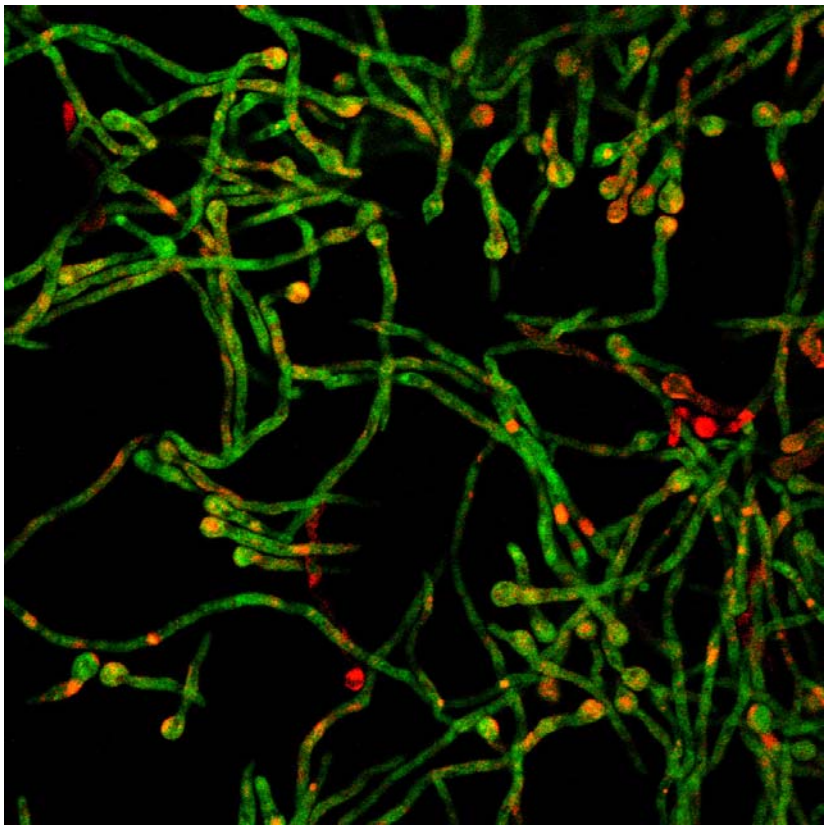


Fig.3.28. *A. nidulans* overnight culture grown on ammonia followed by N-starvation and induction of *niaD* expression by addition of nitrate (super-induced). Overlay of EUK516-Fluos (green) and *niaD*-Cy3 (red) signals.

NonEUB served as a control probe and no unspecific signal was detected. However, additional control experiments are needed to verify the *niaD* signals even in uninduced cultures: Instead of NonEUB, the reverse complement to the antisense *niaD* probe (targeting DNA of the niaD gene) can be used to show the specificity of the *niaD* probe. Additionally, a nonsense probe can serve as a control for unspecific probe binding. Furthermore, RNase treatment prior to hybridisation can serve as an additional confirmation of absence of unspecific probe binding.

The specificity of the *niaD* probe under induced and noninduced conditions may be further evaluated by using an additional strain lacking the *niaD* gene which should not result in any *niaD* signal.

In any case, the actual experiment was intended to act as a preliminary experiment for the establishment of the method. Future experiments are needed and will include the mentioned controls for result verification as well as a formamide series for the determination of a suitable formamide concentration.

4. Discussion

Fungi fulfil a range of important ecological functions. Nevertheless, there is poor understanding of soil fungal community diversity and the specific roles of individual phylogenetic groups present in the environment. In order to address the most fundamental questions of environmental microbiology (who are there? what are they doing?) it is important to establish a diversity census of fungal species present in soils. Subsequently, ecological questions can be addressed: responses of the fungal community or certain functional groups to environmental conditions (e.g. fertilisation, disturbances), interactions among species and ecological processes such as nutrient retention and cycling (functional diversity).

4.1. Fungal community composition in agricultural soils

To study fungal diversity in soil, several methods are available, which broadly can be divided into culture-based and culture-independent methods. Every method suffers from various limitations. No single method reflects “the true” diversity or species composition due to different aspects of diversity or community structure displayed by each method.

Our culture-based approach of assessing fungal diversity is not a classical method in the proper sense, because species identification was achieved by molecular methods rather than by morphological characters. Moreover, it represents an inventory of spores and mycelia present in the soil, which are able to germinate and grow under certain cultivation conditions. The main advantage here is that the cultures are later available for further investigations (*e.g.* analysis of functional genes). Notable disadvantages of culture-based methods are that only cultivable organisms can be detected and fast growing organisms are favoured most of the time. It is well accepted, that only a small proportion of soil bacteria grow on agar plates (0.1 – 10 %) (Torsvik et al*.* 1998), which may also apply to fungi. Many fungi, in particular the obligate biotrophic arbuscular mycorrhizal fungi, cannot be cultured using current culturing techniques (van Elsas et al. 2000). Despite the cultivation-inherent bias, *i.e.* the selectivity of media, pure cultures are still of great importance for the study of functional aspects and interactions of and between fungi and other microorganisms (Hagn et al. 2003).

In order to study functional aspects in microbial ecology, specifically nutrient cycling in agricultural soils as addressed here, cultures of individual species provide the possibility to clone functional genes.

To overcome the limitations imposed by culturability we additionally performed a molecular approach, whereby total soil ITS/LSU amplicons were cloned and identified. The rapid profiling technique ITS/LSU-PCR-RFLP produced a first picture of fungal sequence diversity in ITS/LSU-libraries obtained from different soils. Furthermore, it served as a screening tool for subsequent sequencing reactions.
Molecular tools allow detection of all fungal life stages without the need for cultivation, whereby diverse new taxa throughout the *Fungi* could be uncovered in the last years (Koufopanou et al. 2001; Schadt et al. 2003). Nevertheless, variation in gene copy numbers as well as experimental baises like varying DNA extraction efficiency, PCR biases and artefacts (Becker et al. 2000; Kanagawa 2003) require special attention with regard to data interpretation, in particular quantification. Keeping that in mind, we were able to create a list of fungal species specifically present in the agricultural soils studied in the project. The frequency of an identified fungus only reflects the frequency of a certain RFLP-pattern in the clone library. Therefore, drawing a conclusion from our pattern-frequency to the real abundance of organisms in the soil is not possible (due to the biases mentioned before) and is not discussed here.

4.1.1. Species identification using public databases

Fungal species identification of both methods presented here was performed by DNA sequence comparison, a process known as barcoding. For this purpose, fungal ITS/LSU sequences were subjected to BLAST searches at the International Nucleotide Sequence Database (INSD: GenBank, EMBL, and DDBJ) which is the most widely used sequence repository in the field (Nilsson et al. 2006). The way of sequence-mediated species identification is valuable as it simplifies and standardises the identification of organisms, particularly those with limited morphological characteristics. Nevertheless, a major limitation may be a lack of reference sequences in the database (Anderson et al. 2003). This is especially true for fungal diversity studies of environmental samples, for which it is likely that more diversity is

hidden and new species will still be discovered. In this study, for more than half of the sequences it was only possible to identify them above the genus level.

Identification of sequences from previously undescribed fungal species is almost impossible if only the ITS sequence is available due to its high sequence variability. As a consequence, a more conserved region, in our case the partial LSU, is needed for classification. But still, sequences which allowed no further assignment than to a phylum were encountered. These sequences probably represent novel taxa, which may form additional species, genera or even families in the future.

Another concern regarding barcoding is the problem of insufficiently or even wrongly identified sequences in the database. Nilsson et al. (2006) suggested that about 20 % of the entries of fungal DNA sequences in the INSD may be incorrectly identified to species level, and that the majority of entries lack descriptive and up-to-date annotations. In the course of the sequence analysis we encountered at least 2 sequences, which were obviously incorrectly identified.

4.1.2. Potential biases and concerns

Direct characterisation of microbial diversity from environmental samples typically involves PCR amplification of, most commonly, ribosomal RNA genes. Therefore, by using fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) or the construction of clone libraries require special attention with regard to data interpretation, in particular quantification.

A number of factors exist, which may distort the actual picture of diversity:

- PCR biases result from the heterogeneity of template sequences, limited specificity of PCR primers, formation of secondary structures of the template, formation of chimeric molecules during PCR as well as amplification competition of different templates (Becker et al. 2000; Kanagawa 2003).
- variation in gene copy numbers,
- PCR errors
- as well as experimental errors like varying DNA extraction efficiency. Especially when fungi are investigated, differences in extraction efficiency of mycelium vs. spores may lead to uncertainties whether spores remain intact by using a soft extraction method or released DNA of *e.g.* easily cracked yeast cells may be crushed by tough extraction protocols. However, preliminary results from our lab

show that there is no big error occurring regarding extraction efficiency of mycelium vs. spores (Markus Gorfer, personal communication).

- Another question concerns free DNA available in soil or mycelium of dead fungi, which may or may not be degraded in time.

Potential biases associated with cloning were analysed by Taylor and colleagues (2007) by using two contrasting methods: the Invitrogen TOPO-TA system and the Lucigen PCR-SMART system. After comparison of the two clone libraries, which were not significantly different, they concluded, that there is no bias due to TA cloning (Taylor et al. 2007).

A further constraint is the so called primer bias, which means the possible discrepancies in primer specificity towards certain taxonomic groups (Anderson and Cairney 2004). Hereby, variation of primer affinities for the heterogeneous template molecules during PCR results in a selective amplification. For example, primer ITS1F was suggested to amplify *Ascomycota*, *Basidiomycota* and *Zygomycota*, but not *Chytridiomycota* (Manter and Vivanco 2007). However, 3 sequences affiliated to the phylum *Chytridiomycota* were discovered by the direct PCR approach.

At this point I would like to emphasise on the distinction concerning quantification between clone libraries versus soils. The interpretation of our clone library results are applied on the clone libraries themselves, but are not directly transferable to the soils due to the aforementioned biases.

4.1.3. Cultured fungi from soils

The cultivation approach addressed mainly two issues: 1. How many different and what kind of fungal species present in all five soil samples can be cultivated using the cultivation conditions described for this approach? How different is the cultivated fungal diversity in comparison with the results obtained by the culture-independent approach and is there any overlap? 2. As it turned out that there is a range of different fungal species, which were isolated from all soil samples together, these species were used to build up a strain collection of soil fungi. For this, the primary aim was the generation of a soil fungi collection of as many different cultures of individual species as possible. These isolates are then of particular interest for further investigations regarding functional genes. For example, several nitrate reductase

(NR) sequences were obtained from various kinds of fungi. This vastly enriched the already deposited collection of fungal NR sequences.

In this study, the fungal community of all cultured (under described conditions) fungi of all soil samples consisted of 61 different species.
Fusarium spp., *Penicillium* spp., *Trichoderma* spp. as well as *Verticillium* spp. were frequently isolated, which is in good accordance to Hagn et al. (2003) and references therein. Besides those, a diverse set of mainly *Ascomycota* from the orders *Capnodiales*, *Eurotiales*, *Helotiales, Hypocreales*, *Saccharomycetales* and *Sordariales* as well as some less abundant orders were isolated including previously undescribed species. Accordingly, de Castro et al. revealed by a culture-based analysis of Cerrado (a savanna-like region) soils that most of the identified fungi were *Ascomycota*, pointing at the importance of this phylum in anthropogenic modified areas (de Castro et al. 2008).

As stated by Donnison et al. (2000), fungal community compositions in hay meadows seem to differ from those of arable soils. Meadow soil R from our study, however, did not show significant differences to the other soils by comparing isolated genera, which most probably is due to our pre-selection of fungal colonies.
In addition to filamentous fungi, we were able to identify yeasts in our cultivation-based approach. Yeasts can be found in a broad range of soils, including tropical as well as Antarctic soils (Slavikova and Vadkertiova 2003). They are assumed to be primarily degraders and utilise simple sugars, although their function in soil ecosystems is still unclear (Connell et al. 2008). The fermentative ascomycetous yeast *Candida* sp. as well as two *Cryptococcus* species (basidiomycetous encapsulated yeast) were isolated. These yeast genera have also been detected in agricultural soils by other groups (Slavikova and Vadkertiova 2003; Vishniac 2006).

4.1.4. Molecular sequences vs. morphology

Traditional methods for fungal identification and classification are based on morphological characters. The hierarchical classification of fungi experienced many changes over the last decade. Due to molecular phylogenetic studies based on nucleic acid sequences new insights into an overall fungal classification was achieved. As a consequence, diverse traditional ideas regarding fungal relationships

had to be given up. Organisms which originally have been considered to be fungi were omitted from the kingdom *Fungi* as well as the other way around. Orders and families as well as genera went through a remodelling process and it became clear, that many fungal “species” are several and not one. Examples for such “cryptic” species are *Armillaria mellea s. lat.* containing at least eight species in Europe, or *Fusarium graminearum*, within which nine species have been recognised.
Despite this currently ongoing fundamental reassessment of traditional systematics, molecular approaches are also contributing to our knowledge of unexplored fungal diversity. Species recognition is facilitated by molecular tools, particular when fungi are not sporulating or uncultured. Therefore, this issue is not so much one of “molecules *versus* morphology”, but “molecules plus morphology”. Hence, co-operation of taxonomists and specialists experienced in molecular phylogenetic approaches is becoming the norm (Hawksworth 2006).

4.1.5. Culture-independent assessment of soil fungal diversity

Clone libraries derived from the five soil samples were analysed according to their species composition. Although relative abundances and numbers are very difficult to interpret due to the RFLP approach (difficulties are discussed above), the results from the frequency analyses of the clone libraries (not the soils themselves) are presented here.

Our culture-independent approach yielded a highly different list of species. In total, a number of 122 different RFLP-types were observed for all data.
Among the uncovered species many affiliated to the *Ascomycota*. This phylum predominates (> 75%) in all soils in comparison with *Basidiomycota*, while members of other fungal phyla were recovered at much lower frequencies or were not detected at all. However, investigations on fungal diversity in forest soils gave rise to equal proportions of *Basidiomycota* and *Ascomycota* (O'Brien et al. 2005). This is due to the different sampling sites: ectomycorrhizal fungi are mostly found in forest ecosystems and many ectomycorrhizae belong to *Basidiomycota*.
That *Ascomycota* was clearly the dominant phylum in the clone libraries of soils from a savanna-like region in Brazil has been shown by de Castro and colleagues (2008). Furthermore, the assessment of a sandy lawn soil microbial community structure revealed that within the Fungi, the phylum *Ascomycota* accounted for two thirds of

the ribo-tags (Urich et al. 2008). Ecologically, *Ascomycota* function as primary decomposers of plant materials, but they also are important as plant and human pathogens.

Generally, *Basidiomycota* species were distributed over all libraries, including seven different species like *Cryptococcus* spp., *Coprinellus* sp., *Hebeloma* sp., *Minimedusa polyspora*, *Psathyrella* sp., *Scleroderma bovista*, *Thanatephorus cucumeris* and *Trichosporon dulcitum*.
Basidiomycota could be identified more often by the culture-independent method than in the cultivation approach. Interestingly, the two methods seem to supplement each other in a nice way as far as *Cryptococcus* species are concerned. While those species were isolated from soils M and N, they were detected by direct PCR from P, R and T. Reasons for the complementary rather than overlapping effect of both approaches are discussed below.

Even the more archaic phylum *Chytridiomycota* could be discovered by the molecular method, which was surprising, because of our primer selection for this approach. The primer ITS1F was suggested to amplify all higher fungal species, covering most species of *Ascomycota*, *Basidiomycota* and *Zygomycota*, however, it was not expected to address *Chytridiomycota* (Manter and Vivanco 2007). *Chytridiomycota* are the most primitive, mainly aquatic fungi and belong to the phylogenetically oldest group of fungi.
In the clone libraries N, P and T we found taxa belonging to *Zygomycota*, a traditional phylum, which has long been recognized to be polyphyletic based on molecular analyses. Hibbett et al. (2007) proposed a comprehensive phylogenetic classification of the kingdom *Fungi*, which also included a rearrangement of clades that have traditionally been placed within the *Zygomycota*. Due to pending resolution of relationships among the clades, a deconstruction of the *Zygomycota* and distribution among *Glomeromycota* and several subphyla *incertae sedis* (*Entomophthoromycotina*, *Kickxellomycotina*, *Mucoromycotina*, *Zoopagomycotina*) has been conceptualized (Hibbett et al. 2007).
Our sequences formerly assigned to *Zygomyctoa* turned out to inclusively belong to *Mucoromycotina*, whereby half of the sequences can be further classified as members of the *Mortierellaceae* family.

Already at subphylum level the R library stands out, because it not only harbours the highest diversity on this level compared to the other libraries, but it also contains a newly described subphylum to an extent of 13.4 % of all detected RFLP-types. This subphylum is termed Soil Clone Group I (SCGI) by Porter and co-workers (2008), who postulated this novel subphylum of *Ascomycota* by phylogenetic analyses of unclassified fungal sequences. These sequences from soils of diverse origin (forests as well as grassland) could be combined in a well-supported clade at the base of the *Ascomycota*. SCGI currently represents the only major fungal lineage known exclusively from sequence data. The presence of SCGI in soil R, which derives from a grassland, but its absence from agricultural soils M, N, P and T suggests a preference of SCGI-fungi for undisturbed sites. Presence or absence of SCGI-fungi may in the future be used as an indicator for soil disturbance in a manner similar to the use of indicator organisms for the assessment of water quality (Markus Gorfer, personal communication).

On the class level, all clone libraries have in common that they include ascomycetous species assigned to *Sordariomycetes*, *Eurotiomycetes* and *Dothideomycetes* as well as the basidiomycetous class *Tremellomycetes*.
At the next taxonomic level, it can be shown, that the *Sordariales* and *Hypocreales* are the major orders present in all libraries. The order *Boletales* is unique to M, which contributes 8.4 % to the diversity of M and is composed of *Scleroderma bovista*. Alternatively, the order *Agaricales* is only present in T, which makes up for 6.9 % including the genera *Coprinellus* and *Psathyrella*.
Again outstanding, M holds the largest amount of *Lasiosphaeriaceae* species compared to the other libraries. This family takes 23.2 % of M consisting of species, which could not be further identified than to this level. Another family, which was exclusively detected in M, is *Cortinariaceae*, which is represented by a *Hebeloma* species.

Furthermore, the PCR-based method revealed that different RFLP patterns resulted in the same sequence, which was designated as *Tetracladium*-related (96 to 97% sequence identity). In both clone libraries P and R approximately one quarter of each 96 clones inspected led to this result, additionally *Tetracladium* was detected in N

and T to a minor extent, while the sequence could never be detected in M. *Tetracladium* is an aquatic hyphomycete and has often been isolated from soils (Ronhede et al. 2005). Unfortunately, very little information on *Tetracladium* is available at the moment.

Of all clone libraries M displays some conspicuous characteristics: it has the maximal frequency of identical patterns from RFLP analysis, its diversity encompasses only 2 different phyla and generally it holds the lowest values at all taxonomic levels (Tab.3.12). Furthermore, *Scleroderma bovista* as well as *Hebeloma* sp. exclusively occur in M. Also notable is the fact that M shows the lowest species richness compared to the other libraries (Fig.3.13). Reasons for the outstanding characteristics of M are difficult to find. The presence of mycorrhizae like *Scleroderma* and *Hebeloma* species indicates a forest close to the sampling site, and indeed there are trees located around the field. The low species richness may also be connected with the location of the sampling site, the soil characteristics or the growing of certain crops.

In general, while most sequences could be attributed to a species or a higher level of taxonomic rank, some could only be assigned to a phylum, which is simply a result of a lack of reference sequences, which is either due to the fact, that no sequences of already existing cultures are available in databases yet, or the sequences represent novel lineages.

Since the primers applied in this study do not target *Glomeromycota* species, this phylum was not detected by our approach. However, this group of fungi play significant functional roles in soil. Mycorrhizal fungi (*Glomeromycota*) are specialised to form close associations with plant roots and act as an extension of the plant root system. This aids in the uptake of almost all plant nutrients and is particularly important in the uptake of phosphates. Phosphates typically have low solubility in the soil solution and therefore exist at low concentrations. Mycorrhizal fungi are endemic in most soils and form extensive networks of fungal hyphae that can connect different plant species. Arbuscular mycorrhizae (AM fungi) penetrate the cortical cells of plant roots and form unique structures (arbuscules). In agroecosystems they play important roles: AM fungi are known to stabilise soil aggregates and provide soil

nutrients, particularly phosphorus, to the plant. Mycorrhizae are a well described group of fungi (Dighton et al. 2005).

There is only limited information in the literature on the ecological function, especially regarding nitrogen cycling, of fungal communities in soils. In general, soil fungal communities are understudied in agricultural sciences.

Recent developments in understanding the roles of fungi in nitrogen transformation have been reviewed by Hayatsu et al. (2008). Fungi were shown to be involved in both nitrification and denitrification. Denitrifying activity occurs in various fungal phyla, including *Ascomycota* such as *Cylindrocarpon tonkinense* and *Gibberella fujikuroii* as well as the basidiomycetous *Trichosporon cutaneum*. In soils, denitrifying fungi may contribute significantly to the N_2O and N_2 production under both aerobic and anaerobic conditions. For example, in forest soils, grasslands (Laughlin and Stevens 2002) and semiarid regions (McLain and Martens 2006) fungi have been found to be the dominant denitrifiers. While bacterial denitrification activity is suppressed by excess of oxygen, denitrification activity by fungi is induced in the presence of nitrate or nitrite and significant amounts of oxygen, but not in oxygen excess (Hayatsu et al. 2008).

Furthermore, *Fusarium solani* and *C. tonkinense* produced N_2 in the presence of amino acids by co-denitrification. Hereby nitrogen atoms from nitrite and other nitrogen compounds (co-substrates) such as azide and ammonium are combined under denitrifying conditions. In *Fusarium oxysporum* the denitrification enzyme P450nor was shown to catalyse the co-denitrifcation reaction without an electron donor such as NADH. To distinguish between denitrification and co-denitrification ^{15}N-labeling experiments are required. In grassland soil, the relative contribution of fungal co-denitrification and denitrification was estimated to be ~92 % (for co-denitrification) (Hayatsu et al. 2008).

Also, nitrification (oxidation of nitrite to nitrate) has been revealed in fungal species, including *Aspergillus wentii* and *Penicillium* spp. The contribution of heterotrophic microorganisms to nitrification has been recognised in soil. Especially, fungal nitrification has been observed in acidic soils such as forest soils, but the biochemical mechanisms remain unclear (Hayatsu et al. 2008).

4.1.5.1 Analysis of species richness

Species diversity is defined as the number of species within a community as well as the relative abundance of individuals in that community. Species richness is the simplest diversity index to interpret. Due to differences in collection size, it is hard to compare different samples. Rarefaction is a procedure which addresses the problem of comparing the species richness of different habitats. Thereby the expected species richness based on random subsamples of individuals is calculated. In this way, a large rarefied sample can be directly compared with smaller collections, because the species richness of both collections is then based on an identical number of individuals (Gotelli and Graves 1996).

By comparing the rarefaction-species-accumulation curves of our different clone libraries, M shows the lowest species richness, while the other libraries reached a higher range, N and R with the highest species richness (Fig.3.14). The same trend can be observed by viewing the richness estimator Chao2. This estimator is especially advantageous for mycological studies, because it does not require precise information on the number of individuals per sample. Chao2 is an incidence-based estimator, which relies on the number of unique units and duplicates and not on the number of singletons and doubletons. Units refer to species found in only one and two sample units, whereas singletons and doubletons mean species present with one and two individuals (or sequences) (Unterseher et al. 2008). Regarding our data, all Chao2 curves continued to rise, whereby M being an exception with an alternating curve (Fig.3.9 – Fig.3.13). Additionally, the estimated species richness using Chao2 was the lowest for M (22 ± 2). T and P were located in the middle (33 ± 5 and 35 ± 6), while N (43 ± 8) and R (47 ± 12) reached the highest species richness estimated with Chao2. These data show once again that M possesses unique characteristics compared to the other libraries.

Generally, we can expect that the species richness calculated by the different estimators represent an underestimation. This is due to the fact that the estimation relied on the RFLP analysis, which itself was an underestimation of diversity as we found out that some RFLP patterns turned out to comprise more than one species. To circumvent this particular problem it is advisable to analyse the sequences of all available clones. Nevertheless, the low estimated species richness was surprising,

but the numbers also seem realistic, when compared with species richness of highly diverse forest soils (Taylor et al. 2007).

Furthermore, the observed diversity of RFLP-types ranges from 64 to 91 % of the estimated species richness in the respective library (Tab.4.1). One would expect that the observed species richness of a library harbouring the lowest diversity is closer to its estimated species richness than as it would be for a library with a higher diversity. And precisely this is, what the ratios between observed and estimated species richness show (Tab.4.1): M has the lowest diversity and 91 % of the expected species have already been recovered. This is further reflected by the rarefaction species-accumulation curve, which reaches an asymptote.
For the other libraries the ratio is lower, whereas the library with the highest estimated species richness (R) has the lowest value (64 %). Therefore, increasing the sampling effort, (reflected by the number of clones in the library), would result in a better ratio of recovered to expected species.
All in all, our data show that we were able to capture most of the expected fungal organisms from agricultural soils by the culture-independent approach.

Tab.4.1 Estimated and observed species richness in clone libraries.

Clone library	Estimated species richness (Chao2)	Observed RFLP-types	Observation/ estimation ratio [%]
M	22	20	91
N	43	32	74
P	35	28	80
R	47	30	64
T	33	28	85

4.1.6. Overlap of culture-dependent and -independent approaches

The amplified sequence regions for identification varied between the culture-dependent and –independent approaches: For identification of cultured fungi the ITS-region covering both, ITS1 and ITS2 region was sequenced. Well characterised reference sequences were expected for cultured fungi in the INSD. The complete ITS-region is ideal for species (sometimes even subspecies) identification with sufficient reference data. Conversely, from the culture-independent approach less well characterised reference sequences were expected. In this case the LSU served

as a good marker with improved resolution at higher taxonomic levels. Therefore, the overlapping sequence from the two different approaches mainly covers the ITS2 region (~ 300 bp), which is enough to find identical species from both methods by sequence analysis.

Altogether, the overlapping set of sequences of both approaches within the same soil was diminutive. Only 5 sequences uncovered by both methods, actually derived from the same soil sample. These are *Fusarium oxysporum* (clone library derived from soil R), *Helicodendron* sp. (T), *Davidiella tassiana* (T), *Cercophora/Apodus* sp. (T) and *Bionectria ochroleuca* (P). Still, some other species, e.g. *Cryptococcus aerius*, also show up to 100 % identity within the overlapping sequence region, but originate from different soil samples. This is due to our culture-based approach, which was performed to obtain a picture of the diversity of cultivable fungi from all soil samples together. The results of this approach therefore should be seen on the whole and should not be differentiated into the individual soil samples.

Interestingly, *Aspergillus*, *Penicillium* and *Trichoderma* species which were frequently isolated from all soil samples could not be found in the culture independent approach. Furthermore, plates with rapidly growing and heavy sporulating *Mucorales* were discarded from our culturing approach, since subculturing from such plates was hardly possible.

It is expected that increasing both, the culturing effort and the number of sequenced clones from clone libraries would result in a higher number of species found by both approaches. Thus, the overlap of species found by both approaches may be larger in reality than only 5. In total, we counted 14 different species, which possess an overlap of more than 98 % sequence identity, although a comparison is not totally correct due to different soil origins. Nevertheless, apart from the difference in soil origins, the overlapping 14 species make up for only 8.6 % of identified species by both methods taken together. The majority of species were detected by the culture-independent procedure (62.6 %), while 28.8 % of all species were found by the culturing-approach exclusively. It is surprising, that more than a quarter of all species was cultivated under our conditions, but never detected by the cloning technique. This means, that either these organisms occurred in a very low amount, and might thus have escaped detection in the clone libraries, or these fungi were present as

spores, which were able to germinate, but withstood our DNA extraction procedure. On top of that, the possibility of undersampling should be taken into account.

The analysis clearly demonstrates that both methods are complementary rather than overlapping. Therefore, to cover fungal diversity more broadly, both approaches, displaying different features of diversity or community structure, are needed. However, even using multiple techniques we may be far from reaching the true magnitude of fungal diversity.

4.2. Nitrate reductase sequences from soil fungi

Nitrate is a significant nitrogen source for plants and microorganisms. Nitrate assimilation is a key process in the global nitrogen cycle, whereby nitrate is reduced via nitrite to ammonium which is incorporated into cell material (see 1.1.7. Nitrate assimilation).

To gain insight into specific contribution of fungi in soil N-cycling and possible fluxes of nitrogen between the different members of the soil microbial community, nitrate reductase (NR) encoding genes (henceforward called *niaD* in analogy to the gene from *Aspergillus nidulans* (Garrett and Cove 1976)) from soil fungi were studied. From many studies with different fungi it is well established that NR is under transcriptional control (Marzluf 1997). Ammonia and glutamine repress the expression of NR, whereas nitrate is necessary for induction. However, there are some exceptions: *Gibberella fujikuroi* (*Fusarium fujikuroi*) seems to be independent of nitrate induction (Mihlan et al. 2003). In addition, NR expression of some ectomycorrhizal fungi is de-repressed when N becomes limited without additional induction by nitrate.

It is assumed that detection of fungal *niaD*-mRNA in soil would be indicative of nitrate uptake and utilisation by soil fungi. Sequencing of soil *niaD*-mRNA would additionally provide information about the identity of the fungal species utilising the nitrate.

Since the investigated soils mainly contained *Ascomycota*, the focus was laid on the amplification of ascomycetous *niaD* sequences from soil. Basidiomycetous *niaD* sequences deviate highly from ascomycetous *niaD* sequences so that the development of degenerate primers allowing amplification of both, ascomycetous and basidiomycetous *niaD* genes, is hardly possible. *Trichoderma* species seem to have obtained their *niaD* genes by horizontal gene transfer from a basidiomycetous host

(Slot and Hibbett 2007). *Trichoderma niaD*-genes can therefore only be amplified with *Basidiomycota*-specific PCR-primers.

On the basis of 29 *Ascomycota* of which full length *niaD* sequences were available in Genbank, a multiple alignment of protein sequences was generated to design a set of primers for the amplification of a ~ 400 bp long stretch of the *niaD* gene. Due to the diverse range of organisms included in the alignment we gained a good coverage of mainly *Pezizomycotina* within the *Ascomycota*, whereas the *Saccharomycotina* were excluded. This is because the ability of nitrate assimilation in *Saccharomycotina* is restricted to only a few species (*e.g. Hansenula polymorpha*) (Siverio 2002). No *niaD* sequences from the *Taphrinomycotina* are known.

The primers were preliminary tested with two laboratory strains known to contain nitrate reductase by growth tests on minimal medium with nitrate as the only nitrogen source. In this way, PCR conditions were optimised. However, it became apparent that *niaD* amplification was more challenging when the heterogeneous soil DNA was used. After adaptation of PCR conditions (higher primer concentrations, addition of enhancers like Betaine, *etc.*) a nested PCR gave rise to strong bands for all 4 samples. As the results of our *niaD* clone libraries indicate, degeneracy of primers did not lead to loss of specificity, since almost every insert could be assigned to fungal *niaD*.

In silico analysis revealed that in a portion of sequences derived from P1 and P2, the amplified coding region is interrupted by a single intron. Therefore, sequences of mainly two lengths were obtained: 367 bp without an intron and 419 or 442 bp, respectively, containing a single intron; all sequences coding for 122 amino acids.

The size of the whole nitrate reductase protein may vary from 864 amino acids for *Penicillium chrysogenum* to 982 amino acids for *Neurospora crassa*. It is known that the numbers and positions of introns can differ between species. Furthermore, similarities regarding intron organisation within the gene is associated with taxonomic relationships (Cutler et al. 1998). In fact, the nitrate reductase gene displays substantial sequence similarity between fungi, algae and higher plants, which offers the utilisation of *niaD* as a classification tool. Moreover, phylogenetic trees based on *niaD* correspond well with the phylogeny of the organisms determined from alternative molecular studies and systematics (Zhou and Kleinhofs 1996). Indeed, the development of diagnostic kits for the detection of certain *Verticillium fungicola* strains by usage of nitrate reductase sequence data was proposed by Amey et al.

(2007). Furthermore, phylogenetic analysis of the nitrate reductase protein sequence revealed that the fungal group of eukaryotic nitrate reductases were arranged following taxonomic classification: fungi appeared to be divided into 2 distinct subclades with *Ascomycota* and yeast forming one, and the *Basidiomycota* the other (Stolz and Basu 2002). Due to its conservation and the correlation to taxonomic lines, the eukaryotic nitrate reductase represents a valuable phylogenetic marker. However, all the aforementioned studies rely onto data not including the *niaD* sequence of *Trichoderma reesei (Hypocrea jecorina)*, an ascomycetous mold. According to Slot and Hibbett (2007) *T. reesei* obtained the nitrate assimilation cluster, which consists of a high affinity nitrate transporter, a nitrate reductase and a ferredoxin-independent assimilatory nitrite reductase, by horizontal gene transfer from a basidiomycete. This may have corresponded to a change in nutritional mode, improving the fitness of *T. reesei* in a new niche. Supported by phylogenetic studies, the horizontal transmission is a plausible scenario since some *Trichoderma* species are intracellular parasites of basidiomycetes (Slot and Hibbett 2007).

Sequence analysis of our own data in combination with *niaD* sequences available at Genbank as well as partial *niaD* sequences obtained from several isolated fungi from our strain collection give a similar picture, whereby sequences obtained from *Trichoderma* species cluster together with *Basidiomycota*. Our results therefore confirm the observations of Slot and Hibbett (2007). This is an interesting finding, because horizontal gene transfer shatters the tendency of *niaD* acting as a molecular marker for phylogeny, although *niaD* sequences of many other *Ascomycota* still substantiates a taxonomic classification in accordance to rRNA genes/ITS phylogeny.

Nevertheless, owing to our still increasing *niaD* sequence collection, it became possible to group many of our clone library-derived sequences close to cultivated species. For example, one of the sequences obtained from the *niaD* library was identical with a *Verticillium nigrescens niaD* sequence. Other unknown sequences clustered together with *Plectopshaerella cucumerina* or *Acremonium* sp., respectively. Furthermore, there are some *niaD* sequences derived solely from the soil P clustering together with species from the order *Pezizales*. Interestingly, our diversity study detected *Pezizales* species only in the P clone library, not in N.

Although many *niaD* sequences are available from *Aspergillus* and *Penicillium* species, no *niaD* sequence from our libraries groups within the cluster of *Eurotiomycetidae*.

Generally, for the majority of fungi a more accurate assignment is not possible at the moment. This is due to the shortness of the amplified *niaD* sequence stretch, which does not contain enough variability. Therefore, a new set of primers was designed, amplifying a longer fragment of the *niaD* gene. For future studies the assignment of certain *niaD* sequences to the corresponding species is of particular interest. For example, soil samples treated with different fertiliser conditions can then be analysed at the activity level by quantifying *niaD* mRNA. This may give us information about the actual induction of the fungal nitrate reductase, and furthermore may identify the active species. A *niaD* microarray containing several dozens of *niaD* probes targeting a diverse group of fungi would provide a means for simultaneous analysis of gene expression of many species revealing who is active up to what extent, under which condition and at what time. Furthermore, a combination with pool measurements of ^{15}N labelled substrates would allow an estimation of fungal nitrate assimilation activity. How fungi compete with bacteria and plants in terms of nitrate assimilation as well as dissimilatory activities under diverse conditions are important questions addressed by the “Nitro-Genome”-project.

4.3. Fluorescence *in situ* hybridisation

The determination of diversity and functional capability of microbial communities in the environment is facilitated by molecular techniques. PCR-based fingerprinting techniques, such as denaturing- or temperature-gradient gel electrophoresis (DGGE or TGGE) or terminal restriction fragment length polymorphism (T-RFLP) are commonly used to rapidly profile fungal populations in an ecosystem (Kennedy and Clipson 2003). However, fingerprinting techniques generally lack sensitivity and, even more important, harbour a number of problems associated with preceding PCR. Due to over- or under-amplification of particular sequences, PCR-based techniques are therefore not suitable for quantitative data interpretation (Kennedy and Clipson 2003; Lau and Liu 2007). Since the construction of clone libraries is also based on PCR, our diversity analysis acts more like a demonstration what kind of species can be found by the methods we applied. Also, the quantification we performed for our

different soil clone libraries is always referred to the clone libraries themselves, not directly to the soils. As a result, an additional method, independent from PCR, is required.

Fluorescence *in situ* hybridisation (FISH) not only enables visualisation and phylogenetic identification of single cells, but also the direct quantification of microorganisms. If mRNA is targeted, the question of linking function to diversity can be addressed.

The FISH experiment was performed to test the method on filamentous fungi; in our case a pure culture of the model organism *Aspergillus nidulans* was used. There are not many protocols for the application of FISH on filamentous fungi in the literature; occasionally a permeabilisation of the cell wall with lysing enzymes is suggested (Sterflinger et al. 1998; Teertstra et al. 2004). For *A. nidulans* no enzymatic treatment prior to hybridisation was required to yield strong fluorescence signals. Initially, parameters like culture conditions, the sample fixation method, treatment of slides as well as hybridisation and washing buffer compositions were tested using the 18S rRNA-targeted probes EUK516-Fluos.

These pre-experiments of rRNA-FISH were performed using an overnight culture grown on an AMM-plate. Two fixatives were tested: formaldehyde-fixed samples appeared to work better than ethanol-fixed ones. Uncoated slides were sufficient and H_2O_2-treatment did not negatively affect the outcome. Furthermore, a 10 % formamide concentration seemed to be suitable for hybridisation and both buffers, Tris- and SSC-buffer, worked well.

The most suitable conditions (*i.e.* highest signal intensity) were then selected for the actual mRNA FISH using the probe which locates *niaD* mRNA. In order to avoid mRNA degradation by endogenous and exogenous RNases several precautions were taken (*e.g.* DEPC treatment).

It could be shown that no enzymatic treatment prior to hybridisation is needed for *Aspergillus nidulans* in order to yield a strong, clear and specific signal for the detection of rRNA as well as *niaD* mRNA. While rRNA appeared to be evenly distributed in the cytoplasm, *niaD* mRNA were unevenly distributed in the cells.

It is known, that *niaD* gene expression can either be induced by nitrate (NO_3^-) or repressed by ammonium tartrate. Subsequent to a short N-starvation period after germination followed by induction with nitrate, the expression level of *niaD* can even

be more induced (“super-induced”) than without preceding N-starvation (Todd et al. 2005).

Using the same microscopic settings, induced and super-induced *A. nidulans* cultures as compared to the uninduced ones generated a higher fluorescence intensity for the *niaD*-targeted probe. Interestingly, the induced sample appeared brighter than the super-induced sample. However, the intensity of the signal was not quantified.

In the course of these experiments, we initially focused on the establishment of the FISH protocol applied for *Aspergillus nidulans* as a proof of principle. Furthermore, during this preliminary test, the optimisation of FISH conditions for the detection of *niaD* mRNA was attempted. Fortunately, our results look promising and form the basis of future studies, dedicated to the investigation of fungal *niaD* under different conditions; first studied in pure cultures which can then be expanded to fungi in environmental samples.

4.4. Summary and perspectives

Fungi fulfil a range of important ecological functions. There is poor understanding of soil fungal community diversity and the specific roles of individual phylogenetic groups present in the environment.

Molecular techniques open new possibilities to obtain information on fungal diversity and the importance of fungi in ecosystems (Hawksworth 2006).

By the determination of fungal diversity in agricultural soils as well as one grassland soil using two different approaches, we gained a picture of a diverse range of fungal species. This inventory, connected with physiological information, will allow spanning a bridge between community structure and functional activities. Besides the phylogenetic marker ITS/LSU for community description, we focused on the functional gene *niaD*, coding for fungal nitrate reductase. This enzyme catalyses the reduction of nitrate to nitrite, which is the first reaction in nitrate assimilation. Investigation of the *niaD* gene allows the determination of diversity and functional potential, meaning the simple detection of fungi containing the gene without knowing their activity status. Quantification on the DNA level thus only allows counting population numbers, or more precisely gene copy numbers. This is particularly interesting if environmental changes cause a variation in the microbial population numbers (Saleh-Lakha et al. 2005). However, environmental changes that affect the

gene expression in the same microbial population may be even more important. Exploration of the actual transcriptional activity of a functional gene is achieved by the analysis of the respective mRNA. A functional gene array containing *niaD* probes could be used to monitor the physiological status and functional activity of the *niaD* expressing fungal community. Although, the most sensitive method for the detection of low abundance mRNA is real-time PCR (Saleh-Lakha et al. 2005). Despite the precision and accuracy of real-time PCR, which has already been successfully applied in quantifying the abundance of a gene in environmental samples, both techniques, real-time PCR as well as microarrays, are limited by the low mRNA extraction efficiencies from environmental samples (Saleh-Lakha et al. 2005).
In the course of the “Nitro-Genome”-project time experiments with barley and different fertiliser treatments were conducted. Meanwhile, protocols for RNA extraction were performed and between 0.5 and 2.5 µg RNA per g dry weight soil can now routinely be obtained. The quantity and quality of RNA appeared to be sufficient for reverse transcription and an optimised protocol for this reaction was developed. So far, we have got all the tools available to characterise and quantify the *niaD* expressing population. Future studies will therefore focus on real-time PCR experiments and the development of a *niaD* microarray.

Linking biodiversity with function is an important issue when soil ecosystems are investigated. Therefore, experimental approaches for simultaneous identification of taxa within a community and the functional processes performed by them are valuable. Analysis of the total RNA pool of a community using highly parallel sequencing provides the opportunity to study mRNA and rRNA molecules simultaneously from the same sample. The ‘‘double-RNA’’ approach by (Urich et al. 2008) yielded qualitative and quantitative information on both structure and function of a soil community. However, pyrosequencing is very costly and produces only short reads of around 100 bp.

Fluorescence *in situ* hybridisation (FISH) additionally enables the visualisation of single microbial cells directly in the environmental sample. Furthermore, the phylogenetic identification of cells combined with detection of mRNA addresses the question of linking function and diversity.
PCR-based fingerprinting techniques, such as D/TGGE or T-RFLP offer the determination of diversity and functional capability of microbial communities in the

environment. These techniques are commonly used to rapidly profile fungal populations in an ecosystem (Kennedy and Clipson 2003). T-RFLP analysis of the *niaD* gene, for example, allows the recognition of shifts in soil fungal community structure in response to different fertiliser treatments.

There is a variety of research possibilities for the future by applying additional methods as well as refinements of already performed techniques, which lead to an increased understanding of the diversity and structure of fungal communities. Furthermore, this may contribute to an improvement of methods for sustainable agriculture and restoration of ecosystems at disturbed sites (Midgley et al. 2007), as well as to the enhancement of nitrogen fertiliser efficiency as the final aim of this investigation, which would be for the benefit of ecologists, agronomists, industry and the whole environment.

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Zusammenfassung

Pilze spielen eine zentrale Rolle im Ökosystem Erde. Dennoch ist nach wie vor wenig über die Pilzdiversität in Böden und die spezifischen Funktionen einzelner phylogenetischer Gruppen bekannt.

Vier verschiedene Ackerböden (Maissau, Niederschleinz, Purkersdorf, Tulln) sowie eine Graslanderde (Riederberg) wurden auf ihre Pilzdiversität untersucht. Hierfür wurden ein Kultivierungsansatz sowie eine direkte molekularbiologische Methode verwendet. Die Pilze wurden aufgrund ihrer DNS-Sequenz der ITS/LSU ribosomalen RNS Genregion identifiziert. Es wurden 61 verschiedene Pilzarten isoliert, die hauptsächlich zum Phylum *Ascomycota* gehören, darunter zB *Fusarium* spp., *Penicillium* spp., *Trichoderma* spp., sowie bislang unbeschriebene Arten. Im Rahmen des kultivierungsunabhängigen Ansatzes wurde eine Klon-Bibliothek erstellt. Danach wurde eine RFLP-Analyse durchgeführt. Auch hier dominierten Spezies der *Ascomycota*, die Artenzusammensetzung unterschied sich jedoch erheblich vom Kultivierungsansatz. Vertreter der *Basidiomycota* traten hierbei öfter und in allen fünf Bibliotheken auf. Die Zusammensetzung der Pilzgemeinschaften wies deutliche Unterschiede innerhalb der einzelnen Klon-Bibliotheken auf. Die Artenzahl (species richness) wurde mit verschiedenen Methoden berechnet. Chao2 zeigte für die untersuchten Böden eine gute Abdeckung der erwarteten Pilz-Artenzahl.

Lediglich 8,6% der identifizierten Arten konnten mit beiden Verfahren detektiert werden. Die Mehrzahl der Arten (62,6%) wurde mittels des kultivierungs-unabhängigen Verfahrens ermittelt, während 28,8% ausschließlich mit Hilfe des Kultivierungsansatzes identifiziert wurden. Hierdurch wird deutlich, dass beide Methoden sich vielmehr ergänzen, als überlappen. Die vom Grasland Riederberg entstammende Klon-Bibliothek enthielt die größte Diversität und wies als einzige das neu beschriebene *Ascomycota* Subphylum Soil Clone Group I (SCGI) auf, was eventuell darauf hindeuten könnte, dass SCGI-Pilze bevorzugt an unberührten Standorten vorkommen. Die Maissau Klon-Bibliothek zeigte den geringsten Artenreichtum und auch andere spezifische Eigenschaften grenzen diesen Standort von den anderen ab.

Der zweite Teil dieser Studie befasste sich mit der Erforschung der Rolle von Pilzen in N-Transformationsprozessen. Hierfür wurden die Gene, welche für die pilzliche Nitratreduktase kodieren (*niaD*) untersucht. Dabei stand die Erstellung eines Sequenz-Sets im Vordergrund, welches partielle *niaD* Sequenzen von Isolaten sowie unkultivierten Pilzen enthält. Auf Grund dieser Erkenntnis soll es möglich werden,

transkriptionelle Aktivitäten von Pilz-Populationen, welche dieses Gen exprimieren, unter verschiedenen Umweltbedingungen zu beobachten. Zusätzlich wurde Fluoreszenz *in situ* Hybridisierung (FISH) mit dem Modelorganismus *Aspergillus nidulans* durchgeführt. Im Rahmen eines Vorversuchs wurden die FISH Parameter optimiert und die Detektion der *niaD* mRNA angestrebt. Unsere viel versprechenden Ergebnisse stellen die Basis für zukünftige Studien dar, welche die Expression der Pilz-Nitratreduktase unter verschiedenen Umweltbedingungen erforschen sollen.

Curriculum vitae

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Scholarships

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Publications / Poster Presentations

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„Es ist wichtiger, dass sich jemand über eine Rosenblüte freut, als dass er ihre Wurzeln unter das Mikroskop legt.“

Oscar Wilde