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Diversity and taxonomy of fungi inhabiting extremely acidic and saline soils of natural and anthropogenic origin in the Czech Republic

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Ph.D. Thesis

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Declaration: I hereby declare that I have written this thesis independently, using the listed references or in cooperation with other paper coauthors (for this author's contributions to particular papers, see chapter 2). I have not submitted this thesis, or any of its parts, to acquire any other academic degree.

Prague 26th April 2015 ....

Martina Hujslová

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Further aknowledgements, relevant to particular papers, can be found in the included manuscripts (chapter 2).

Abstract: Highly acidic environments represent some of the most extreme habitats for the microbial growth. For a long time it has been assumed that these sites are populated exclusively by prokaryotes. However recently, eukaryotic organisms including fungi have been found to be abundant and important component of acidophilic communities. Concerning fungal diversity only fragmentary data are available indicating that highly acidic sites harbour specific and low-diversified fungal communities dominated by dematiaceous fungi. In the present work we focused on the cultivable mycobiota occupying highly acidic (pH < 3) soils which are at the same time saline (sulfate-rich) and are located at geographically isolated localities in the Czech Republic. This study should provide a deep insight into the diversity and biogeographical pattern of acidophilic/tolerant fungi. A combination of classical and specialized cultivation techniques was successfully applied since it significantly contributed to the broadening of the detected fungal spectrum. The revealed fungal assemblages inhabiting highly acidic sites worldwide are closely similar and differ from the ones known from less acidic habitats. The core of the fungal assemblage under study consisted of phylogenetically unrelated and often globally distributed fungi exclusively inhabiting highly acidic habitats as well as taxa known from less acidic and often extreme environments. High numbers of specialized species we have detected indicate that highly acidic environments provide suitable conditions for the evolution of specialist species. The occurrence of ubiquitous fungi in highly acidic substrates points to the principal role of competition in colonization of highly acidic environments. In spite of both morphological and molecular markers used for identification we have detected numerous unknown taxa which show that fungal community of highly acidic habitats is more diversified than it has been supposed so far. During two taxonomical studies several dominant members of the fungal community were identified and their phylogenetic relationships have been resolved. The four of them are here described as a new genera and species. The detected taxa did not require low pH to survive, because they can grow in a broad range of pH. The present work primarily advances our understanding of fungi inhabiting highly acidic habitats. In addition, it provides a great deal of isolates of specialized fungi from which several are known for their enormous biotechnological potential which indicate that this type of environment has a great potential of finding new biotechnologically interesting fungi.

Abstrakt: Silně kyselé substráty (pH < 3) představují pro život mikroorganismů jedno z nejextrémnějších prostředí. Dlouhou dobu panoval názor, že tento typ habitatu osidlují výhradně prokaryotní organismy. Nicméně výsledky recentních studií ukázaly, že mikrobiální společenstvo silně kyselých substrátů je tvořeno z velké části rovněž eukaryotickou složkou zahrnující vedle řas a protist také houby. O diverzitě acidofilních/tolerantních hub, je prozatím k dispozici jen málo informací, které ukazují, že zdejší houbové společenstvo je druhově chudé, specifické s převahou demáciových druhů. Předkládaná práce se zabývá studiem diverzity a taxonomie kultivovatelných druhů hub osidlujících silně kyselé a zároveň zasolené (sulfátové) půdy, a to na několika geograficky izolovaných lokalitách v rámci České republiky. Porovnání našich dat s dalšími dostupnými informacemi o houbách izolovaných z podobných silně kyselých míst ve světě umožní odhalit druhy tvořící základ zdejšího společenstva a jejich případnou podobnost, a zároveň pomohou objasnit otázku rozšíření acidofilních/tolerantních hub. S cílem rozšířit detekované houbové spektrum byla pro izolaci úspěšně použita kombinace standardně užívaných a speciálních metod. Naše výsledky ukázaly, že silně kyselé substráty po celém světě skrývají velmi podobné houbové společenstvo, které je odlišné od společenstev známých z míst s vyšším pH. Základem zdejšího společenstva jsou fylogeneticky nepříbuzní zástupci osidlující jednak výhradně extrémně kyselá místa a jednak méně kyselé převážně extrémní substráty. Tyto výsledky ukazují na kosmopolitní rozšíření acidofilních/tolerantních hub, a zároveň na zásadní roli kompetice při kolonizaci tohoto typu substrátu. Velký podíl výlučně acidofilních zástupců ukazuje na fakt, že extrémně kyselé habitaty představují vhodné prostředí pro evoluci acidofilních hub. Přestože identifikace byla založena na morfologických i molekulárních znacích, řadu taxonů se určit nepodařilo, což naznačuje, že studované společenstvo je druhově bohatší, než se dosud předpokládalo. V rámci dvou taxonomických studií se podařilo identifikovat a zařadit některé dominantní zástupce zdejšího společenstva. Čtyři z nich jsou zde popsáni jako nové rody hub. Výsledky růstových testů ukázaly, že všechny detekované druhy hub jsou vysoce adaptované na extrémně nízké hodnoty pH, nicméně jsou schopny růst i při vyšším pH, což ukazuje na vysokou míru plasticity, která je pro houby typická. Souhrnně lze říci, že předkládaná práce přináší jednak nové informace o houbách osidlujících silně kyselé habitaty a jednak velký počet izolátů hub s výrazným biotechnologickým potenciálem využitelných pro další studie.

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1 INTRODUCTION

1.1 Origin and characteristics of extremely acidic environments

Highly acidic environments (pH < 3) are of both natural and anthropogenic origin. The first ones are typically geothermal and volcanic areas where elemental sulfur is oxidized to sulfuric acid, which, if not neutralized by basic minerals can contribute to the lowering of the pH (Johnson 1998). The second group of habitats is connected to mining activity which is responsible for exposure of sulfide minerals and thus for their oxidation (Baker and Banfield 2003). Consequently in locations where rocks have low buffering capacity highly acidic solutions, acid rock drainage (ARD) or acid mine drainage (AMD) are generated (Baker and Banfield 2003). Moreover sulfide minerals largely encompass metals such as Au, Ag, Cu, Zn, and Pb, thus these solutions are often metal-rich (Baker and Banfield 2003). In all highly acidic habitats microbial activity increases the rate of acidity generation as well as the rate of release of metals and sulfur to the environment (Baker and Banfield 2003).

Hence highly acidic habitats are characterized by high concentrations of hydrogen ions, heavy metals and dissolved sulfates, low concentration of dissolved organic carbon and often extreme temperatures as a consequence of the close connection with volcanic activity and exothermic nature of the reactions (Brock 1978; Johnson 1998; Zak and Wildman 2004). As a result of such harsh conditions these sites represent some of the most extreme habitats for the microbial growth.

1.2 Fungi in acidophilic microbial community

For a long time it has been assumed that these substrates are populated exclusively by prokaryotes. The interest in biodiversity of microorganisms inhabiting highly acidic environments has increased at the end of the past century when the evidence of the eukaryotes including fungi has been suggested (Cooke 1976; Johnson 1998; Gross and Robbins 2000). Recently due to the application of new culture-independent approaches (see Rincon-Florez et al. 2013) high degree of hidden eukaryotic diversity has been detected (Amaral Zettler et al. 2002, 2003). Besides algae and protists, fungi have been found to represent an abundant and important component of the acidophilic communities (López-Archilla and Amills 1999; Robbins et al. 2000; López-Archilla et al. 2001, 2004; Amaral Zettler et al. 2002, 2003; Baker et al. 2004, 2009; Gadanho and Sampaio 2006; Bühring et al. 2012; Zirnstein et al. 2012). Fungal hyphae have been found to be the dominant eukaryotic element of acidophilic

biofilms. They provide the backbone for these structures, anchor them to the sediments and serve as a surface for prokaryotes. In acidophilic food webs, the main role of fungi is decomposition (López-Archilla et al. 2001; Das et al. 2009). Recently, Mosier and coauthors (2013) looked at acidophilic microbial community from the metabolomics point of view and identified key metabolites associated with microorganisms inhabiting acidophilic biofilms. They found that fungi are involved in the metabolism of lipids and taurine, the compound likely providing protection from osmotic stress in the biofilms. Since important fraction of the eukaryotic diversity likely has not yet been documented (Amaral Zettler et al. 2002, 2003; Gadanho and Sampaio 2006) a lot of novel data on the function and structure of acidophilic community might be expected.

1.3 Fungal diversity in highly acidic habitats

Concerning fungal diversity in highly acidic environments limited information is still available. Fungal diversity has been studied marginally as a part of the microbial (López-Archilla and Amills 1999; López-Archilla et al. 2001; Gherman et al. 2007) or the eukaryotic (Amaral Zettler et al. 2002, 2003; Baker et al. 2004, 2009; Gadanho and Sampaio 2006; Bühring et al. 2012; Zirnstein et al. 2012; Amaral-Zettler 2013) diversity and only a few investigations analysed fungal communities in more detail (Cooke 1976; López-Archilla et al. 2004; Vazquéz-Campos et al. 2014) have been performed. Moreover, in most of the studies mentioned above, the accurate identification of the detected taxa is missing which make the comparison of the fungal assemblages difficult. It was shown that fungal communities of highly acidic sites are similarly low-diversified and dominated by a few dematiaceous fungal species (López-Archilla and Amils 1999; López-Archilla et al. 2001, 2004; Baker et al. 2004, 2009; Amaral-Zettler 2013; Vazquéz-Campos et al. 2014). In addition to the dark pigmented fungi, the occurrence of the members of the class Eurotiomycetes (López-Archilla and Amils 1999; López-Archilla et al. 2001, 2004; Baker et al. 2004) and Urediniomycetes (Baker et al. 2009) have been reported. To date, five acidophilic/tolerant fungal species which are not known from less acidic habitats have been identified (Table 1). This fact points to the specificity of the acidophilic fungal community. However further data on fungal diversity from geographically distinct localities might help to elucidate the composition of fungal assemblages inhabiting these sites and moreover the distribution pattern. From this point of view the detailed information is available from hypersaline environments where the detected fungal assemblage is worldwide similar and composed of cosmopolitan halophilic as well as

ubiquitous fungi (Gunde-Cimerman and Zalar 2014). This is in agreement to general hypothesis of cosmopolitan distribution of the majority of fungal taxa (Tedersoo et al. 2014).

Table 1 The list of the fungal species known exclusively from highly acidic habitats. The pH of all substrates from which fungi were isolated was less than 3.

Species	Isolated from	Reference
Acidiella uranophila	highly acidic water from uranium mine (Australia)	Vazquéz-Campos et al. (2014) Kolařík et al. (2015)
Acidomyces acidophilus	acidophilic algae, acid drainage (Germany) soil near sulfur pile (Canada) sulphuric acid (Denmark) volcanic soil (Iceland) acidic industrial water (The Netherlands) AMD water (USA)	Selbmann et al. (2008)
Coniochaeta fodinicola	highly acidic water from uranium mine (Australia) acidic waste water from uranium mine (China)	Vazquéz-Campos et al. (2014)
Hortaea acidophila	lignite (Germany)	Hölker et al. (2004)
Teratosphaeria acidotherma	highly acidic hot springs (Japan)	Yamazaki et al. (2010)
	highly acidic water from uranium mine (Australia)	Vazquéz-Campos et al. (2014)

The discovered diversity of fungi largely depends on the methods used for the study (Bills et al. 2004). It is known that traditional culturing methods and culture-independent molecular ones yield different results with respect to the detected fungal taxa (Rao et al. 2013). The different fungal spectra are also obtained when various isolation and culturing approaches are applied compared with a single method (Bills et al. 2004; Collado et al. 2007). The observed diversity can be also affected by the method of a strain's selection and identification (Bills et al. 2004). The problems with identification can be partially overcome by analysis of large number of isolates, their dereplication using PCR fingerprinting techniques and by sequencing of rRNA regions or other genes (Collado et al. 2007). Usability of these data for identification of a particular strain is limited by the fact that different rRNA regions have been used by each study (Fell et al. 2006; Fierer et al. 2007; Lesaulnier et al. 2008). Therefore a combination of various approaches is recommended to provide a more accurate assessment of the biodiversity of the studied habitat (Kirk et al. 2004; Rincon-Florez et al. 2013).

1.4 Adaptability and mechanisms that allow fungi to thrive in highly acidic habitats

The five fungi known from highly acidic environments were found to be highly adaptable to the extreme conditions where they live, i.e. high acidity, sulfates and various heavy metals like Al, Fe, Cu or U (Starkey and Waksman 1943; Gould et al. 1974; Gimmler et al. 2001; Hölker et al. 2004; Yamazaki et al. 2010; Vazquéz-Campos et al. 2014). Except *Acidiella uranophila*, the following four species were able to grow at pH 1 (*Coniochaeta fodinicola*, *Hortaea acidophila* and *Teratosphaeria acidotherma*) or even lower (*Acidomyces acidophilus*) which is a rare phenomenon in fungi (Magan 2007). Their growth optima (ranging from 3 to 5) also point to their moderately acidophilic behaviour (Johnson 2012). Nevertheless they do not require high acidity for viability, since they maintain the ability to grow at less acidic pH values (Starkey and Waksman 1943; Gimmler et al. 2001; Hölker et al. 2004; Yamazaki et al. 2010; Vazquéz-Campos et al. 2014). A similar pattern of adaptation to a broad amplitude of an ecological factor is reported for most of the fungal species inhabiting hypersaline habitats which can grow under the whole salinity range (Gunde-Cimerman and Zalar 2014) as well as for rock-inhabiting meristematic black fungi from the Antarctic showing optimal growth at temperatures higher than those to which they are exposed in nature (Selbmann et al. 2005). Gostinčar et al. (2010) defined these species as adaptive specialists in contrast to obligate ones which have narrow ecological amplitude. The second profile is typical for prokaryotes however from hypersaline substrates a halophilic fungus *Wallemia ichtyophaga* with this rare behaviour is known (Gostinčar et al. 2010) and genome sequencing data show that this fungus differs from others in mechanisms which allow it to thrive in hypersaline environment (Gostinčar et al. 2014). Further data dealing with fungi in highly acidic environments might help to reveal possible representatives with such specific ecology.

Which mechanisms are responsible in fungi for the adaptability to extremely low pH values is unclear. It is known that high concentration of hydrogen ions affect ionic state and thus metabolic activity of the cells (Magan 2007). Fungi inhabiting such harsh environments possess intracellular pH controlling system which allows them to maintain their cytoplasm at neutral pH or close to it (Hesse et al. 2002; Bignell 2012). This state is achieved by efficient control of proton movement into and out of the cells mediated by ATPase (Pam1) actively pumping protons out of the cell and vacuolar V-ATPase mediated influx of protons into vacuole (Bignell 2012). Recently, the involvement of secondary transporter (PfMFS) in acid resistance and intracellular pH homeostasis has been revealed (Xu et al. 2014). In addition,

Gimmler et al. (2001) detected the close connection between acid and heavy metal resistance and the main role of membrane and zeta potentials in control of the uptake of heavy metals and thus in the resistance to these elements. Moreover, the importance of vacuole in tolerance to high concentrations of arsenic and other metals has been confirmed (Cánovas et al. 2003). In addition, melanins should play an important role due to their ability to bind various metals as is discussed by Vazquéz-Campos et al. (2014) and López-Archilla et al. (2004).

1.5 Evolution trends in extreme environments

Based on the data yielded from scarce studies mentioned above, the acidophilic/tolerant mycobiota seems to be polyphyletic (Amaral-Zettler et al. 2002; Baker et al. 2004, 2009). This corresponds with situation in hypersaline and alkaline habitats, where mycobiotas composed of phylogenetically unrelated fungi have been found (Gunde-Cimerman and Zalar 2014; Grum-Grzhimaylo et al. 2015). Grum-Grzhimaylo et al. (2015) explain this fact by independent evolution and several origins of the alkaliphilic trait in filamentous fungi. The hypothesis of the evolution of specialist species in extreme environments is described in Gostinčar et al. (2010) who mentioned that specialists developed from generalist species which have the ability to persist across various environments due to their robust genotypes. These fungi are more successful in stressful environments contrary to moderate ones due to the limited competition. The authors suggest that these species serve as a genetic reservoir of potential candidates for the evolution of specialists in extremes. To confirm this hypothesis in context to highly acidic environments more data on diversity, taxonomy and ecology are needed.

1.6 Biotechnological potential of acidophilic/tolerant fungi

Recently, an enormous biotechnological potential of fungi inhabiting extremely acidic habitats has been revealed. Several isolates of the four known fungi (*Acidomyces acidophilus*, *Coniochaeta fodinicola*, *Hortaea acidophila*, *Teratosphaeria acidotherma*) were found to yield a great deal of novel enzymes which are potentially exploitable in various industrial applications (e. g. Tetsch et al. 2005; Wang et al. 2009; Luo et al. 2010; Zhao et al. 2010; Yang et al. 2011; Boonen et al. 2014). In addition, due to their abilities, these fungi might serve as good candidates for the development of bioremediation programs (Selbmann et al. 2013). A great deal of novel metabolites with anticancer activity has been described from several isolates obtained from highly acidic metal-rich mine waste in Berkeley Pit lake system (North America) (Stierle et al. 2006, 2007, 2012a,b). Therefore further investigations of these

underexplored sites might provide isolates with unique characteristics which might be potentially exploitable.

1.7 Fungal nomenclature news: one fungus = one name

According to the International Code of Nomenclature for algae, fungi and plants (McNeil et al. 2012) fungi may no longer have more than one scientific name. In connection with this change the classical terms anamorph and teleomorph have been replaced by asexual state/morph and sexual state/morph (Rossman 2014). In the present work this novel concept has been applied.

1.8 Research objectives of the thesis

In the present thesis, we focused on the cultivable mycobiota inhabiting highly acidic (pH < 3) and saline (sulfate-rich) soils at geographically isolated localities in the Czech Republic which should provide a deep insight into the diversity and distribution pattern of fungal assemblages populating this hostile habitat.

The main objectives of the research were:

- to investigate the diversity of cultivable filamentous fungi inhabiting saline (sulfate-rich) soils with various pH values (paper I) and exclusively highly acidic ones (paper IV) using classical and specialized cultivation techniques and the combination of morphological and molecular markers for identification
- to resolve taxonomy and phylogenetic relationships of fungal species forming the core of the acidophilic fungal community (paper II, III)
- to reveal the distribution pattern of fungi predominantly inhabiting highly acidic habitats (paper II – IV)
- to determine the extent of adaptations of dominant species by testing their growth response to different salinities (paper I, II) and pH values in laboratory conditions (paper I - III)
- to evaluate the usefulness of the application of various cultivation approaches on the detected fungal spectrum (paper I, IV).

1.9 Thesis outline

Diversity and distribution of acidophilic/tolerant fungi

The diversity of cultivable filamentous fungi inhabiting saline (sulfate-rich) soils with various pH values (paper I) and exclusively highly acidic ones (paper IV) has been studied. For paper I most of the data were obtained during diploma thesis research (Hujslová 2006). To cover the broadest range of species and to detect acidophilic ones a combination of classical and specialized techniques for cultivation were successfully applied (discussed bellow). Our results showed that fungal assemblage detected in sulfate-rich soils is considerably different from those typical for saline habitats (paper I) and the comparison of fungal communities detected from sites with neutral or slightly acidic pH and highly acidic ones yielded considerable differences showing that highly acidic sites harbour low diversified fungal community (paper I). The detailed investigation of fungi in soils with highly acidic pH revealed an apparent similarity of fungal assemblages from these sites (Czech Republic) and the ones reported from highly acidic environments elsewhere (paper IV). The core of the fungal assemblage under study consisted of phylogenetically unrelated and often globally distributed fungi exclusively inhabiting highly acidic habitats as well as taxa known from less acidic and often extreme environments (paper IV). The dominant representatives of the community include different dematiaceous species of Dothideomycetes (paper II), several penicillia (paper IV) as well as different hyaline species belonging to Leotiomycetes and Sordariomycetes (paper III, IV). The large number of identified specialized species indicates that highly acidic environments provide suitable conditions for the evolution of specialist species (paper IV). The occurrence of ubiquitous fungi in highly acidic substrates points to the principal role of competition in colonization of highly acidic environments (paper IV). In spite of both morphological and molecular markers used for identification we have detected numerous unknown taxa which show that fungal community of highly acidic habitats is more diversified than it has been supposed so far (paper IV). During two taxonomical studies several dominant members of the fungal community were identified and their phylogenetic relationships have been resolved (paper II, III).

Taxonomy and phylogeny of acidophilic/tolerant fungi

Two groups of dominant and mostly sterile isolates were studied using phenotype, RAPD fingerprinting and nuclear ribosomal DNA sequences (ITS region, SSU, LSU) (paper

II, III). In paper II, 82 dark pigmented isolates taken from 20 samples from Czech Republic and Iceland have been investigated. Based on phylogenetic analyses, they were accommodated by three lineages within the family Teratosphaeriaceae (Dothideomycetes, Ascomycota). Phylogenetic position and ecology of the first lineage lead to the description of a new genus and species *Acidiella bohemica*. The two remaining lineages were placed in sister clades of two acidophilic fungi *Acidomyces acidophilus* and *Teratosphaeria acidotherma*. The detailed phylogenetic study showed that these two sister species form well-defined cluster and the second species was located at a large distance from other *Teratosphaeria* species. According to the natural generic concept, *Teratosphaeria acidotherma* has been ascribed to the genus *Acidomyces* (paper II).

In paper III, 89 hyaline isolates obtained from 16 samples of highly acidic soils in the Czech Republic and an isolate from Antarctic alkaline soil were resolved. Based on phylogeny they belonged to three lineages within two different classes Sordariomycetes and Leotiomycetes (Ascomycota). Their phylogenetic position as well as ecology and physiology lead to the description of three new genera and species *Acidothrix acidophila*, *Acidea extrema* and *Soosiella minima* (paper III). On the whole, our results indicate the placement of acidophilic/tolerant fungi in extremotolerant (paper II, III) as well as mesophilic (paper III) clades and they showed that fungi forming the core of acidophilic community are phylogenetically diverse (paper I - IV).

Adaptability of acidophilic/tolerant fungi

The extent of the adaptations of dominant and tentatively new species (paper I - III) was determined by testing their growth response to different pH values (1 – 8) and salinities (0 – 0.5M Na_2SO_4 or 0 – 5M NaCl). In paper I, all tested species were able to tolerate high concentration of sulfate. Majority of fungi obtained from less acidic sites grew at pH range of 4 to 8 contrary to the species isolated from acidic sites which were able to grow at pH 3 and lower (paper I - III). These results correspond with the pH values of the soil from which they were isolated except for the *Soosiella* isolate obtained from pH 2 soil sample and *Acidea* isolate SH26-1 originating from alkaline soil with pH 8 both of which were not able to grow at these pH values in laboratory conditions (paper III). This phenomenon pointing to the behaviour typical for generalist species is discussed in paper IV. Moreover eight species were able to grow at pH 2 and five of them could even grow at pH 1, which is a rare phenomenon in fungi (paper I, III). Majority of tested species exhibited unimodal growth pattern with optimal growth in the pH range from 3 to 5 which indicate their moderately acidophilic

behaviour (paper I – III). Two species, *Acidea extrema* and *Acidothrix acidophila* showed besides unimodal also bimodal growth response with growth optima at pH 2 or 3 and at pH 6 which indicates their acidotolerant characteristic (paper III). This phenomenon has been already detected in fungi (discussed in paper III). Three species showed slightly halophilic behaviour (paper III). On the whole, our results showed high adaptability of all tested species to the extreme conditions of the substrate where they live. However, it is apparent that the tested taxa did not require low pH to survive, because they can grow in a broad range of pH (paper I – III) and that none of the detected taxa had obligately acidophilic characteristic (paper IV).

Usefulness of methodological approaches applied

To cover the broadest range of species and to detect acidophilic ones a combination of classical and specialized cultivation methods were applied. In addition to isolation procedures favouring highly sporulating fungi (paper I), the soil washing technique which allows isolation of actively growing members (paper IV) was applied and the obtained results indicated that the dominant taxa are present in studied soils in both stages thus they can be considered indigenous inhabitants of the studies soils (paper IV). Besides, 2% malt agar (MA2), a medium with high content of sugar and incubation at 24°C commonly used for isolation of soil fungi (paper I, IV), media simulating oligothrophic and acidophilic conditions (paper I, IV) as well as incubation temperatures 5 and 37°C were applied and permited isolation of several slow growing fungi due to the absence or limited growth of fast growing species (paper I, IV). The same effect was detected in case of using dilution series in soil suspension plating method (paper I). Moreover, the application of specialized media was successful aiming at the isolation of fungi with acidophilic behaviour (paper I – IV). Our results showed that the application of both classical and specialized cultivation methods was successfully applied since it significantly contributed to the broadening of the detected fungal spectrum (paper I, IV)

2 ORIGINAL PAPERS

I. Hujslová M, Kubátová A, Chudíčková M, Kolařík M (2010) Diversity of fungal communities in saline and acidic soils in the Soos National Natural Reserve, Czech Republic. Mycol Prog 9:1-15

II. Hujslová M, Kubátová A, Kostovčík M, Kolařík M (2013) *Acidiella bohemica* gen. et sp. nov. and *Acidomyces* spp. (Teratosphaeriaceae), the indigenous inhabitants of extremely acidic soils in Europe. Fungal Divers 58:33-45

III. Hujslová M, Kubátová A, Kostovčík M, Blanchette RA, de Beer ZW, Chudíčková M, Kolařík M (2014) Three new genera of fungi from extremely acidic soils. Mycol Prog 13:819-831

IV. Hujslová M, Bukovská P, Kubátová A, Chudíčková M, Kolařík M. Extremely acidic soils are dominated by species-poor and highly specific fungal communities. Manuscript

Author's contributions

Paper I. Alena Kubátová and I jointly planned the study. I had done the sampling, processing the samples, isolation of fungal strains. A. Kubátová helped me with morphological identification and Miroslav Kolařík made molecular identification of the isolates. I and M. Kolařík made statistical analyses and figures. Milada Chudíčková helped me with growth tests. We jointly wrote the paper.

Paper II. Miroslav Kolařík and I jointly planned the study. I had done the isolation and morphological identification of fungal strains. M. Kolařík helped me analyze molecular data. Martin Kostovčík submitted sequences to GenBank database and together with M. Kolařík made phylogenetic analyses. M. Kolařík and I made the figures and tables. I wrote the paper and A. Kubátová and M. Kolařík helped me with the final improvements of the manuscript.

Paper III. Miroslav Kolařík and I jointly planned the study. I had done the isolation and morphological identification of fungal strains. M. Kolařík helped me analyze molecular data. Robert Blanchette provided the Antarctic strain to the study and finally improved the text of the manuscript. Martin Kostovčík submitted sequences to GenBank database and together with M. Kolařík made phylogenetic analyses. I wrote the paper and A. Kubátová, M. Kolařík and Wilhelm de Beer helped me with the final improvements of the manuscript.

Paper IV. I had planned the study, done the sampling, processing the samples and isolated fungal strains. Alena Kubátová helped me with morphological identification and Miroslav Kolařík helped me with molecular identification of the isolates. Milada Chudíčková made DNA isolation. Petra Bukovská helped me with statistical analyses and made figures. I wrote the paper and A. Kubátová and M. Kolařík helped me with the final improvements of the manuscript.

On behalf of all of the co-authors, we declare the keynote participation of Martina Hujslová in the research of fungi inhabiting extremely acidic environments and the writing the papers, as described above.

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Alena Kubátová Miroslav Kolařík

2.1 Paper I

Hujslová M, Kubátová A, Chudíčková M, Kolařík M (2010) Diversity of fungal communities in saline and acidic soils in the Soos National Natural Reserve, Czech Republic. Mycol Prog 9:1-15

Diversity of fungal communities in saline and acidic soils in the Soos National Natural Reserve, Czech Republic

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Abstract

During 2003-2005, the diversity of culturable filamentous soil microfungi in saline and acidic soils of the Soos National Natural Reserve (Czech Republic) was studied. Altogether, 28 soil samples were collected from four sampling sites and were processed by various approaches. In total, 92 fungal taxa were identified using classical and molecular markers. Several detected species were known from similar substrata worldwide; however, the overall fungal spectrum was distinct, as shown by comparison to similar studies. All methodological approaches increased the observed fungal diversity. The different fungal communities observed on the four sampling sites were influenced by the complex effects of environmental factors. The growth response of selected strains to different salinities and pH values was determined. The results of the growth tests showed high adaptability of all tested species to the extreme conditions of the studied substrate. Two acidophilic species (*Acidomyces acidophilus*, *Sporothrix* sp.) were isolated.

Keywords

microscopic fungi, *Penicillium*, *Acidomyces*, molecular identification, acidotolerance, halotolerance

Introduction

Estimates of the global fungal diversity vary from 0.7 to 9.9 million species (Hawksworth 1991, 2004; Schmit and Mueller 2007), but only 80 000 species have been described to date (Schmit and Mueller 2007). The number of unexplored habitats, which have

proven to be rich in specialised and unique fungi, is still enormous (Suryanarayanan and Hawksworth 2005). In addition to tropical forests, extreme habitats such as extremely acidic and saline soils and waters also fall into this category.

Recently, the interest in studying extreme environments has increased. These habitats may accommodate new species, which may be used as unique sources of enzymes or secondary metabolites of biotechnological or pharmaceutical potential. The species found in extreme environments may also produce novel chemical compounds (Stierle et al. 2006). Moreover, some of these undiscovered species could have the potential to remediate contaminated sites (Gadd 2001). In addition, organisms adapted to extreme environments have received more attention because of the specific mechanisms of adaptation that allow them to thrive under exceptional conditions (Magan 1997; Gunde-Cimerman et al. 2005).

Although extremely acidic (pH < 3) and saline soils and waters represent some of the most extreme environments, they appear to be suitable biotopes for several groups of microorganisms, including fungi (Baker and Banfield 2003; Gunde-Cimerman et al. 2005). Investigations of the fungi in saline biotopes have been done in many places all over the world. In addition to oceanic habitats, numerous continental saline sites such as salt lakes (Anastasiou 1963; Buchalo et al. 1998; Buchalo et al. 2000; Steiman et al. 2004), salterns (Gunde-Cimerman et al. 2000; Zalar et al. 1999; Cantrell et al. 2006), salt marshes (Pugh 1962; Hendrarto and Dickinson 1984) and saline soils (Moubasher et al. 1990; Guiraud et al. 1995; Steiman et al. 1995; Kubátová et al. 2002; Grishkan et al. 2003) have been investigated. The pH values of these studied substrates ranged from slightly acidic to slightly alkaline. Conversely, extremely acidic saline habitats (pH ≤ 3) are quite rare (Zak and Wildman 2004), and fungi have been studied there mostly marginally (Joseph 1953; Satake and Saijo 1974; Cooke 1976; López-Archilla and Amils 1999; Redman et al. 1999; Amaral Zettler et al. 2002; Baker et al. 2004; López-Archilla et al. 2004).

The discovered diversity of fungi largely depends on the method of isolation and culturing used (Bills et al. 2004). Various isolation and culturing approaches, including use of different isolation methods, different culture media and different incubation temperatures, have demonstrated that inventories using a single method detect only a portion of the organisms present (Bills et al. 2004; Collado et al. 2007). The observed diversity can be also affected by the method of a strain's selection and identification (Bills et al. 2004). The problems with identification can be partially overcome by comparison of strains by PCR fingerprinting techniques and by sequencing of rRNA regions (Collado et al. 2007). Also, the sequences generated during studies of uncultured fungal diversity have provided data for

database comparison. Usability of these data for identification of a particular strain is limited by the fact that different rRNA regions have been used by each study (Fell et al. 2006; Fierer et al. 2007; Lesaulnier et al. 2008).

The aims of the present study were to investigate the diversity of culturable filamentous fungi inhabiting saline and acidic soils of the Soos National Natural Reserve and to determine the extent of their adaptation to the extreme environment by testing their growth response to different salinities and pH values. The Soos National Natural Reserve is located in the Czech Republic and is unique because of its diverse soil types with high salinity and extremely low pH values (1-2). Two different cultivation methods, two types of isolation media and three incubation temperatures were used to cover the broadest range of species. To maximise the possibility of indentifying fungi using published sequences, three rDNA regions and the β-tubulin gene were used to characterise the selected morphologically unidentifiable strains.

Materials and methods

Description of the location and the sampling sites

The Soos National Natural Reserve is situated near Františkovy Lázně in Western Bohemia, Czech Republic (Fig. 1). It is located at 50°09‘ N, 12°24‘ E, at an altitude that ranges from 430 to 441 m. Soos belongs to a mild humid climate region with mild winters. The average annual temperature and precipitation are 7 °C and 589 mm, respectively.

In a relatively small area (211 ha), a rare combination of different vegetation types like mineral fens and peat bogs can be found in close contact with salt marshes (Chocholoušková and Vaněčková 1998). The existence of the rare geological phenomenon called moffettes, caused by carbon dioxide as well as the occurrence of numerous mineral springs with high concentrations of salts, contributes to the uniqueness of this locality (Hájek and Vízdal 1998). The mineral salts accumulated in the soil have caused the substrate conditions to become extreme. However, many former human activities, such as draining, intensive pasture, or clay, peat and fen extraction have influenced the natural development of this area (Brož 1998). Sulphuric acid, occurring as a pyrite oxidation product, as well as extremely acidic “vitriolic waters” (pH 1-2) have originated at several places as a consequence of mining activities. These places have become extremely acidic and lack vegetation cover.

Four permanent sampling sites that had different types of vegetation cover, conductivity, pH values and moisture content were established in the southwestern part of Soos (Fig. 1, Table 1).

Sampling

Altogether, 28 soil samples were collected from four sampling sites in Soos during 2003-2005. In November 2003, a pilot study on four samples was done to choose suitable treatments. During 2004-2005, the main investigation was completed with collections 3 times per year (May, August, November), and altogether, 24 soil samples were obtained. The soil samples were collected at 5-10 cm depth and placed into sterile plastic bags.

Isolation and identification

Fungal strains were isolated using two methods: method 1 (M1) – direct inoculation of soil (five ca. 1 mm^3 soil particles per plate) and method 2 – soil suspension plating in two different soil dilutions (M2 - a soil/water ratio $1/10^2$, M3 – a soil/water ratio $1/10^4$). Two different isolation media were used: 2 % malt agar (MA2) and soil agar with rose Bengal and glucose (SEA) (Fassatiová 1986). To simulate the specific conditions of the studied substrates, SEA was prepared using the substrates of the respective sampling sites. Streptomycin was added to all media (0.1 g/l) to suppress bacterial growth. The plates were incubated at 5°C (in the refrigerator), 25°C and 37°C. After several days, the emerging colonies were transferred to identification media.

Taxonomic identification of fungal isolates was based on morphological characteristics using the current taxonomic publications. The selected sterile mycelia or strains that could not be identified according to their phenotypes were subjected to molecular identification. These strains were typed to groups using PCR-fingerprinting using RAPD and characterised by their ribosomal DNA (ITS region, SSU and LSU-rDNA) and β-tubulin sequences. Representative fungal strains have been deposited in the Culture Collection of Fungi (CCF), Department of Botany, Faculty of Science, Charles University in Prague, Czech Republic or the personal collection of the senior author (Table 2; electronic supplementary material, ESM, Table 5).

Determination of environmental parameters

In all soil samples, the following parameters were determined: pH (pH meter - pH315i), conductivity (WTW Multilab 540 microprocessor conductometer), concentration of SO_4^{2-} anions (isotachophoretic capillary electrophoresis on the analyser ZKI 01 Villa-Labeco, Slovakia) and moisture content (weight difference between native samples and samples desiccated at 20°C).

DNA analyses

Genomic DNA was isolated from 3-7 day old cultures using a Microbial DNA isolation kit (MoBio Laboratories Inc.). RAPD was performed with primers 8F (5´-GCTCTGAGATTGTTCCGGCT-3´) and 10R (5´-GGCCAGTGTGAATATGC-3´) according to the procedure described by (Kolařík et al. 2004). Strains with identical RAPD patterns were considered to belong to identical genetic lineages, and representative strains were characterised by their DNA sequences. Nuclear rDNA from the 5.8S region and the D1-D2 domains of the 28S region containing internal transcribed spacers (ITS1 and ITS2) was amplified with primers ITS1 and ITS5 or ITS1F and NL4 (Gardes and Bruns 1993; O'Donnell 1993). The SSU rDNA gene was amplified using primers NS1 and NS24. The primers ITS1F, ITS4, NL1, NL4, NS4, NS5 and NS24 (White et al. 1990) were used for sequencing. The reaction mixtures, amplification protocols and DNA visualisation were done according to (Hulcr et al. 2007). Partial ß-tubulin gene sequences were amplified and sequenced using primers Bt2a and Bt2b according to the protocol of (Glass and Donaldson 1995). Custom purification of the PCR products and sequencing of the DNA was done at Macrogen Inc. (Seoul, Korea). GenBank accession numbers are given in Table 3. Sequences were compared with data from GenBank using a Blast similarity search and by phylogenetic analysis with neighbouring taxa using MEGA 4.1 software (Tamura et al. 2007).

Physiology

The effects of pH and salinity on the growth of 22 selected dominant or tentatively new species were determined by measuring colony diameters on MEA agar (malt extract 20 g, glucose 20 g, peptone 1 g, agar 20 g, H_2O 1 L). Eight different pH values ranging from 1 to 8 and three salt concentrations (0 M, 0.25 M and 0.5 M) were used. The pH and salinity of the medium were adjusted with concentrated H_2SO_4 and NaOH and with Na_2SO_4, respectively. We used Na_2SO_4 instead of NaCl, which is commonly used, to remain consistent with the character of the mineral salts in the studied area. To gel the agar at extremely acidic pH values (1, 2), double quantities of agar were used and acidification of the medium was done after sterilisation. The plates were inoculated with spore suspensions or mycelial segments. Each variant was done in three replicates. The plates were incubated at 24°C for two weeks. The colony size was measured every other day or every day in case of fast growing strains.

Data analyses

For the species isolated during the main investigation, the total frequency of occurrence was calculated as the percent of all samples (n=24) in which a particular species was identified. Moreover, the frequency of occurrence at an individual site and in a single sample was calculated as the percent of all plates for an individual site (n=180) and all plates for a single sample (n=30) in which a particular species was identified. The frequency of occurrence was not counted for the species isolated during the pilot study. The species from the pilot study were also removed from the datasets prior to all analyses. The analysis of diversity was based on the Shannon-Weaver index (H). To compare fungal assemblages from the four sampling sites, the Bray and Curtis similarity index (C) was used (Bray and Curtis 1957). The C index ranges from 0 (no overlap between assemblages) to 1 (total overlap). Both indices were calculated using PAST (Hammer et al. 2001). Species accumulation curves were generated for fungal assemblages from each sampling site using EstimateS (Colwell 2006). Multivariate statistical analysis was conducted using CANOCO 4.5 for Windows (Ter Braak and Šmilauer 1998). Two modified datasets, which were restricted to consider only those species that were recovered more than ones, were used. The first dataset was used to estimate the influence of the methodological approaches on the species composition using redundancy analysis (RDA). The second one was used to determine the influence of the environmental variables on the species composition using canonical correspondence analysis (CCA). Detrended correspondence analysis (DCA) was performed to obtain information on the inner structure of the data. Significance levels of the indicator values were calculated by applying a Monte-Carlo permutation procedure (1000 permutations).

Results

Species composition and identification

Altogether, 92 taxa, representing 39 genera, were identified from 799 isolates obtained from 28 soil samples. Of these species, 11 were recorded exclusively during the pilot study (4 samples). During the main investigation (24 samples), 81 taxa representing 32 genera were obtained. The prevailing genus was *Penicillium* (24 species). Only 1 species, *Penicillium* sp. 1 (CCF 3782), was found at a frequency exceeding 50 % of all samples. The majority of other species were recorded at frequencies less than 12.5 % of all samples. In total, 261 plates of the 720 plates remained sterile (Table 2).

Taxonomic identification of all 92 taxa was based on morphological characteristics and 41 of them was characterised also by molecular genetics methods (Table 3). Altogether, 150

isolates were typed to groups using RAPD analysis. In total, 67 strains were characterised by ribosomal DNA (ITS region, SSU and LSU-rDNA) and/or β-tubulin sequences. On the whole, 55 taxa were linked with known species and 37 remained undetermined (Table 2). Twenty two of the taxa studied also by DNA sequences were not determined to the species (Table 3) and four of them belong to the principal taxa with overall frequency of 20.8% and more (Table 2).

Methodological approaches

The observed fungal diversity was affected by the incubation temperature, the isolation procedure and the isolation medium (Table 2, Fig. 2 – 4). In addition, the influence of these variables on the species composition was statistically significant (temperature: $p = 0.001$; isolation procedure: $p = 0.003$; medium: $p = 0.01$). The incubation temperature explained the highest percent of the variance (44.8 %). The isolation procedure and the medium explained 36.6 % and 12.9 %, respectively. All of the most abundant species were obtained on both isolation media and were found at least by two isolation procedures (M1, M2, M3). The majority of the frequently isolated species grew successfully at a minimum of two of the incubation temperatures. Seven dominant species were isolated at all temperatures. Species isolated exclusively from a single incubation temperature, isolation procedure or medium belonged to the rarely recorded species (with a maximum frequency of 16.7 % of all samples) (Table 2).

Fungal communities at the four sampling sites

The fungal communities of the four sampling sites differed considerably (Table 2 and 4, Fig. 5). The sampling site had a strong and statistically significant influence ($p = 0.002$) on the species composition (Fig. 5). The highest species richness was recorded at site 1 and the lowest at site 2 (Table 2). The similarity of the fungal communities among the different sites ranged from 0, (site 2 vs. site 1 and site 4) to 0.23 (site 1 vs. site 3) (Table 4). None of the species was obtained from all sampling sites. A total of 28 taxa were isolated from at least two sites: 9 species were isolated from 3 sites and 19 taxa were obtained from 2 sites. More than half of all species (50 species) were isolated only from 1 sampling site. At sites 2, 3, and 4, our sampling of the fungal community was statistically complete: the estimated richness based on bootstrap analyses fell within the 95 % confidence interval for the observed species richness (Fig. 6). At site 1, the estimated richness significantly exceeded the richness captured

by our sampling (Fig. 6). The diversity of the fungal species recovered from the sampling sites increased from site 2 (H = 0.3) to site 3 (H = 2.4), site 4 (H = 2.6) and site 1 (H = 2.9).

In addition to low spatial distribution, most of the species also had a low frequency of temporal occurrence (ESM, Table 5). Only four species, namely *Acidomyces acidophilus*, *Fusarium sporotrichioides*, *Penicillium* sp. 1 and *Talaromyces helicus* were recovered from all six samples obtained from a single site over the course of the investigation.

Physiology

All tested isolates were capable of growing on media with and without salt (0 – 0.5 M MEA) except for *Sporothrix* sp., which grew at 0.5 M MEA only after a long incubation period (9 days) (Table 6). Most of the tested species showed optimal growth on medium without salt. *Fusarium culmorum*, *Penicillium chrysogenum* and *Penicillium* sp. 6 "complex" had optimal growth on 0.25 M MEA, and *Penicillium coprobium* had optimal growth on 0.5 M MEA (Table 6).

The majority of the tested species grew over a pH range from 3 to 8. Only six species (*Acidomyces acidophilus*, *Paecilomyces* sp., *Penicillium* sp. 3, *Penicillium* sp. 4, *Sporothrix* sp. and *Talaromyces helicus* var. *helicus*) were able to grow at extremely low pH values (1, 2) (Table 6). Most of the strains showed their optimal growth in range of pH 4 - 8. Two species showed a growth optimum at pH 3 (Table 6).

Discussion

Species composition and identification

The fungal communities that we sampled consisted of several dominant taxa accompanied by species that had low frequencies of spatial and temporal occurrence. In total, 92 taxa were found. The dominance of several species suggests that these species are highly adaptable to the extreme conditions typical of the studied substrate (Table 6).

Several species that create the Soos fungal community are found in similar substrata worldwide. *Penicillium* sp. 1, *Talaromyces helicus* var. *helicus* and *T. helicus* var. *major* belong to the most abundant taxa isolated from Soos. *Penicillium* sp. 1 is identical to *Penicillium* cf. *varians* isolated from saline soil (Kubátová et al. 2002), and *T. helicus* was also found in this habitat (Stolk and Samson 1972). According to the phylogenetic analyses, *Penicillium* sp. 1 was the most closely related to *T. helicus* and to an ex-type strain of *P. varians* sequenced during this study (data not shown). These three sister taxa form a well defined group sharing ecological preferences for saline soils.

We also isolated other fungi in high frequencies, such as *Penicillium simplicissimum*, *Trichoderma harzianum* and *Fusarium sporotrichioides*, that have been found previously in saline soils (Steiman et al. 1995; Kubátová et al. 2002).

The dark sterile fungus prevailing at the extremely acidic site 2 had identical rDNA sequences to strain CBS 335.97, which belongs to the recently described taxon *Acidomyces acidophilus* (Selbmann et al. 2008).

Three taxa isolated here were at least distantly related to other fungi known from similar habitats. The morphologically unique *Paecilomyces* sp. was related to the strain *Paecilomyces* sp. AK18/95 (= CCF 3680) [FJ430757] that was obtained from saline soil by (Kubátová et al. 2002) according to the morphology and their DNA sequences. The white ascomycete CCF 3830 was closely related to an uncultured fungus from a highly acid river in Spain (Amaral Zettler et al. 2002), as shown by comparison of the phylogenetic position of the most related SSU sequences. Finally, a dematiaceous chlamydosporic fungus *Lulwoana* sp. lacking the *Zalerion* anamorph typical of other related species showed 98% similarity to SSU RNA sequences of *L. uniseptata*. According to the phylogenetic analyses, our fungus belonged to the *L. uniseptata* clade (Campbell et al. 2005), a group that contains marine fungi.

In general, we found taxa like *Acidomyces acidophilus*, *Talaromyces helicus* and some penicillia, which are often found in similar extreme habitats worldwide. The other listed taxa show that the recorded fungal spectrum is distinct at least judging by other published fungal spectra. The uniqueness of the fungal spectrum could be explained by the scarcity of studies on fungi from other extremely acidic sites and by the fact that majority of the data from the saline biotopes, like those described by (Moubasher et al. 1990; Guiraud et al. 1995; Steiman et al. 1995; Buchalo et al. 1998; Buchalo et al. 2000; Gunde-Cimerman et al. 2000; Grishkan et al. 2003), were obtained in hot, arid areas, a climate that affected the composition of the fungal communities.

Great care was taken to accurately identify the isolated fungi, especially in case of the 41 taxa that were determined using both morphology and DNA sequences. The 22 species from this set differed from the published species and probably represent novel taxa. Another 15 species studied only by morphology failed to be linked to published ones (Table 2). This suggests that a large number of the species that we isolated in the studied habitat are little known or undescribed species.

Fungal communities at the four sampling sites

Although the sampling sites were separated only by a few meters or less (Fig. 1), their fungal communities differed considerably (Table 2 and 4, Fig 5). This fact was also supported by the results of the statistical analysis, which showed that the sampling site had the largest influence (47.6%) on the species composition of the variables we analysed. Because they differed in both abiotic and biotic characteristics such as soil pH, moisture, conductivity and abundance of vegetation cover (Table 1), we suggest that the species composition of the fungal community is shaped by the complex effects of these factors. Concerning the sampling effort, at sites 2 and 4 our sampling was statistically complete. However, in the case of the remaining two sites, the estimated richness significantly exceeded the richness captured by our sampling (site 1), and the species accumulation curve remains non-asymptotic (site 3); therefore, more samples should be collected.

Methodological approaches

We used two isolation techniques in the present study: direct inoculation (M1) and soil suspension plating (M2, M3). The second method is the most commonly used method in the study of soil fungi (Gams 1992), and with various modifications, this method is widely used in studies of saline substrates (Guiraud et al. 1995; Steiman et al. 1995; Grishkan et al. 2003; Steiman et al. 2004). Both direct inoculation and soil suspension plating were used by Kubátová (2002). As expected, almost the same number of species was obtained using M1 and M2, while fewer taxa were recovered using M3 (Table 2). However, as Fig. 2 shows, all isolation procedures contributed to an increase in the fungal spectrum.

Regarding the isolation media used, 2 % malt agar is widely used in studies of soil fungi (Gams 1992; Bills et al. 2004). A similar medium (MEA) with various modifications has been used in the majority of studies of extreme habitats (Guiraud et al. 1995; Steiman et al. 1995; Buchalo et al. 2000; Grishkan et al. 2003; Steiman et al. 2004). Concerning more specialised media, Zalar (1999) and Gunde-Cimerman (2000) prepared MEA as a mixture of distilled water and the water from the Dead Sea. In our case SEA was prepared from the substrate of the sampling sites. Almost the same number of species was obtained on both media used in the present study (Table 2), but they both contributed to the broadening of the observed species composition of the community (Fig. 3).

The plates were incubated at three cultivation temperatures. Predictably, almost the same number of species was isolated at 5°C and 24°C, and lower species richness was recorded at 37°C (Table 2). The first two temperatures were used by Kubátová (2002). The

third temperature (37°C) was used in studies dealing with fungi from similar substrates from subtropical and tropical regions (Guiraud et al. 1995; Steiman et al. 1995; Grishkan et al. 2003). In our case, this incubation temperature was used to obtain the broadest spectrum of soil micromycetes. This approach was successful since several species, such as *Chromocleista* sp. and *Chrysosporium* sp., were isolated exclusively at 37°C (Fig. 4). Moreover, the absence of fast-growing species at this temperature allowed for easier isolation of other species; for example, *Lulwoana* sp. and *Paecilomyces* sp. were isolated at both 24 and 37°C (Fig. 4).

Effect of salinity and pH

The results of the growth tests confirmed high adaptability of the tested species to extreme salinity and pH. All tested species grew on all salinities (0 – 0.5 M MEA) (Table 6), and, therefore, they should be classified as halotolerant. These results coincide with higher values of conductivity in the substrate of the studied sites (Table 1). More than half of the tested species were capable of growth at a pH of 3 or lower (Table 6); therefore, they may be classified as acidotolerant (Zak and Wildman 2004). Six species even could grow at an extremely low pH (1 - 2) (Table 6), which coincides with the pH values of the soil at the sampling sites from which they were isolated. Two species (*Acidomyces acidophilus*, *Sporothrix* sp.) shifted their growth optimum at pH 3; hence, they may be considered acidophilic (Cavicchioli and Torsten 2000). Numerous fungi such as *Aspergillus*, *Eurotium*, *Fusarium* and *Penicillium* spp. can grow down to pH 2 (Magan 1997), but only a few fungal species like *Acidomyces acidophilus* (Selbmann et al. 2008), *Acontium velatum* Morgan (Starkey and Waksman 1943) and *Hortaea acidophila* Hölker, Bend, Pracht, Tetsch, Tob. Müll., M. Höfer & de Hoog (Hölker et al. 2004) tolerate pH 1 or lower. In addition to *Acidomyces acidophilus*, two other species (*Paecilomyces* sp., *Penicillium* sp. 4) isolated in the present work belong to this small group.

Conclusions

In general, altogether 92 fungal taxa were recorded during the present study. Several taxa forming a core of the Soos fungal community belong to fungal groups known from similar substrata worldwide. However, the overall fungal spectrum is distinct in comparison to the fungal spectra described in similar studies. All methodological approaches used contributed to the broadening of the observed fungal spectrum. In spite of using both phenotypic and molecular approach, 37 of the 92 taxa failed to be determined to the species level; therefore, it can be expected that we isolated many new taxa. The considerably different

fungal communities observed from the four sampling sites were influenced by the complex effects of biotic and abiotic factors. The results of the growth tests indicate high adaptability of all tested species to the extreme conditions of the studied substrates. Moreover, two acidophilic species (*Acidomyces acidophilus*, *Sporothrix* sp.) were isolated.

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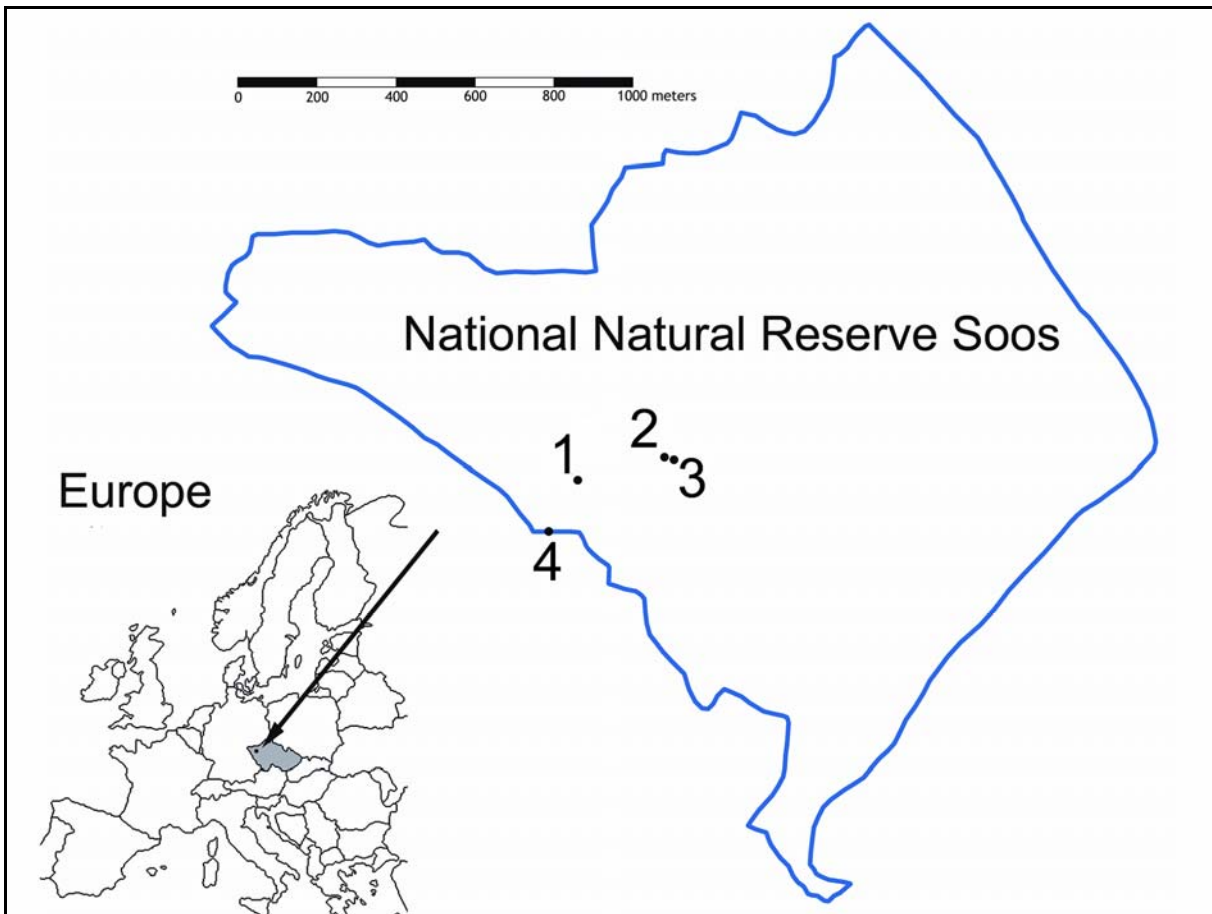

Fig. 1 Map of the Soos National Natural Reserve showing the four sampling sites.

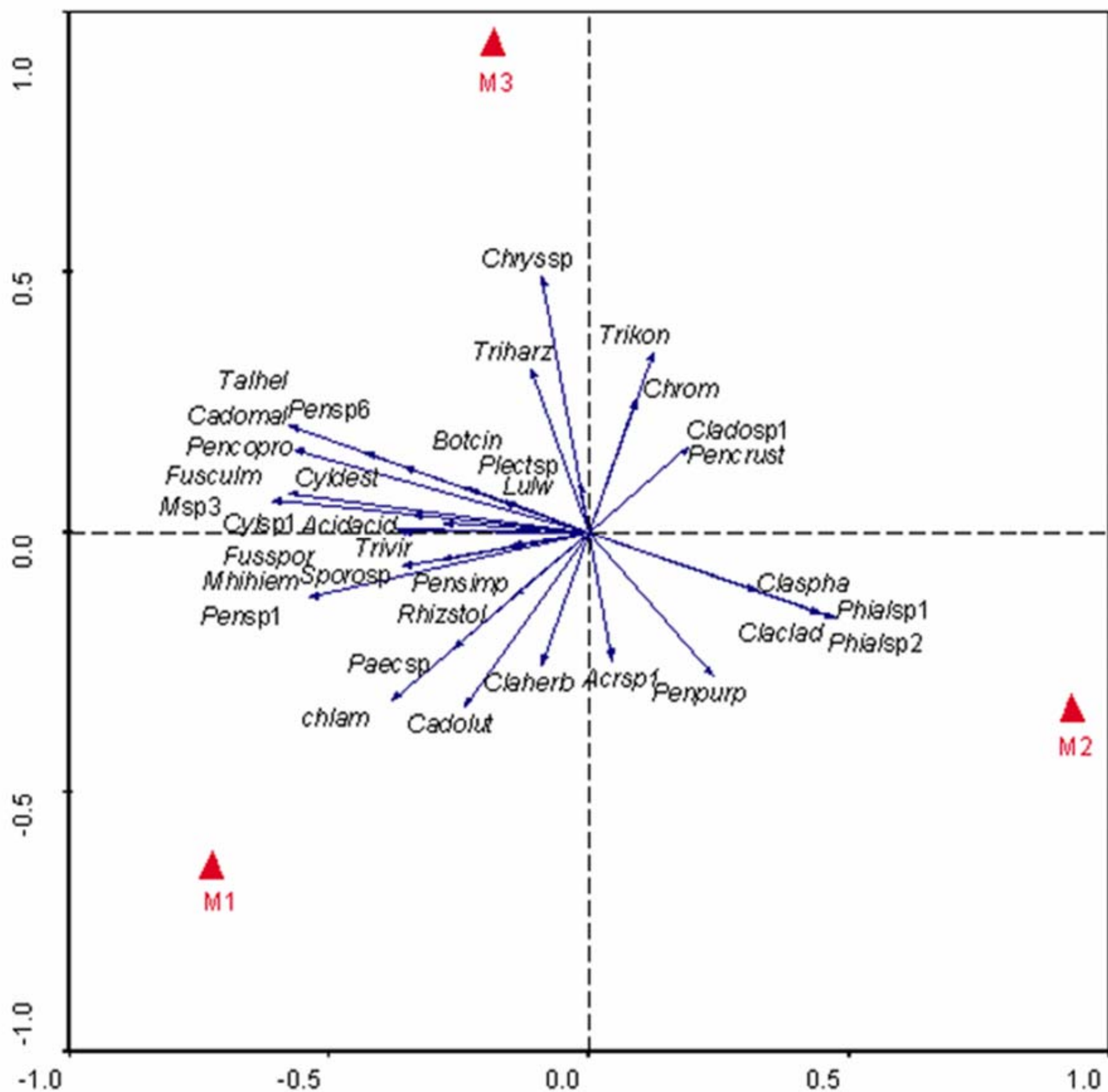

Fig. 2 Ordination diagram RDA showing the position of the species and the isolation procedure (M1, M2, M3) in the plane formed by first two axes. The figure shows relationships of fungal species (arrows) with environmental variable – isolation procedure (triangles). The majority of the species were obtained by M1 and M2 (species on the left side of the picture), while fewer species were isolated using M3 (species on the right side of the picture).

Fig. 3 Ordination diagram RDA showing the position of the species and the isolation medium (MA2, SEA) in the plane formed by first two axes. The figure shows relationships of fungal species (arrows) with environmental variable - medium (triangles). Species are distributed in the picture almost uniformly, what suggest the weak affinity of the fungal species to the single medium.

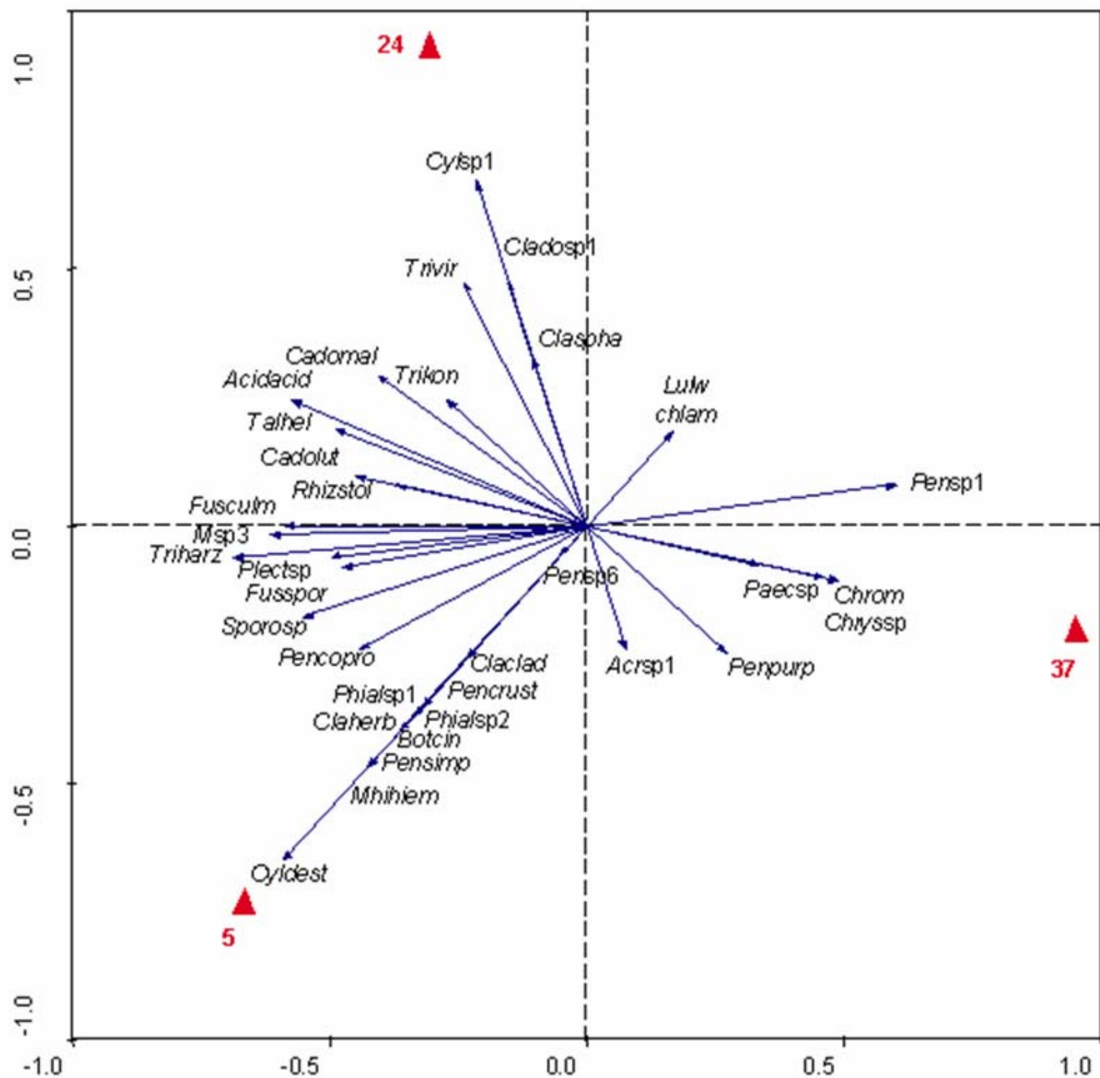

Fig. 4 Ordination diagram RDA showing the position of the species and the incubation temperature (5, 24, 37°C) in the plane formed by first two axes. The figure shows relationships of fungal species (arrows) with environmental variable – incubation temperature (triangles). The majority of the species were obtained at 5 and 24°C (species on the right side of the picture), while fewer species were isolated at 37°C (species on the right side of the picture).

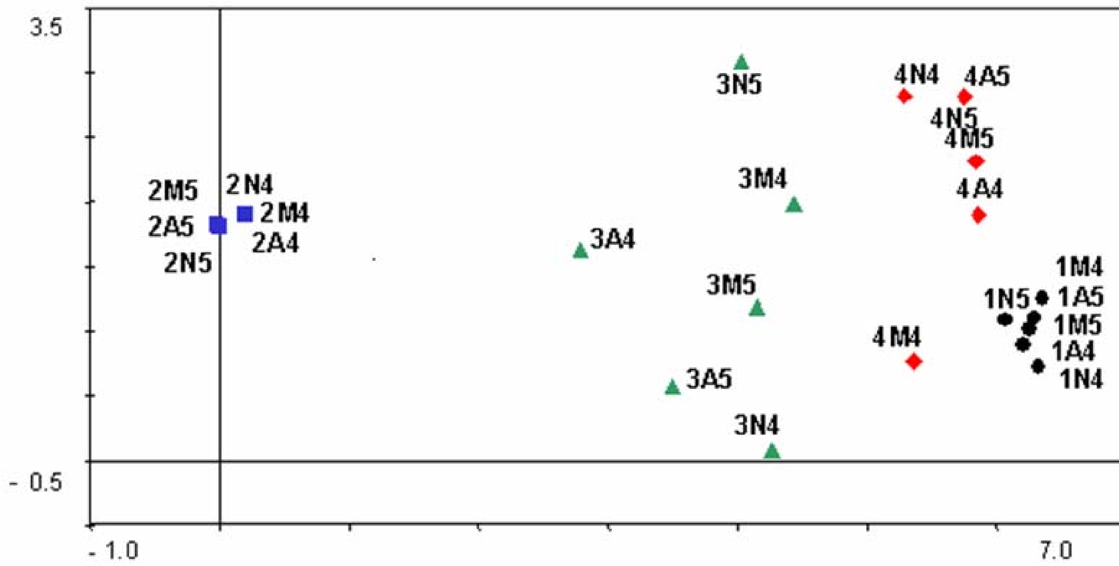

Fig. 5 Ordination diagram DCA showing the position of the samples in the plane formed by first two axes. The figure shows tendency to split the samples into groups according to the sampling sites (1-site1, 2-site2, 3-site3, 4-site4); M-May, A-August, N-November; 4-2004, 5-2005.

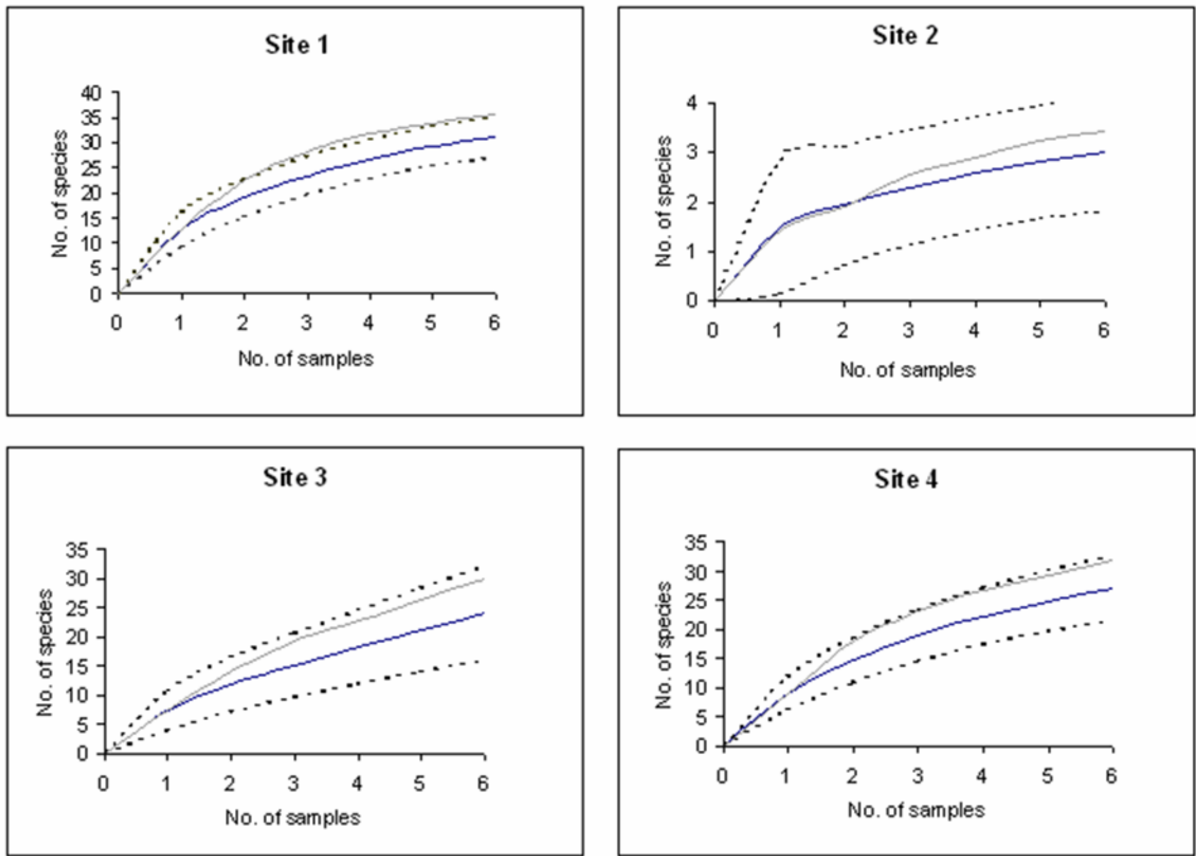

Fig. 6 Species accumulation curves and bootstrap estimates for each sampling site. The solid black line indicates observed richness, the black dashed lines indicate the 95 % confidence interval around the observed richness and the grey line indicates the bootstrap estimates of total species richness inferred using EstimateS.

Table 1 Description of the four selected sampling sites in Soos; values were based on seven measurements during 2003-2005.

Sampling site	**Vegetation cover**	**pH**	**Conductivity** (mS)	**SO_4^{2-} in the solid phase** (mg/1000g)	**Moisture in the soil samples** (in %)
1.	Dominance of *Schoenoplectus tabernaemontani*	5.6 – 7	1.6 – 8.7	5901.6 – 27936	82.6 – 92.4
2.	Bare soil	1.6 – 2.2	13.8 – 24.2	93872.9 – 251660.8	53.4 – 62.9
3.	Zone between the bare soil and vegetation (*Poa* spp., *Typha* spp., *S. tabernaemontani*)	1.9 – 2.7	9.1 – 12.7	79807.4 – 116516.9	59.3 – 70.8
4.	Zone between the pool and vegetation (mosses, *Poa* spp., *Rumex* spp., *S. tabernaemontani*, young trees)	6.8 – 7.2	3.9 – 7.4	19848 – 39236.1	75.1 – 82.2

Table 2 The list of the isolated species with frequencies of their occurrence and preferences to methodological approaches used. The species which were isolated only during the pilot study (November 2003) are marked with (+). The species which were identified by both morphological and molecular approach are marked with (*).

Species	Temp.	Medium	Methods	Sampling site				Total F %[a]
				S1 %[b]	S2 %[b]	S3 %[b]	S4 %[b]	
Absidia coerulea Bainier	5	MA	M1	0.6	-	-	-	4.2
Acidomyces acidophilus (Sigler & J. W. Carmich.) Selbmann, de Hoog & De Leo CCF 3785, 3786*	all	both	all	-	33.3	1.1	-	29.2
Acremonium sp. 1 CCF 3791, 3792*	all	both	M1, M3	2.2	-	-	-	8.3
Acremonium sp. 2	24	SEA	M1	0.6	-	-	-	4.2
Alternaria alternata (Fr.: Fr.) Keissler	5, 24	both	M1, M2	0.6	-	0.6	0.6	12.5
Aphanocladium album (Preuss) W.Gams [d]	24	SEA	M2	+	-	-	-	-
Botrytis cinerea Pers.	5	SEA	M1, M2	-	-	1.7	-	4.2
Cadophora luteo-olivacea J.F.H. Beyma CCF 3794, 3795, 3796*	5, 24	both	all	2.8	-	0.6	3.3	20.8
Cadophora malorum (Kidd et Beaumont) W. Gams CCF 3783, 3784*	5, 24	both	M1, M2	-	-	5	-	16.7
chlamydosporical ascomycete CCF 3810*	24.37	MA	M1	-	-	-	2.8	8.3
Chaetomium funicola Cooke [d]	37	SEA	M1	+	-	-	-	-
Chromocleista sp. CCF 3793, 3816*	37	both	M2 M3	-	-	3.9	-	8.3
Chrysosporium sp. CCF 3789, 3790, 3822*	37	both	M2	2.2	-	-	-	8.3
Cladophialophora sp. 1	24	SEA	M3	-	-	0.6	0.6	8.3
Cladophialophora sp. 2	5, 24	SEA	M1, M2	-	-	0.6	0.6	8.3
Cladosporium cladosporioides (Fresen.) de Vries	5	MA	M3	-	0.6	0.6	0.6	12.5
Cladosporium herbarum (Pers.: Fr.) Link	5	MA	M2, M3	-	-	0.6	2.2	16.7
Cladosporium sphaerospermum Penz.	24	MA	M3	0.6	-	0.6	0.6	12.5
Coniothyrium sp.	24	SEA	M2	-	-	-	0.6	4.2
Cylindrocarpon destructans Zinssm. CCF 3832, 3833*	5	both	all	4.4	-	-	-	12.5
Cylindrocarpon obtusisporum (Cooke et Harkn.) Wollenw.	5	MA	M1	-	-	-	1.7	12.5
Cylindrocarpon sp. 1 CCF 3801, 3802*	24	both	M1, M2	-	-	-	2.8	8.3
Cylindrocarpon sp. 2 CCF 3803*	24	MA	M1	-	-	-	0.6	4.2

Emericellopsis terricola J.F.H. Beyma CCF 3815*	37	MA	M2	-	-	0.6	-	4.2
Epicoccum nigrum Link CCF 3818*	5, 24	MA	M1, M2	0.6	-	-	0.6	8.3
Exophiala sp.	37	MA	M2	0.6	-	-	-	4.2
Fusarium avenaceum (Corda) Sacc. CCF 3804*	24	SEA	M1	-	-	1.1	-	4.2
Fusarium culmorum (W.G.Sm.) Sacc. CCF 3548	5, 24	both	M1, M2	11.7	-	-	-	16.7
Fusarium solani (Mart.) Sacc. CCF 3547	37	SEA	M2	0.6	-	-	-	4.2
Fusarium sporotrichioides Sherb. CCF 3549	5, 24	both	all	12.8	-	-	0.6	29.2
Fusarium spp. [e]	5, 24	both	M1, M2	9.4	-	-	0.6	25
Gibellulopsis nigrescens (Pethybr.) Zare, W. Gams & Summerb.	5	SEA	M2	0.6	-	-	-	4.2
Hypholoma fasciculare (Huds.) P.Kumm.[d] CCF 3827*	24	MA	M1	-	+	-	-	-
Lecanicillium muscarium Petch	5	MA	M3	-	-	-	0.6	4.2
Lecanicillium sp.	24	SEA	M2	-	-	-	0.6	4.2
Lulwoana sp. CCF 3787, 3788*	24, 37	SEA	M1 M2	-	-	-	2.2	8.3
Mammaria echinobotryoides Ces. CCF 3537	24	SEA	M2	-	-	-	0.6	4.2
Metarhizium anisopliae Metschn. CCF 3414 [d]	24	SEA	M2	+	-	-	-	-
Mortierella spp. [d]	5, 24	both	M1, M2	+	-	-	-	-
Mucor hiemalis (Wehmer) f. *corticolus* (Hagem) Schipper	5, 24	both	M3	1.1	-	0.6	-	8.3
Mucor hiemalis (Wehmer) f. *hiemalis*	5	both	M1, M2	0.6	-	1.1	-	8.3
Mucor sp. 1	5	MA	M2	0.6	-	-	-	4.2
Mucor sp. 2	5	MA	M2	-	-	-	0.6	4.2
Mucor sp. 3 CCF 3774, 3775*	all	both	M1, M2	2.2	-	15	2.8	41.7
Paecilomyces sp. *	24, 37	MA	M1	-	-	2.8	-	8.3
Penicillium allii Vincent et Pitt*	5	SEA	M3	0.6	-	-	-	4.2
Penicillium brevicompactum Dierckx	5	SEA	M1	0.6	-	0.6	-	8.3
Penicillium charlesii G. Sm. CCF 3797*	5	SEA	M3	0.6	-	-	-	4.2
Penicillium chrysogenum Thom CCF 3704*	37	MA	M3	0.6	-	1.1	-	8.3
Penicillium citrinum Sopp	24	MA	M2	-	-	-	0.6	4.2
Penicillium coprobium Frisvad CCF 3707, 3706, 3705*	5, 24	both	M1, M2	1.1	-	-	2.8	20.8
Penicillium crustosum Thom	5	both	M2, M3	-	-	-	2.2	8.3
Penicillium expansum Link	5	SEA	M1	-	-	1.1	-	4.2

Penicillium griseofulvum Dierckx	24	MA	M3	-	-	0.6	-	4.2
Penicillium montanense M.Chr. et Backus	5	both	M1, M3	1.1	-	-	0.6	12.5
Penicillium piceum Raper et Fennell	37	MA	M1	-	-	0.6	-	4.2
Penicillium purpurogenum Stoll	5, 37	both	M1, M3	0.6	-	-	1.7	8.3
Penicillium purpurogenum var. *rubrisclerotium* Thom	24	MA	M1	-	-	-	0.6	4.2
Penicillium roseopurpureum Dierckx [d]	24	SEA	M3	+	-	-	-	-
Penicillium rugulosum Thom [d]	5	SEA	M3	-	-	+	-	-
Penicillium simplicissimum (Oudem.) Thom	all	both	all	1.7	-	8.9	1.7	33.3
Penicillium spinulosum Thom CCF 3823*	5	SEA	M2	-	-	-	0.6	4.2
Penicillium sp. 1 CCF 3782, 3829*	all	both	all	15	-	16.1	3.9	58.3
Penicillium sp. 2 [d] CCF 3781*	5	MA	M2	-	-	+	-	-
Penicillium sp. 3 CCF 3812*	5	MA	M1	-	-	0.6	-	4.2
Penicillium sp. 4 [d] CCF 3828*	24	SEA	M1	-	+	-	-	
Penicillium sp.5* [d]	24	SEA	M1	+	-	-	-	-
Penicillium sp. 6 “complex” CCF 3776, 3777, 3778, 3405, 3779, 3780*[c]	all	both	all	-	-	16.7	-	20.8
Phialophora sp. 1	5	both	M3	2.2	-	-	-	8.3
Phialophora sp. 2	5	both	M3	1.1	-	-	0.6	12.5
Phialophora sp. 3	24	MA	M1	-	-	-	0.6	4.2
Phialophora sp. 4	24	SEA	M2	0.6	-	-	-	4.2
Phoma sp. 1 [d] CCF 3798*	24	SEA	M1	-	-	+	-	-
Plectosphaerella sp. CCF 3811*	5, 24	both	all	2.8	-	1.1	6.7	37.5
Pleosporales sp. *	24	SEA	M1	-	-	0.6	-	4.2
Preussia sp. CCF 3831*	37	SEA	M1	-	-	-	0.6	4.2
Rhizopus stolonifer (Ehrenb.: Fr.) Vuill.	5, 24	both	all	3.3	-	-	-	4.2
Sordaria fimicola Roberge ex Desm.	37	SEA	M1	0.6	-	-	-	4.2
Sporothrix sp. CCF 3799, 3800*	5, 24	both	M1, M2	-	2.2	-	-	8.3
Stachybotrys chartarum (Ehrenb.) S. Hughes	24	MA	M1, M2	1.1	-	-	0.6	12.5
Stilbella fimetaria (Pers.) Lindau	24	SEA	M1	-	-	0.6	-	4.2
Talaromyces helicus var. *helicus* (Raper et Fennell) C.R.Benj. CCF 3770, 3771* *Talaromyces helicus* var. *major* Stolk et Samson [c] CCF 3772, 3773*	all	both	M1, M2	0.6	-	0.6	25.6	33.3

Trichoderma atroviride P. Karst.	24	MA	M2	0.6	-	-	0.6	8.3
Trichoderma fertile Bissett	24, 37	SEA	M2	1.1	-	-	-	8.3
Trichoderma harzianum Rifai	5, 24	both	all	17.2	-	-	0.6	29.2
Trichoderma koningii Oudem. CCF 3814, 3834*	5, 24	both	M2	1.7	-	-	-	4.2
Trichoderma koningiopsis CCF 3813*	24	SEA	M3	0.6	-	-	-	4.2
Trichoderma virens (Miller et al.) Arx	5, 24	both	M1, M2	4.4	-	-	-	16.7
Trichoderma spp. [e]	5, 24	both	M1, M2	10.6	-	-	1.7	37.5
White ascomycete CCF 3830*	24	MA	M1	-	+	-	-	-
Total No. of species				**50**	**6**	**35**	**40**	**92**
sterile plates				12.8	61.1	37.2	33.9	100

Table 3 The results of the molecular analysis of 41 taxa including accession numbers of DNA and β-tubulin sequences and the closest matches from the GenBank database; the similarities above 95% for ITS and β-tubulin, 98% LSU and 99% for SSU rDNA are shown; no other values for rDNA regions are given in case of full identity in ITS rDNA.

Species	Genbank Accession No.				Blast
	ITS	LSU	SSU	β-tubulin	
Acidomyces acidophilus AK72/03	FJ430711				AJ244237 *Acidomyces acidophilus*, 450/450. 100 % ITS
Acremonium sp. 1 CCF 3791	FJ430713			FJ430785	AF289655 *Illosporium carneum*, 1632/1643, 99% SSU
Cadophora luteo-olivacea J.F.H. Beyma CCF 3794	FJ430741				AY371510 *Cadophora luteo-olivacea*, 543/543, 100% ITS
Cadophora malorum (Kidd et Beaumont) W. Gams CCF 3784	FJ430743				DQ317328 *Cadophora malorum*, 555/555, 100% ITS
chlamydosporical ascomycete CCF 3810, MH 576	FJ430718, FJ430719				DQ493955 *Magnaporthe grisea*, 1573/1597, 98% SSU
Chromocleista sp. MH 345, CCF 3816, MH 355	FJ430750, FJ430751, FJ430752				AY753348 *Chromocleista cinnabarina*, 474/490, 97% ITS AB075359 *Chromocleista cinnabarina*, 546/547, 99% LSU
Chrysosporium sp. MH 687, CCF 3790, MH 686, CCF 3789	FJ430724, FJ430725, FJ430726, FJ430727				AB361655 *Chrysosporium keratinophilum*, 586/598, 97% AB359444 *Ch. keratinophilum*, 594/595, 99% LSU
Cylindrocarpon destructans Zinssm. MH 337, MH 315, MH 484, CCF 3833, MH 651	FJ430728, FJ430729, FJ430730, FJ430731, FJ430732				AY295330 *Neonectria radicicola*, 490/490, 100% ITS
Cylindrocarpon obtusisporum (Cooke et Harkn.) Wollenw. CCF 3820	FJ430736			FM991733	AY677292 *Cylindrocarpon obtusisporum*, 455/455, 100% ITS AM419088 *Cylindrocarpon obtusisporum*, 408/418, 97% β-tub
Cylindrocarpon sp. 1 CCF 3802	FJ430733			FM865808	AY295335 *Cylindrocarpon* sp., 516/520, 99% ITS AB237468 *Neonectria veuillotiana*, 346/359, 96% β-tub
Cylindrocarpon sp. 2 CCF 3803, MH 730	FJ430734, FJ430735			FM865810	AB237468 *Neonectria veuillotiana*, 325/335, 97% β-tub
Emericellopsis terricola J.F.H. Beyma CCF 3815	FJ430737				U57676 *Emericellopsis terricola*, 566/567, 99% ITS
Epicoccum nigrum CCF 3818	FJ430776, FM991735				FJ424264 *Epicoccum nigrum*, 502/502,100% ITS

Fusarium avenaceum (Corda) Sacc. CCF 3804	FJ430738		EU255802 *Gibberella avenacea*, 504/504, 100% ITS
Hypholoma fasciculare (Huds.) P.Kumm.[d] CCF 3827, MH 85	FJ430716, FJ430717		AM504126 *Hypholoma fasciculare*, 600/600, 100% ITS
Lulwoana sp. MH 585, CCF 3787, MH 630, CCF 3788	FJ430720, FJ430721, FJ430722, FJ430723		AY879034 *Lulworthia uniseptata*, 1568/1584, 98% SSU
Mucor sp. 3 CCF 3774	FJ430740		AJ271061 *Mucor racemosus*, 564/574, 98% ITS AF113467 *Mucor circinelloides* f. *lusitanicus*, 648/648, 100% LSU X54863 *M. racemosus*, 1728/1728, 100% SSU
Paecilomyces sp. MH 393, MH 443	FJ430755, FJ430756	FM865816	AB176628 *Talaromyces ryukyuensis*, 454/459, 98% ITS AB047229 *Paecilomyces pascua*, 599/600, 99% LSU AY753358 *Paecilomyces aerugineus*, 463/472, 98% β-tub
Penicillium allii Vincent et Pitt MH 210	FJ430766		AJ005484 *Penicillium allii*, 567/567, 100% ITS
Penicillium charlesii G. Sm. CCF 3797, MH 247	FJ430768, FJ430769		AY742707 *Penicillium charlesii*, 557/558, 99% ITS AF033400 *Penicillium charlesii*, 506/506, 100% LSU
Penicillium chrysogenum Thom CCF 3704	FJ430774	FM865818	AY213669 *Penicillium chrysogenum*, 545/545, 100% ITS EU128566 *Penicillium chrysogenum*, 398/398, 100% β-tub
Penicillium coprobium Frisvad CCF 3707, 3706, 3705, MH 470	FJ430770, FJ430771, FJ430772, FJ430773	FM865817	DQ339559 *Penicillium coprobium*, 551/551, 100% ITS AY674424 *Penicillium coprobium*, 383/383, 100% β-tub
Penicillium spinulosum Thom CCF 3823	FJ430767		AY373933 *Penicillium spinulosum*, 560/563, 99% ITS AF033410 *Penicillium spinulosum*, 595/595, 100% LSU
Penicillium sp. 1 CCF 3782	FJ430760		AB176623 *Talaromyces helicus* var. *major*, 486/491, 98% ITS AF033396 *Talaromyces helicus*, 589/592, 99% LSU
Penicillium sp. 2 [d] CCF 3781	FJ430754	FM865815	AF380354 *Penicillium minioluteum*, 548/557, 98% ITS
Penicillium sp. 3 CCF 3812	FJ430775	FM865819	DQ486649 *Penicillium piscarium*, 475/478, 99% β-tub AY213675 *Penicillium ochrochloron*, 553/561, 98% ITS
Penicillium sp. 4 [d] CCF 3828	FJ430753	EU021663	AF245269 *Penicillium purpurogenum*, 1040/1045, 99% SSU AF245237 *P. purpurogenum*, 550/553, 99% ITS EF198559 *Penicillium* sp. NRRL 35637, 377/398, 95% β-tub
Penicillium sp. 5 [d] MH 30	FJ430744	FM991734	L14505 *Penicillium minioluteum*, 466/468, 99% ITS

Penicillium sp. 6 “complex” CCF 3778, MH 792, 276, 53, 272	FJ430745, FJ430746, FJ430747, FJ430748, FJ430749	FM865811 FM865812 FM865813	DQ123663 *Penicillium oxalicum*, 592/601, 98% ITS AY213620 *Penicillium oxalicum*, 547/554, 98% LSU
Phoma sp. 1 [d] CCF 3798	FJ430739		AY183371 *Phoma glomerata*, 503/506, 99% ITS AY904062 *Phoma pomorum*, 503/506, 99% ITS
Plectosphaerella sp. CCF 3811, MH 727	FJ430714, FJ430715	FM865809	AF176951 *Plectosphaerella cucumerina*, 1685/1691, 99% SSU DQ227288 *P. cucumerina*, 531/537, 98% ITS U17399 *P. cucumerina*, 456/461, 98% LSU
Pleosporales sp.	FM995624		AF250818 *Phaeosphaeriopsis nolinae*, 1660/1666, 99% SSU AF422976 *Stagonospora* sp., 383/397, 96% ITS
Preussia sp. CCF 3831, MH 12	FJ430777, FJ430778		U42478 *Sporormia lignicola*, 1639/1650, 99% SSU EU551212 *Preussia minima*, 494/505, 97% ITS DQ678056 *Preussia minima*, 548/552, 99% LSU
Sporothrix sp. MH 566, CCF 3799	FJ430780, FJ430781		< 95% SSU < 82% DQ069034 *Leptodontidium* sp., 163/182, 89% ITS
Stilbella fimetaria (Pers.) Lindau MH 178	FJ430712		AY952467 *Stilbella fimetaria*, 573/574, 99% ITS
Talaromyces helicus var. *helicus* (Raper et Fennell) C.R.Benj. CCF 3771, AK 71/03	FJ430759, FJ430761		AB176621 *Talaromyces helicus* var. *helicus*, 487/487, 100% ITS
Talaromyces helicus var. *major* Stolk et Samson [c] CCF 3773, MH 480	FJ430764, FJ430765		AB176623 *Talaromyces helicus* var. *major*, 459/459, 100% ITS
Trichoderma koningii Oudem. CCF 3814, 3834	FJ430782, FJ430783		AJ301990 *Hypocrea koningii*, 800/800, 100% ITS
Trichoderma koningiopsis CCF 3813	FJ430784		DQ379015 *Trichoderma koningiopsis*, 565/565, 100% ITS
white ascomycete CCF 3830	FJ430779	AJ877055	AY082984 Uncultured eukaryote, 1556/62, 99% SSU EU700254 Helotiales sp. MK9 460/476, 96% ITS DQ267629 *Lemonniera cornuta* 525/537, 98% LSU

Table 4 Similarity on fungal communities as a function of study sites (Bray-Curtis index).

	Site 1	**Site 2**	**Site 3**	**Site 4**
Site 1	1	0	0,23	0,22
Site 2	0	1	0,03	0
Site 3	0,23	0,03	1	0,17
Site 4	0,22	0	0,17	1

Table 5 The list of the isolated species with frequencies of their occurrence at an individual site and in a single sample (ESM).

Species	Site 1							Site 2							Site 3							Site 4						
	M4[b]	A4[b]	N4[b]	M5[b]	A5[b]	N5[b]	%[a]	M4[b]	A4[b]	N4[b]	M5[b]	A5[b]	N5[b]	%[a]	M4[b]	A4[b]	N4[b]	M5[b]	A5[b]	N5[b]	%[a]	M4[b]	A4[b]	N4[b]	M5[b]	A5[b]	N5[b]	%[a]
Absidia coerulea Bainier	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acidomyces acidophilus acidophilus (Sigler & J. W. Carmich.) Selbmann, de Hoog & De Leo	-	-	-	-	-	-	-	40	6.7	50	10	63.3	30	33.3	-	6.7	-	-	-	-	1.1	-	-	-	-	-	-	-
Acremonium sp. 1	-	-	-	-	3.3	10	**2.2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acremonium sp. 2	3.3	-	-	-	-	-	**0.6**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alternaria alternata (Fr.: Fr.) Keissler	-	-	-	3.3	-	-	**0.6**	-	-	-	-	-	-	-	3.3	-	-	-	-	-	**0.6**	-	-	-	-	3.3	-	**0.6**
Aphanocladium album (Preuss) W.Gams [c]							+																					
Botrytis cinerea Pers.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	**1.7**	-	-	-	-	-	-	-
Cadophora luteo-olivacea J.F.H. Beyma	-	-	-	-	3.3	13.3	**2.8**	-	-	-	-	-	-	-	3.3	-	-	-	-	-	**0.6**	-	10	-	6.7	-	3.3	**3.3**
Cadophora malorum (Kidd et Beaumont) W. Gams	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.7	6.7	3.3	13.3	-	-	**5**	-	-	-	-	-	-	-
chlamydosporical ascomycete	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.7	6.7	-	-	-	-	**2.8**
Chaetomium funicola Cooke [c]							+																					
Chromocleista sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13.3	10	-	-	-	**3.9**	-	-	-	-	-	-	-
Chrysosporium sp.	-	-	-	6.7	-	6.7	**2.2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cladophialophora sp. 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	**0.6**	-	3.3	-	-	-	-	**0.6**
Cladophialophora sp. 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	**0.6**	-	-	-	-	3.3	-	**0.6**

Cladosporium cladosporioides (Fresen.) de Vries	-	-	-	-	-	-	**-**	3.3	-	-	-	-	-	**0.6**	-	-	-	-	3.3	-	**0.6**	-	-	3.3	-	-	-	**0.6**
Cladosporium herbarum (Pers.: Fr.) Link	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	3.3	-	-	**0.6**	-	-	3.3	-	3.3	6.7	**2.2**
Cladosporium sphaerospermum Penz.	-	-	-	-	3.3	-	**0.6**	-	-	-	-	-	-	**-**	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	3.3	**0.6**
Coniothyrium sp.	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	3.3	-	-	-	-	-	**0.6**
Cylindrocarpon destructans Zinssm.	-	-	6.7	-	6.7	13.3	**4.4**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Cylindrocarpon obtusisporum (Cooke et Harkn.) Wollenw.	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	3.3	3.3	3.3	-	-	-	**1.7**
Cylindrocarpon sp. 1	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	10	-	-	6.7	-	-	**2.8**
Cylindrocarpon sp. 2	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	3.3	-	**0.6**
Emericellopsis terricola J.F.H. Beyma	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	3.3	-	-	-	-	**0.6**	-	-	-	-	-	-	**-**
Epicoccum nigrum Link	-	3.3	-	-	-	-	**0.6**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	3.3	-	-	-	**1.1**
Exophiala sp.	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Fusarium avenaceum (Corda) Sacc.	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	6.7	-	-	-	**1.1**	-	-	-	-	-	-	**-**
Fusarium culmorum (W.G.Sm.) Sacc.	-	16.7	10	-	16.7	26.7	**11.7**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Fusarium solani (Mart.) Sacc.	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Fusarium sporotrichioides Sherb.	10	20	3.3	23.3	10	10	**12.8**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	3.3	-	-	-	-	-	**0.6**
Fusarium spp. [f]	6.7	10	13.3	13.3	13.3	-	**9.4**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	3.3	-	-	-	-	-	**0.6**
Gibellulopsis nigrescens (Pethybr.) Zare, W. Gams	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**

& Summerb.																												
Hypholoma fasciculare (Huds.) P.Kumm. [c]	-	-	-	-	-	-	**-**	-	-	-	-	-	-	+	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Lecanicillium muscarium Petch	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	3.3	**0.6**
Lecanicillium sp.	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	3.3	-	-	-	-	-	**0.6**
Lulwoana sp.	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	3.3	-	-	-	6.7	-	**2.2**
Mammaria echinobotryoides Ces.	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	3.3	-	-	-	-	-	**0.6**
Metarhizium anisopliae Metschn. [c]	-	-	-	-	-	-	+	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Mortierella spp. [c]	-	-	-	-	-	-	+	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Mucor hiemalis (Wehmer) f. *corticolus* (Hagem) Schipper	-	-	6.7	-	-	-	**1.1**	-	-	-	-	-	-	**-**	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	-	**-**
Mucor hiemalis (Wehmer) f. *hiemalis*	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	-	**-**	-	-	6.7	-	-	-	**1.1**	-	-	-	-	-	-	**-**
Mucor sp. 1	-	-	-	-	3.3	-	**0.6**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Mucor sp. 2	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	3.3	-	-	-	-	**0.6**
Mucor sp. 3	-	6.7	-	3.3	-	3.3	**2.2**	-	-	-	-	-	-	**-**	3.3	3.3	23.3	20	40	-	**15**	13.3	-	-	-	-	3.3	**2.8**
Paecilomyces sp.	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	6.7	10	-	-	**2.8**	-	-	-	-	-	-	**-**
Penicillium allii Vincent et Pitt	-	3.3	-	-	-	-	**0.6**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Penicillium brevicompactum Dierckx	-	-	-	-	-	3.3	**0.6**	-	-	-	-	-	-	**-**	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	-	**-**
Penicillium charlesii G. Sm.	-	3.3	-	-	-	-	**0.6**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Penicillium chrysogenum Thom	-	3.3	-	-	-	-	**0.6**	-	-	-	-	-	-	**-**	-	-	6.7	-	-	-	**1.1**	-	-	-	-	-	-	**-**

Penicillium citrinum Sopp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	**0.6**
Penicillium coprobium Frisvad	-	-	3.3	-	-	3.3	**1.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	3.3	10	-	-	**2.8**
Penicillium crustosum Thom	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	10	**2.2**
Penicillium expansum Link	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.7	-	-	-	**1.1**	-	-	-	-	-	-	-
Penicillium griseofulvum Dierckx	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	-	**0.6**	-	-	-	-	-	-	-
Penicillium montanense M.Chr. et Backus	-	3.3	-	-	3.3	-	**1.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	**0.6**
Penicillium piceum Raper et Fennell	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	-	-
Penicillium purpurogenum Stoll	3.3	-	-	-	-	-	**0.6**	-	-	-	-	-	-	-							-	-	-	-	-	-	6.7	**1.7**
Penicillium purpurogenum var. *rubrisclerotium* Thom	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	-	-	**0.6**
Penicillium roseopurpureum Dierckx[c]	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium rugulosum Thom [c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Penicillium simplicissimum (Oudem.) Thom	-	-	-	-	3.3	6.7	**1.7**	-	-	-	-	-	-	-	6.7	3.3	-	10	-	33.3	**8.9**	-	-	3.3	-	-	6.7	**1.7**
Penicillium spinulosum Thom	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	-	-	**0.6**
Penicillium sp. 1	10	10	13.3	20	20	16.7	**15**	-	-	-	-	-	-	-	6.7	10	36.7	36.7	-	6.7	**16.1**	16.7	-	-	3.3	3.3	-	**3.9**
Penicillium sp. 2 [c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-

Penicillium sp. 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	-	-	**0.6**	-	-	-	-	-	-	-
Penicillium sp. 4 [c]	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium sp. 5 [c]	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium sp. 6 “complex” [d]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	43.3	-	3.3	6.7	4.4	**16.7**	-	-	-	-	-	-	-
Phialophora sp. 1	-	6.7	-	-	-	6.7	**2.2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phialophora sp. 2	-	-	-	-	3.3	3.3	**1.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	**0.6**
Phialophora sp. 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	**0.6**
Phialophora sp. 4	-	-	3.3	-	-	-	**0.6**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phoma sp. 1 [c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Plectosphaerella sp.	3.3	10	-	-	-	3.3	**2.8**	-	-	-	-	-	-	-	6.7	-	-	-	-	-	**1.1**	6.7	20	3.3	6.7	-	3.3	**6.7**
Pleosporales sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	-	-	**0.6**	-	-	-	-	-	-	-
Preussia sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	**0.6**
Rhizopus stolonifer (Ehrenb.: Fr.) Vuill.	-	-	20	-	-	-	**3.3**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sordaria fimicola Roberge ex Desm.	-	3.3	-	-	-	-	**0.6**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sporothrix sp.	-	-	-	-	-	-	-	-	-	-	-	10	3.3	**2.2**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stachybotrys chartarum (Ehrenb.) S. Hughes	-	-	-	3.3	3.3	-	**1.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	-	-	**0.6**
Stilbella fimetaria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	-	-	-	-	-	**0.6**	-	-	-	-	-	-	-
Talaromyces helicus var. *helicus* (Raper et Fennell) C.R.Benj. *Talaromyces helicus* var. *major* Stolk et Samson [e]	-	-	-	-	-	3.3	**0.6**	-	-	-	-	-	-	-	-	-	-	3.3	-	-	**0.6**	3.3	20	20	36.7	40	33.3	**25.6**
Trichoderma atroviride P. Karst.	-	-	-	-	-	3.3	**0.6**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.3	**0.6**
Trichoderma fertile	-	3.3	-	-	3.3	-	**1.1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Bissett																												
Trichoderma harzianum Rifai	20	10	10	20	30	-	**17.2**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	3.3	**0.6**
Trichoderma koningii Oudem.	-	10	-	-	-	-	**1.7**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Trichoderma koningiopsis	-	-	-	3.3	-	-	**0.6**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Trichoderma virens (Miller et al.) Arx	3.3	-	10	6.7	6.7	-	**4.4**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**
Trichoderma spp. [f]	10	10	10	23.3	3.3	6.7	**10.6**	-	-	-	-	-	-	**-**	-	-	-	-	-	-	**-**	3.3	-	-	-	3.3	3.3	**1.7**
white ascomycete [c]	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total No. of species	**7**	**15**	**15**	**9**	**15**	**14**		**2**	**1**	**1**	**1**	**2**	**2**		**10**	**9**	**16**	**8**	**4**	**3**		**17**	**7**	**10**	**6**	**10**	**13**	
	28			**25**				**2**			**2**				**26**			**10**				**25**			**22**			
	42 (20)							**4 (4)**							**30 (14)**							**38 (21)**						
sterile plates	30	6.7	13.3	10	16.7	13.3		43.3	86.7	43.3	90	33.3	70		43.3	10	6.7	40	40	53.3		6.7	26.7	60	46.7	43.3	20	

Table 6 Effect of salinity and pH on the growth of selected fungal strains; colony diameter [mm] was measured after 7 or 3 days in case of fast growing species (*); values corresponding with the optimum are in bold; MH indicate personal collection.

Tested strains	MEA	0.25M MEA	0.5M MEA	pH1	pH2	pH3	pH4	pH5	pH6	pH7	pH8
Acidomyces acidophilus CCF 3785	**10.8**	4.8	1.7	10.7	11.5	**12.3**	10.8	10	10	5.25	3
Cylindrocarpon sp. 1 CCF 3802	**18.3**	13	11.5	-	-	-	8.3	15.25	15.5	15.3	**17.8**
Cylindrocarpon sp. 1 CCF 3801	**21.3**	12.8	10	-	-	-	13.2	18	20.2	22.3	**24**
Cylindrocarpon sp. 2 CCF 3803	**16.8**	9	8.3	-	-	3.8	14.7	**19.8**	17	11.5	12.4
Chrysosporium sp. CCF 3789	**26**	21.2	11.3	-	-	-	-	17.3	16.3	23.2	**23.5**
Chrysosporium sp. CCF 3790	**21.5**	19	15.3	-	-	-	-	17.7	17.3	19.5	**20**
Fusarium culmorum * MH 217	45.8	**50.8**	28	-	-	-	15	50.8	47.3	**60**	**60**
Fusarium sporotrichioides * MH 216	**53.3**	53.2	31.5	-	-	-	12.8	49	46.3	**54**	51
Mucor sp. 3 * CCF 3775	**67.8**	60.5	41	-	-	-	18	**78**	62	64.3	61.3
Paecilomyces sp. MH 393	**42.3**	25.3	26.5	3.3	27.25	36.3	**43**	41.5	42.8	41	39.5
Penicillium sp. 1 MH 163	**39.2**	39	38.5	-	-	12.3	36	34.8	34	38.3	**39.7**
Penicillium chrysogenum CCF 3704	35.5	**59**	56.5	-	-	-	11.3	38.7	34.7	37.7	**42**
Penicillium coprobium MH 23	23.8	30.7	**31.7**	-	-	-	8.5	22.3	22.7	**25.7**	**25.7**
Penicillium coprobium CCF 3705	25.8	32.3	**35.5**	-	-	-	4.8	23.7	22.5	**24.3**	23.8
Penicillium sp. 2 CCF 3781	**31.3**	22.5	15.5	-	-	3	**26.5**	21.7	24.8	17.5	10.8
Penicillium sp. 4 CCF 3828	**26.3**	19	13.5	7.7	21.5	26	**31.7**	23.7	25.3	23.2	16.3
Penicillium sp. 3 CCF 3812	**57.8**	34.8	27.8	-	20.75	37.25	50	**55.5**	34	35.25	33.7
Penicillium sp. 6 "complex" CCF 3405	49.8	**61.3**	54.7	-	-	25.7	47.2	45	42.5	53	**56**
Sporothrix sp. CCF 3799	**21.5**	6.5	0	-	25.5	**35**	31	28.5	6.5	0	0
Talaromyces helicus var. *helicus* CCF 3771	**31.5**	16.3	8.3	-	2.8	31.3	33.5	35.3	**35.5**	26.5	13
Talaromyces helicus var. *major* CCF 3772	**33.6**	22.3	12.8	-	-	7.5	**41**	34.8	35.7	16.3	5
Trichoderma harzianum * MH 694	**80**	46	23.7	-	-	2.8	50.3	**66.3**	57	49.3	47.7

2.2 Paper II

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Acidiella bohemica gen. et sp. nov. and *Acidomyces* spp. (Teratosphaeriaceae), the indigenous inhabitants of extremely acidic soils in Europe

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Abstract

Extremely acidic soils (pH < 3) harbour mycobiota that are different from less acidic habitats and are dominated by a small number of fungal species. During investigation of the mycobiota of highly acidic soils in the Czech Republic and Iceland, a group of melanised fungal isolates was obtained. Based on phenotype and nuclear ribosomal DNA sequences (ITS region, SSU, LSU), the isolates were accommodated by three phylogenetic lineages within the family Teratosphaeriaceae (Capnodiales, Dothideomycetes). The first lineage is here described as a new acidotolerant genus and species *Acidiella bohemica* gen. nov. et sp. nov. The most closely related species to this new clade are other extremotolerant fungi isolated from rocks and lichens collected in the Alps and Antarctica as well as leaf-spotting species and opportunistic human pathogens. The isolates belonging to the second and the third lineages were identified as the recently described acidophilic fungi *Acidomyces acidophilus* and *Teratosphaeria acidotherma*, respectively. The taxonomic status of both sister species is discussed and the latter species is ascribed here to the genus *Acidomyces*.

Keywords
black yeast, acidotolerant, *Acidomyces acidophilus*, *Teratosphaeria acidotherma,* soil fungi, Ascomycota

Introduction

Highly acidic soil and water (pH < 3) represent some of the most extreme environments and exceptional living conditions for microorganisms. It has been generally assumed that eukaryotic microorganisms (including fungi) are unable to adapt to these extreme living conditions and that these substrata are populated exclusively by bacteria (Oren 2002). However, an unexpected degree of eukaryotic diversity in such extreme habitats, along with the abundance and importance of fungi within acidophilic microbial communities, has recently been revealed (López -Archilla and Amills 1999; López-Archilla et al. 2001; Amaral Zettler et al. 2002, 2003; Baker et al. 2004, 2009).

Only fragmentary data are available on fungal diversity in extremely acidic environments (pH < 3), but it is apparent that black meristematic fungi are common inhabitants of such habitats (Baker et al. 2004; López-Archilla et al. 2004; Hujslova et al. 2010) as well as other extreme environments (Gunde-Cimerman et al. 2005). Due to the specific mechanisms of adaptation such as meristematic growth, melanisation, alterations of cell wall structure or optimisation of the volume-to-surface ratio, this group of fungi, known as black yeasts, can thrive under exceptional conditions (Sterflinger et al. 1999; Gunde-Cimmerman et al. 2005). Because they are characteristically polymorphic and exhibit morphological plasticity, the identification of black yeasts should involve the combination of morphological data with nutritional and physiological tests and molecular methods (Gunde-Cimmerman et al. 2005).

To date, only three acidophilic fungi have been identified (Hölker et al. 2004; Selbmann et al. 2008; Yamazaki et al. 2010). Taxonomically, they belong to the family Teratosphaeriaceae (Capnodiales, Dothideomycetes, Ascomycota), which encompasses saprobes, leaf-spotting species, opportunistic human pathogens and lichens, as well as extremotolerant species (Crous et al. 2007, 2009a,b,c) found in various extreme substrata such as saline environments (Gunde-Cimmerman et al. 2005), Antarctic cold deserts (Onofri et al. 1999; Selbmann et al. 2005, 2008), rock surfaces (Sert et al. 2007, 2011), or heat-treated soil (Seifert et al. 2004).

Based on the distinct morphological features of its members as well as its phylogenetic position within the Capnodiales, the family Teratosphaeriaceae was separated from Mycosphaerellaceae (Crous et al. 2007). Recently, it has been shown that the family is too widely defined and incorporates many diverse genera and families, many of which may still eventually be removed from the Teratosphaeriaceae as additional taxa and sequence data are added (Crous et al. 2009a, b,c).

The modern generic concept was applied by Crous et al. (2009a) in an attempt to provide a more natural classification for the genera in Mycosphaerellaceae and Teratosphaeriaceae. A single generic name fulfilling standard nomenclatural priority rules is used for each monophyletic phylogenetic lineage, regardless of whether the lineage is anamorphic or teleomorphic (Crous et al. 2009a).

We are interested in the comparative analysis of the mycobiota inhabiting extremely acidic soils (pH < 3) in geographically isolated localities of small areas. These localities were found to be occupied by specific species-poor communities dominated by dematious filamentous fungi. Here, we provide a taxonomic analysis of melanised fungal isolates obtained during this study in the Czech Republic and Iceland. Based on phenotype and nuclear ribosomal DNA (ITS region, SSU, LSU) sequences, the isolates can be placed in three phylogenetically isolated positions within the Teratosphaeriaceae. The first group of isolates is here described as a new genus, and its response to different pH values has been determined. The remaining two dominant species, identified as recently described sister species of the acidophilic fungi *Acidomyces acidophilus* and *Teratosphaeria acidotherma*, are combined to form the single genus *Acidomyces*.

Materials and methods

Sampling, isolation, morphological and cultural characterisation

Altogether, 20 samples of extremely acidic soil (pH < 3) were collected from six sampling sites in the Czech Republic and Iceland in May, July and November 2007 (Fig. 1, Table 1). The samples were processed using two methods and three types of isolation media. The methods were direct inoculation of soil (M1) (Fassatiová 1986) and the soil washing technique (M2) (Kreisel and Schauer 1987), and the media were 2% malt agar (MA2), acidified 2% malt extract agar (MEA-pH2) and acidified soil agar with rose Bengal and glucose (SEA-pH2) (Pitt 1980; Fassatiová 1986). The pH of the MEA and SEA was adjusted to 2 with concentrated H_2SO_4; furthermore, SEA was prepared from the substrata of the respective sampling site. Streptomycin was added to all media (0.1 g/l) to suppress bacterial

growth. The plates were incubated at 5°C, 24°C and 37°C. After 7-14 days, the emerging black colonies were transferred to identification media.

All measurements and observations were performed using fungal structures grown for one month on MEA-pH2 and incubated in the dark at 24°C. Other media used for colony description were malt extract agar (MEA) and potato-dextrose agar (PDA) (Booth 1971). Colour codes were determined according to the Munsell System (1966). Slides were mounted in lactophenol with cotton blue and in Melzer‘s solution (Bills and Foster 2004) and observed using light microscopy. The ex-type and other representative strains have been deposited in the Culture Collection of Fungi (CCF), Department of Botany, Faculty of Science, Charles University in Prague, Czech Republic or in the personal collection of the senior author (Table 2).

DNA and molecular phylogenetic analyses

Genomic DNA was isolated from 14- to 28-day-old cultures using a Microbial DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA USA). The ITS1, 5.8S, and ITS2 regions of the rDNA, together with partial LSU rDNA, were amplified using primer pairs ITS1, ITS5 or ITS1F and NL4 or LR6 (Gardes and Bruns 1993; O'Donnell 1993). The SSU rDNA gene was amplified using primers NS1 and NS24. Custom purification of the PCR products and sequencing of the DNA was performed at Macrogen (Seoul, Korea) using the same primers listed above together with NS2, NS4 and NS5 (White et al. 1990). GenBank accession numbers are provided in Table 2. Sequence manipulations were performed using the BioEdit v. 7.0.4.1 software (Hall 1999). A Blast similarity search (Altschul et al. 1997) was used to find similar sequences in the GenBank database. LSU sequences were included in a pruned alignment of Capnodiales published by Crous et al. (2009a) (TreeBase No. M4375). The LSU and SSU sequences were included in an alignment of capnodialean fungi published by Crous et al. (2009c) (TreeBase No. M4794) and combined with the closest matches from GenBank. Another dataset consisting of ITS, LSU and SSU rDNA sequences was compiled for *Acidomyces acidophilus* and *Teratosphaeria acidotherma* sequences from our study and from GenBank. The input dataset combined the available ITS, LSU and SSU rDNA sequences (Table 3) and included a total of 3040 positions (172 variable, 135 singleton and 37 parsimony-informative sites). A tree was inferred by maximum likelihood analysis (with default input parameters using all positions and 500 bootstrap replicates in evolutionary analyses) using the MEGA5 software (Tamura et al. 2011). *Teratosphaeria verrucosa* was used as the outgroup (Fig. 5). For the LSU and LSU/SSU analyses, DNA sequences were

aligned using the T-coffee web server, and ambiguous positions were subsequently aligned based on Core analysis of local reliability (Notredame et al. 2000; Poirot et al. 2003). The LSU alignment consisted of 732 positions (286 variable, 67 singletons and 219 parsimony-informative sites), and the combined dataset consisted of 2010 positions (401 variable, 172 singleton and 229 parsimony-informative sites). Phylogenetic relationships were inferred from the maximum likelihood and Bayesian methods. *Phaeobotryosphaeria visci* was used as the outgroup. For both LSU rDNA and a dataset containing concatenated LSU and SSU rDNA, the model of molecular evolution for each separate alignment was assessed using jModelTest (Guindon and Gascuel 2003; Posada 2008). This analysis showed GTR to be the most relevant model, with site-to-site rate variation approximated with a gamma distribution and an estimated proportion of invariable sites. For the likelihood analysis, we used a fast bootstrapping algorithm (Stamatakis 2006) in RAxML (version 7.2.7) conducted on the CIPRES Science gateway Web server (RAxML-HPC2 on TG) (Miller et al. 2010). For the Bayesian analysis, we used MrBayes (version 3.1.2) (Huelsenbeck and Ronquist 2001) run on the same server used above (MrBayes on TG) with 10,000,000 generations, sampling trees every 1000 generations and discarding the first half of the trees as a burn-in. The convergence of two runs with four chains was evaluated by Tracer v. 1.5.0 (Rambaut and Drummond 2003). The resulting files were then combined and a 50% majority-rule consensus tree was computed.

Growth at different pH levels

The effect of pH on the growth of the seven strains of *Acidiella bohemica* (Table 2) was determined by measuring colony diameter on MEA. Eight different pH values ranging from 1 to 8 were used. To permit polymerisation of the agar at pH 1, twice the amount of agar was added. The pH of the medium was adjusted with concentrated H_2SO_4 and/or NaOH after sterilisation. The triplicate plates were inoculated with mycelial segments and incubated at 24°C for one month. The colony size was measured after seven days.

Temperature preference test

The temperature preference of the eight strains of *Acidomyces acidophilus* and *Teratosphaeria acidotherma* was determined by measuring colony diameter on MEA at 37°C (Table 3). The growth at 24°C was used as a control. The plates were inoculated by mycelial segments (in triplicate). The colonies were checked after seven days.

Results

Altogether, 82 black fungal isolates were obtained from 20 soil samples collected from six sampling sites in the Czech Republic and Iceland (Fig. 1, Table 1). All isolates were divided into three groups based on phenotype. Selected isolates from each morpho-group (10 isolates from the first group, 13 from the second and 3 from the third) were characterised by analysis of rDNA sequences (Table 2). Based on the phylogeny data, our isolates were accommodated in three distinct phylogenetic lineages within the family Teratosphaeriaceae (Capnodiales, Dothideomycetes) (Fig. 3, 4).

The first phylogenetic lineage was represented by 10 isolates (the first morpho-group). ITS rDNA sequences from these isolates do not show a high degree of relatedness to any published sequence (the highest similarity score was 91% with *Penidiella rigidophora* EU019276). Sequences of LSU rDNA show 97% similarity with several capnodialean species. Based on SSU rDNA sequences, these strains are most closely related to an uncultured eukaryote clone from highly acidic river (99-99.8%, AY082971), *Catenulostroma chromoblastomycosum* Crous & U. Braun (99.2-99.3%, GU214516) and three unidentified rock-inhabiting fungal isolates from Teratosphaeriaceae (99.1-99.2% similarity, GU250351, GU250318, and GU350240).

The second phylogenetic lineage, represented by 10 isolates (part of the second morpho-group), contained ITS rDNA sequences that are nearly identical to those found in *Acidomyces acidophilus* (Sigler & J.W. Carmich.) Selbmann, de Hoog & De Leo (99.6%, AJ244237). Other published rDNA sequences of *Acidomyces acidophilus* were not available for comparison.

The third phylogenetic lineage (three isolates from the second morpho-group and three isolates from the third) contained ITS and LSU rDNA sequences identical to those found in *Teratosphaeria acidotherma* A. Yamaz., K. Toyama & Nakagiri (AB537901, AB537900, AB537899, AB537898). The SSU rDNA sequences from this lineage were identical to part of the published *Teratosphaeria acidotherma* sequences (AB537895, AB537894) and 99.9% similar to other GenBank entries from this species (AB537897, AB537896), as well as to *Acidomyces* spp. (98.2% similarity; FJ430711, EU090191, AY374298, AY374299, AY374300).

Both the MB and ML analyses of the LSU and LSU-SSU datasets revealed phylogenetic trees that strongly support the placement of the first phylogenetic lineage in a separate cluster (Fig. 3, 4). The first lineage, here described as a new genus and species *Acidiella bohemica* Hujslová & M. Kolařík, clustered in a group with two unidentified

isolates of Teratosphaeriaceae (GU250393, GU250363) (Fig. 3). Based on LSU-SSU sequences, *Acidiella* formed a sister group with the uncultured eukaryote clone RT3n2 (Fig. 4). Among the nearest neighbours were *Catenulostroma chromoblastomycosum* (GU214516, EU019251), three unidentified isolates of Teratosphaeriaceae (GU250351, GU250318, GU350240), *Recurvomyces mirabilis* Selbmann & de Hoog (EU019297 GU250372), *Catenulostroma elginense* (Joanne E. Taylor & Crous) Crous & U. Braun (GU214517, EU019252), *Elasticomyces elasticus* Zucconi & Selbmann (GU250353, GU250375) and *Friedmanniomyces endolithicus* Onofri (GU250326, GU250366) (Fig. 4).

The isolates belonging to the second and the third lineages were located in sister clades representing two acidophilic fungi, *Acidomyces acidophilus* and *Teratosphaeria acidotherma* (Fig. 3, 4). The phylogenetic placement of our isolates with respect to both sister species is shown in Fig. 5. Based on a comparison of LSU rDNA, both acidophilic fungi clustered in a group with *Piedraia hortae* (Brumpt) da Fonseca & de Arêa Leão (AY016366) and two *Penidiella*-like isolates (DQ246234, GQ852625) (Fig. 3). Analysis of the LSU-SSU dataset yielded phylogenetic trees that confirm the placement of the *Acidomyces acidophilus/Teratosphaeria acidotherma* clade in a group with *Teratosphaeria suberosa* (Crous, F.A. Ferreira, Alfenas & M.J.Wingf.) Crous & U. Braun (GU214614, GU214512) and *Readeriella* Syd. & P. Syd. species (incl. *Cibiessia* and *Nothostrasseria*) (FJ493200, GU214521 EU019258, EU754110, EU019291, EF394832, and EU019271) (Fig. 4).

Taxonomy

Acidiella Hujslová & M. Kolařík, **gen. nov.** MB 564518

Ad fungos anamorphos, hyphomycetes pertinens. Coloniae lente crescentes, compactae, in medio acervatae vel cerebriformes, margine planae, velutinae, nigrae; olivaceo-griseae in acido agaro. Mycelium ex hyphis ramosis, septatis, hyalineae vel pallide brunneis pigmentatis compositum, fissione ad septa in conidia disarticulantes. Arthroconidia bacillaria vel inflatae utrinque truncata. Teleomorphosis ignota.

Etymology: Acidiella refers to the occurrence in acidic substrata.

Anamorphic fungi, hyphomycetes. Colonies slow growing, compact, centrally heaped or cerebriform, marginally flat, slightly velvety, black; on acidic medium powdery, moderate olive green (5GY4/4), marginally strong greenish yellow (7.5Y7/10). Mycelium composed of

septate, branched colourless or pale brown hyphae, forming conidia by fragmentation. Arthroconidia oblong or swollen with truncated ends. *Teleomorph*: Unknown.

Type species: ***Acidiella bohemica*** Hujslová & M. Kolařík, sp. nov.

Acidiella bohemica Hujslová & M.Kolařík, **sp. nov.** MB 564519 (Fig. 2)

Coloniae in agaro MEA ad 24°C lente crescentes, compactae, velutinae, in medio acervatae vel cerebriformes, nigrae, margine planae et olivaceo-nigrae, reversum olivaceo-nigrum; post 28 dies 23–28 mm diam. Coloniae in acido agaro ad 24°C post 28 dies 20–23 mm, compactae, in medio acervatae vel cerebriformes, margine planae, olivaceo-griseae, reversum olivaceo-griseae et olivaceae. Mycelium ex hyphis ramosis, septatis, hyalineae vel pallide brunneis pigmentatis, 2–2.3 µm latis compositum. Conidia arthrice secedentia, bacillaria (8.0–16.0 × 1.7–3.0 µm) vel inflatae (4.8-18.0 × 1.8-4.8 µm) utrinque truncata. Teleomorphosis ignota.

Etymology: bohemica refers to the occurrence in Bohemia (Latin name of Czechia).

Colonies on MEA at 24°C, 28 days slow growing, reaching a diameter of 23-28 mm; compact, flat, centrally heaped or cerebriform showing a tendency to crack, surface slightly velvety, black. Colonies on acidic medium (MEA pH 2) achieving diameters of 20–23.5 mm in 28 days at 24°C; centrally heaped or cerebriform with flat margin, compact, powdery; coloured moderate olive green (5GY4/4), marginally strong greenish yellow (7.5Y7/10); reverse moderate olive (10Y3/4), marginally light olive (10Y5/6). On PDA at 24°C, 28 days, only the strain CCF 4120 was able to grow; centrally cerebriform, yeast-like margin; 16 mm in diam. Mycelium composed of 2–2.3 µm wide, septate, pale to mid-brown hyphae, often converting into swelling cells (2.8–5.0 µm). Conidia produced by fragmentation of hyphae are oblong (8.0–16.0 × 1.7-3.0 µm) sometimes longer or swollen (4.8–18.0 × 1.8-4.8 µm) with truncated ends. *Teleomorph*: Unknown.

Habitat: highly acidic soil (pH < 3)

Distribution: Czech Republic

Holotype: Czech Republic, Western Bohemia, kaolin quarry Mírová, 50°15'00" N, 12°46'12" E, alt. 414 m, isolated from extremely acidic soil (pH 2.2–2.5), May 2007, M. Hujslová, holotype PRM 859933 (dried ex-type culture CCF 4119); isotype PRM 859934

Other specimens examined: CCF 4118, CCF 4120, CCF 4121, CCF 4122, and CCF 4123 (PRM 859935, PRM 859936)

Based on the LSU and LSU-SSU trees, *Teratosphaeria acidotherma* clustered in a well-defined clade as a sister species with *Acidomyces acidophilus* (Fig. 3, 4). Moreover, this clade was located at a large distance from other *Teratosphaeria* species including ex-type species *T. fibrillosa* Syd & P. Syd. (Fig. 3, 4). According to the natural generic concept established in this group by Crous et al. (2009a), we propose using the generic name *Acidomyces* for *Teratosphaeria acidotherma*.

Acidomyces acidothermus (A. Yamaz., K. Toyama & Nakagiri) Hujslová & M. Kolařík, comb. nov. MB 564520
Basionym: *Teratosphaeria acidotherma* A. Yamaz., K. Toyama & Nakagiri 2010, Mycoscience 51(6): 444
Description: Yamazaki et al. (2010)

Growth at different pH levels and temperature preference test

All tested isolates were capable of growing over a pH range from 2 to 8 with optimum growth at pH 4. None of the strains grew at pH 1 (Fig. 6). Only two of the six isolates of *Acidomyces acidothermus* grew at 37°C (Table 3). None of the isolates of *Acidomyces acidophilus* were capable of growing at 37°C (Table 3).

Discussion

Based on LSU rDNA phylogeny, the organisms most closely related to the newly described species *Acidiella bohemica* are two rock-inhabiting meristematic isolates of Teratosphaeriaceae (GU250393, GU250363) collected in the Alps and Antarctica (Ruibal et al. 2009) (0.98/81% bootstrap support) (Fig. 3). The LSU-SSU trees generated here show that the most related species to *Acidiella bohemica* is an uncultured eukaryote clone (RT3n2) from the highly acidic Tinto River in Spain (Amaral Zettler et al. 2002) (0.99/98% bootstrap support) (Fig. 4). Other closely related fungi belong to the poorly resolved group of mainly

rock-inhabiting meristematic species, including three fungal species isolated from Antarctica (*Friedmanniomyces endolithicus*, *Recurvomyces mirabilis* and *Elasticomyces elasticus*) (Selbmann et al. 2005, 2008) and three unidentified fungal isolates of Teratosphaeriaceae (GU250351, GU250318, and GU350240) isolated from the Alps and Antarctica (Ruibal et al. 2009); the Teratosphaeriaceae isolates GU250318 and GU250351 are equivalent to isolates GU250363 and GU250393 (identified above based on similarity with the LSU region). The next nearest relative, *Catenulostroma elginense*, is a leaf-spotting species isolated from living leaves of *Protea grandiceps* from South Africa (Crous et al. 2007). As an inhabitant of the hard, leathery leaves of Proteaceae, *Catenulostroma elginense* is considered to be an extremotolerant species (Crous et al. 2009c). The next nearest relative from this extremotolerant clade, *Catenulostroma chromoblastomycosum* (Fig. 4) was isolated from man with chromoblastomycosis in Africa (Crous et al. 2007). Baddley and Dismukes (2011) have mentioned this fungus as one of the etiologic agents of chromoblastomycosis however no proven report is available on its pathogenic abilities as well as on its ecological demands (Najafzadeh et al. 2009).

The results of the growth test confirmed high adaptability of the tested *Acidiella bohemica* isolates to extreme pH. All tested strains were capable of growth at pH levels below 3 (Fig. 6); therefore, the species *Acidiella bohemica* may be classified as acidotolerant (Zak and Wildman 2004). None of the tested isolates grows at pH 1; however, all isolates were capable of growing at pH 2, which coincides with the pH values of the soil from which they were isolated (Table 1, 2).

Ten isolates found in all six locations in both countries conform to phylogenetic lineage representing the species *Acidomyces acidophilus* (ex-type AJ244237, FJ430711), which has been isolated from various highly acidic environments (pH < 3) worldwide (Selbmann et al. 2008; Hujslová et al. 2010) (Fig. 3, 4).

According to phylogenetic analysis of ITS-LSU rDNA sequences, six isolates from four locations in both countries belong to a lineage containing another type of acidophilic fungus, *Teratosphaeria acidotherma*, which has been found in extremely acidic hot springs in Japan (Yamazaki et al. 2010) (Fig. 3, 4).

A detailed phylogenetic study was performed using sequences of our isolates from the *Acidomyces acidophilus*/*Teratosphaeria acidotherma* complex together with all related accessible sequences from relevant databases (Table 3). The results we obtained confirmed two separated lineages identical to *Acidomyces acidophilus* and *Teratosphaeria acidotherma* (Fig. 5). Moreover, it was found that three American isolates invalidly named "*Acidomyces*

richmondensis" (AY374298, AY374299, AY374300; Table 3) (Baker et al. 2004), which were directly linked with *A. acidophilus* by Selbmann et al. (2008), in fact belong to the *Teratosphaeria acidotherma* clade (Fig. 5). Two isolates of "*A. richmondensis*" isolated from the acidic lake Caviahue in Argentina (EU090191, EU090192; Table 3) conform to the *A. acidophilus* clade (Fig. 5).

Based on morphology, our *A. acidophilus* and *T. acidotherma* isolates cannot be distinguished; however, it seems that these two species might be differentiated according to temperature preference. Most of the *Teratosphaeria acidotherma* isolates were obtained from high-temperature environments such as hot springs (Yamazaki et al. 2010), geothermal areas in Iceland (Table 1, 2) and hot acid mine drainage (Baker et al. 2004). Moreover, all but one isolate of *Teratosphaeria acidotherma* from our study were isolated from plates incubated at 37°C, whereas all but one isolate of *Acidomyces acidophilus* were isolated from plates incubated at 24°C (Table 2). To determine whether temperature might be used as a distinguishing feature, growth tests at different temperatures were performed. The results obtained did not confirm that temperature preference is a distinguishing characteristic for these species. Thus, *Acidomyces acidophilus* and *Teratosphaeria acidotherma,* which potentially co-occur at the same environmental sites (as was found in this study), might only be distinguished using molecular data (e.g., rDNA sequences) (Table 3).

The clades most related to *Acidomyces* based on LSU rDNA are a soil fungus *Piedraia hortae* (AY016366) and two leaf-spotting *Penidiella*-like isolates (DQ246234, GQ852625) (Fig. 3). However, this result is supported only by MB analyses (0.93); according to ML analyses, *Penidiella*-like isolates were found to be unrelated (data not shown). *Piedraia hortae* is a keratinophilic soil fungus known from tropical areas causes black piedra, the disease characterized by formation of the dark ascomata on hairs (de Hoog et al. 2000). The phylogenetic position of *Piedraia hortae* (Piedraiaceae) within the family Teratosphaeriaceae was confirmed also by Crous et al. (2009) and Ruibal et al. (2011). However as the authors of both papers have noted more taxa and sequences should be added to provide better resolution of these two families.

The LSU-SSU rDNA trees showed that the *Acidomyces* clade is located in a group with the leaf-spotting species *Teratosphaeria suberosa* and several *Readeriella* species (incl. *Cibiessia* and *Nothostrasseria*) (Fig. 4). As inhabitants of living leaves, these species may be regarded as extremotolerant (Crous et al. 2009c); this fact may explain the placement of these species in an acidophilic cluster, as in the case of *Catenulostroma elginense* in the clade *Acidiella*. However, this result is highly supported only by MB analyses (0.79); according to

ML analyses, the relationship between *Acidomyces* and leaf-spotting species it is poorly resolved.

Conclusions

Recent knowledge based on our own data as well as previously published work (Amaral Zettler et al. 2002, 2003; Baker et al. 2004, 2009; Hujslová et al. 2010) suggests that extremely acidic substrata (pH < 3) harbour a poorly diversified mycobiota that is highly different from that found in less acidic habitats. In addition to the three strictly acidophilic fungi *Acidomyces acidophilus* (Selbmann et al. 2008), *Hortaea acidophila* (Hölker et al. 2004) and *Acidomyces acidothermus* (Yamazaki et al. 2010)*,* the acidotolerant fungus *Acidiella bohemica* is also known to inhabit extremely acidic soils. All of these fungi occupy geographically distant sites and show high adaptability to extreme pH values; thus, they may be regarded as indigenous inhabitants of extremely acidic habitats (pH < 3). *Acidiella bohemica*, here described as a new genus and species, as well as *Acidomyces acidophilus* and *Acidomyces acidothermus*, represent well-defined groups within the family Teratosphaeriaceae.

Acknowledgements

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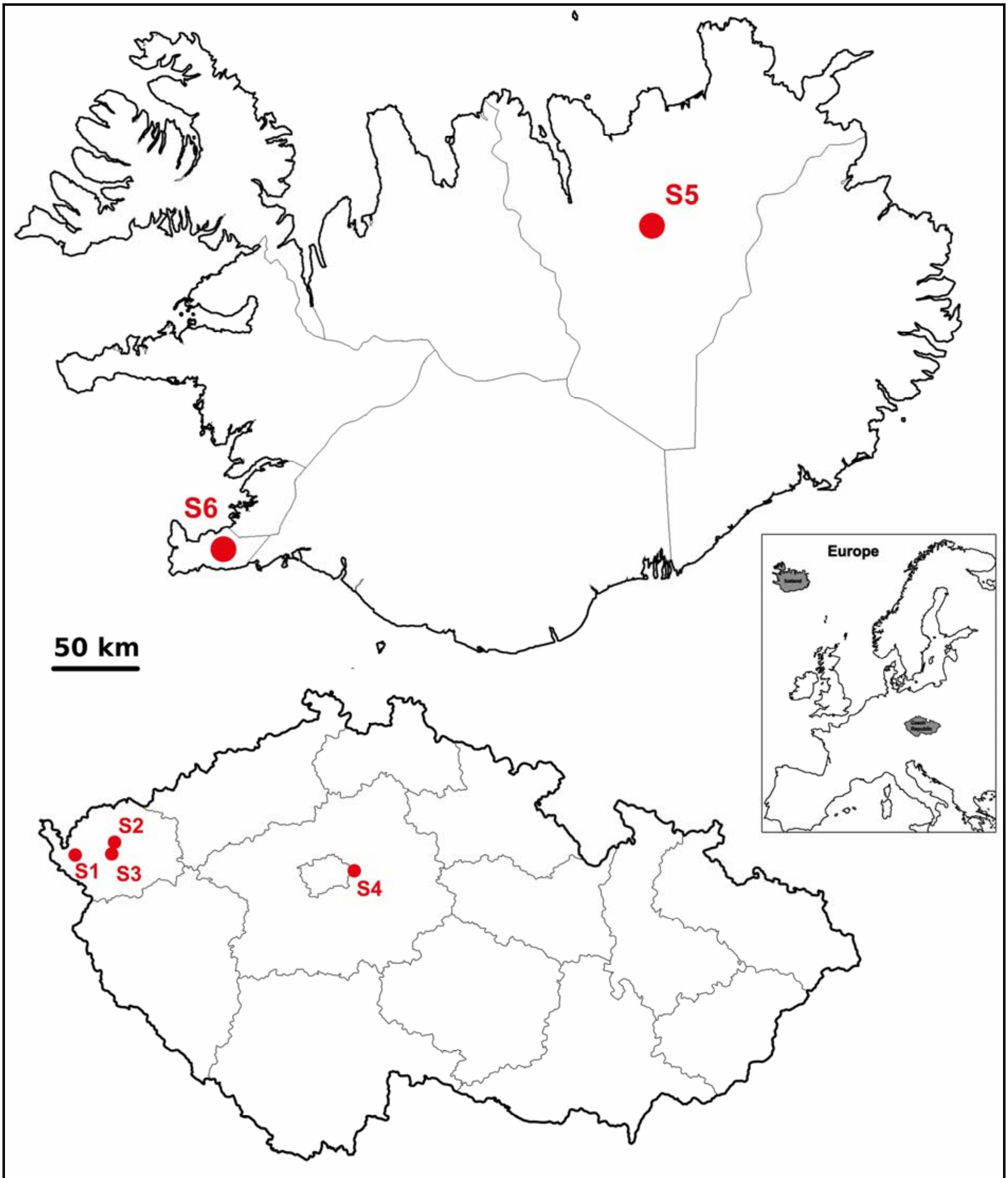


Fig. 1 Map of the Czech Republic and Iceland showing the six sampling sites: S1 - 50°08'60" N, 12°24'00" E, S2 - 50°15'00" N, 12°46'12" E, S3 - 50°15'00" N, 12°46'48" E, S4 - 50°06'36" N, 14°31'48" E, S5 - 65°37'48" N, 16°49'12" W, S6 - 63°55'48" N, 22°06'00" W.

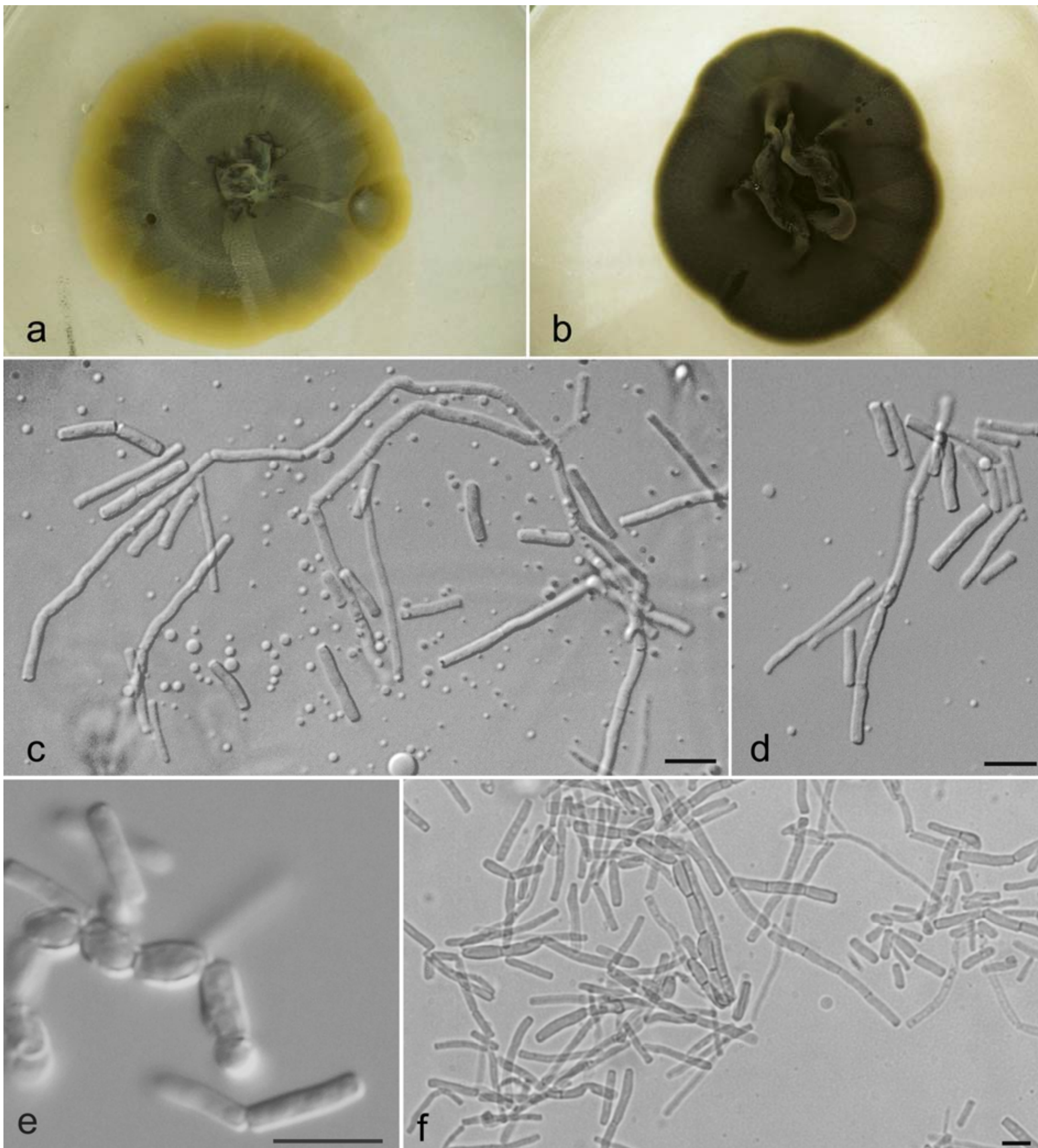


Fig. 2 *Acidiella bohemica*. **a** Colony on MEA pH2 at 24°C, 28 days; **b** Colony on MEA pH 5.5 at 24°C, 28 days; **c, d** Thin walled mycelium fragmented to arthroconidia; **e** Arthroconidia and thick-walled swelling cells; **f** Thin and thick walled mycelium converting into arthroconidia. – Scale bars = 10 μm.

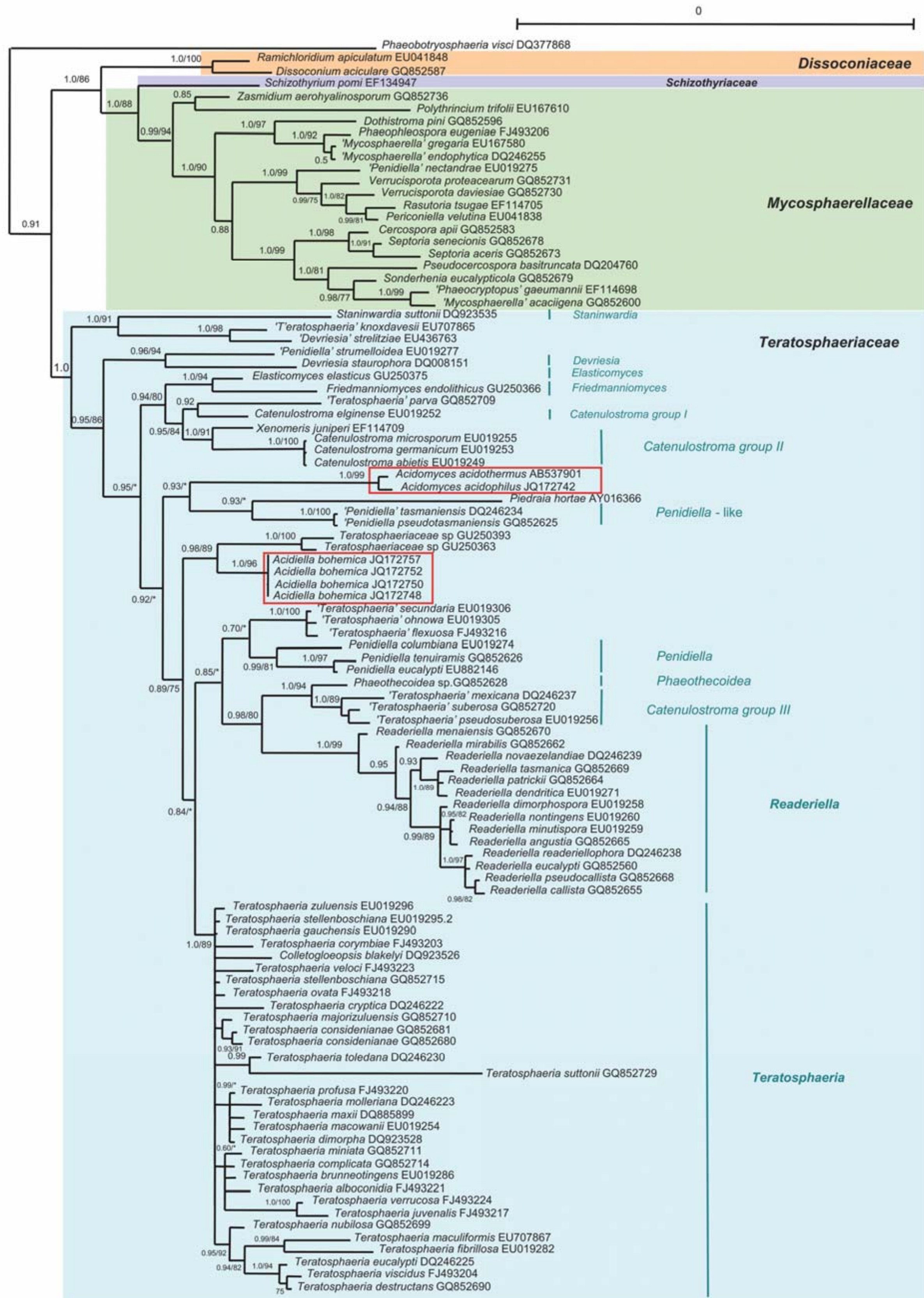


Fig. 3 Phylogenetic tree based on the LSU rDNA sequences. Phylogeny reconstructed using Bayesian inference with Bayesian posterior probabilities (>0.7) noted above individual branches.

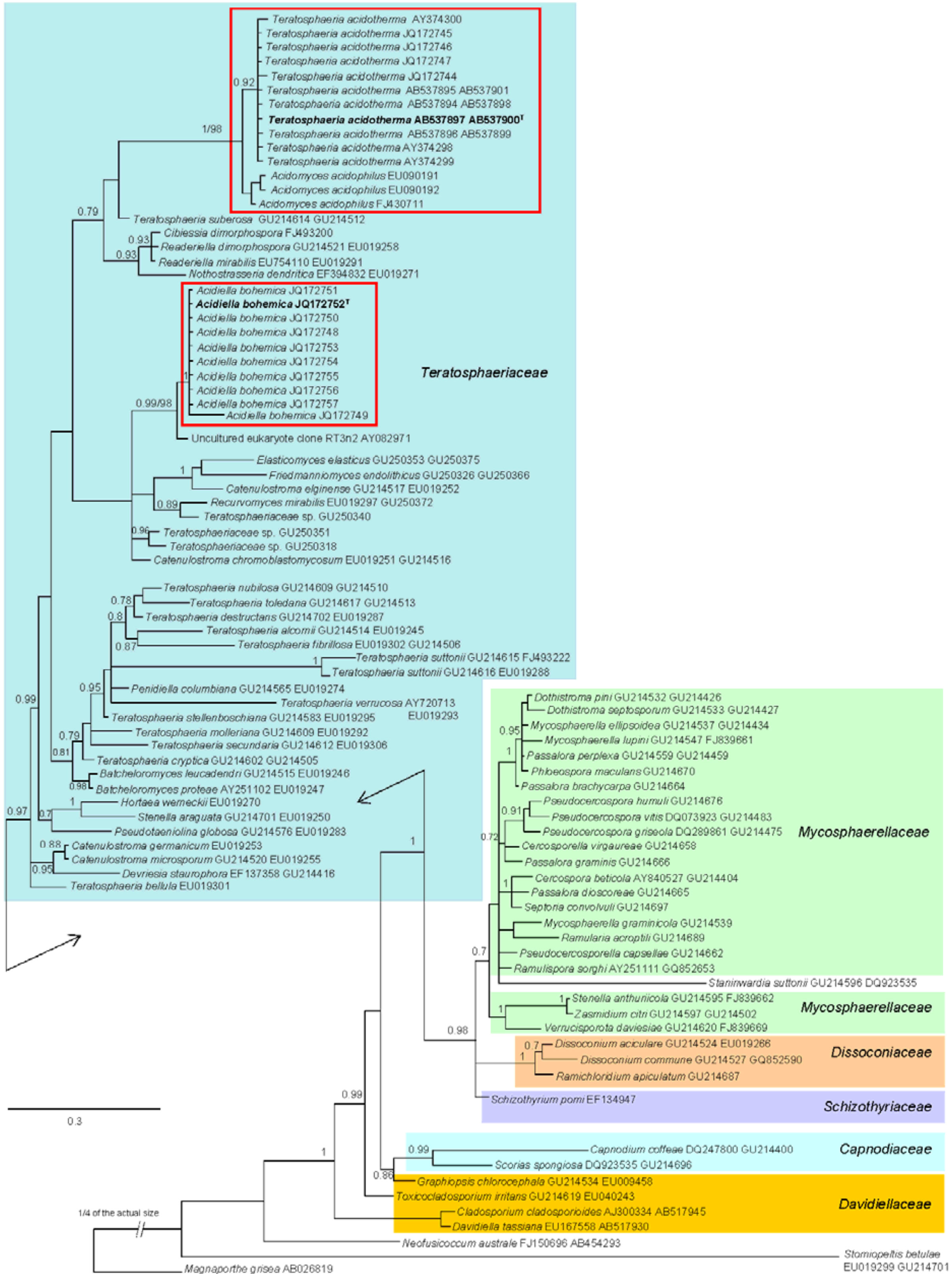
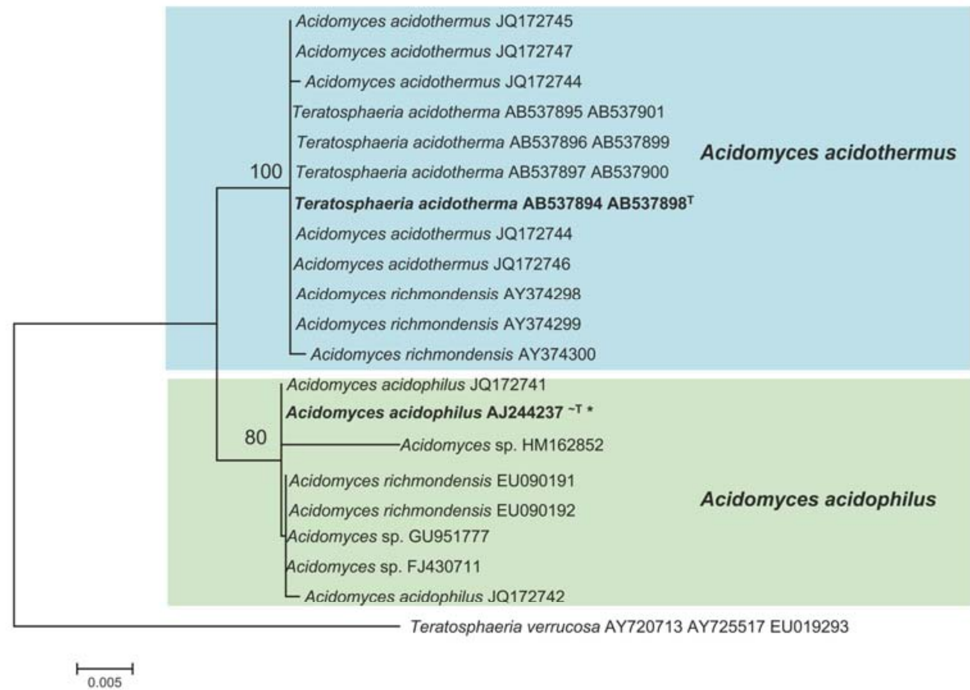


Fig. 4 Phylogenetic tree based on the combined LSU and SSU rDNA sequences. Phylogeny reconstructed using Bayesian inference with Bayesian posterior probabilities (>0.7) noted above individual branches.



Footnotes:
* identical with ex-type of *A. acidophilus* in Selbmann et al. (2008)

Fig. 5 Molecular phylogenetic relationship of *Acidomyces acidophilus* and *Acidomyces acidothermus* obtained from GenBank and from the present work. Input dataset represents a combination of available ITS, LSU and SSU rDNA sequences. Names represent original published names or GenBank descriptions (Table 3). *Teratosphaeria verrucosa* was set as outgroup.

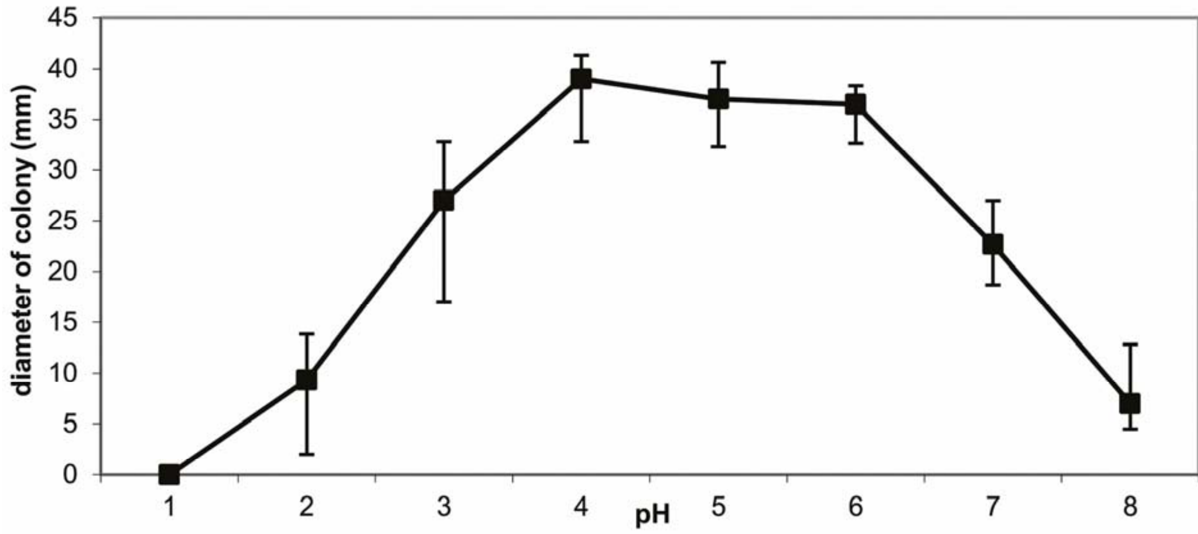


Fig. 6 Growth of the seven strains of *Acidiella bohemica* (Table 2) on MEA at different pH values after 28 days at 24°C.

Table 1 Characterisation of the six sampling sites. Frequency (F %) of isolated fungi was calculated as percentage of positive samples.

Site code	Sampling site	Characterisation	Soil pH	Sampling/No of samples	Frequency %		
					Acidiella bohemica	*Acidomyces acidophilus*	*Acidomyces acidothermus*
S1	Soos National Natural Reserve, Czech Republic	area including peat bogs, mineral fens, salt marshes and highly acidic places	1.3-2	May, November 2007/4	-	50%	-
S2	Mírová, Czech Republic	kaolin quarry with sulfur and humic acids rich brown coal layers	2.2-2.5	May, November 2007/4	100%	100%	50%
S3	Jimlíkov, Czech Republic	kaolin quarry with sulfur-rich brown coal layers	2.9-3	May, November 2007/4	-	50%	25%
S4	Cihelna v Bažantnici National Monument, Czech Republic	area of exposed clay sediments and sulfur-rich brown coal layers	2-2.2	May, November 2007/4	100%	50%	
S5	Námafjall, Iceland	geothermal area	1.1-1.6	July 2007/2	-	100%	50%
S6	Krýsúvik, Iceland	geothermal area	2-2.5	July 2007/2	-	50%	50%

Table 2 List of studied isolates and their accession No; codes of the sampling sites (S1 – S6) are described in Tab 1; M1 – dirrect inoculation of soil, M2 – soil washing technique; MA2 – 2% malt agar, MEA (pH2) – acidified 2% malt extract agar, SEA (pH2) – acidified soil agar with rose Bengal and glucose; MH - personal collection of M. Hujslová; CBS – Centraalbureau voor Schimmelcultures, Utrecht; CCF - Culture Collection of Fungi, Prague; seven strains of *Acidiella bohemica* used for the growth test are in bold.

Species	Strain No.	Sampling site	Isolation method	Isolation medium	Incubation T (°C)	GenBank Accession No. (ITS, LSU, SSU)	Reference
Acidiella bohemica	MH835 = CCF 4118	S2	M2	MA2	24	JQ172750	
Acidiella bohemica	MH857 = CCF 4119 = CBS 132721	S2	M2	MA2	24	JQ172752	
Acidiella bohemica	MH1073 = CCF 4120	S2	M1	SEA (pH2)	5	JQ172753	
Acidiella bohemica	MH1129	S2	M1	SEA (pH2)	24	JQ172756	
Acidiella bohemica	MH1084 = CCF 4121	S4	M1	MEA (pH2)	24	JQ172757	
Acidiella bohemica	MH1131 = CCF 4122	S4	M2	MA2	24	JQ172748	
Acidiella bohemica	MH1160 = CCF 4123 = CBS 132720	S4	M1	MEA (pH2)	24	JQ172754	
Acidiella bohemica	MH1165	S4	M2	MA2	24	JQ172755	
Acidiella bohemica	MHK1	S4	M1	MEA (pH2)	24	JQ172751	
Acidiella bohemica	MH1156	S4	M2	SEA (pH2)	24	JQ172749	
Acidomyces acidophilus	AK72/03 = CCF 3679	S1	-	-	-	FJ430711	Hujslová et al. 2010

Acidomyces acidophilus	MH934	S1	M1	MEA (pH2)	24	JQ172742	
Acidomyces acidophilus	MH1206	S2	M2	MEA (pH2)	24		
Acidomyces acidophilus	MH931	S3	M1	MEA (pH2)	24		
Acidomyces acidophilus	MH933	S3	M1	SEA (pH2)	24		
Acidomyces acidophilus	MH1098	S3	M1	SEA (pH2)	5		
Acidomyces acidophilus	MH1085	S4	M2	MA2	24	JQ172741	
Acidomyces acidophilus	MH1091	S5	M1	MA2	24		
Acidomyces acidophilus	MH1092	S5	M2	MEA (pH2)	24		
Acidomyces acidophilus	MH1109	S5	M1	MA2	24		
Acidomyces acidophilus	MH1102	S6	M1	MEA (pH2)	37		
Acidomyces acidothermus	MH1101	S5	M1	MEA (pH2)	37	JQ172743	
Acidomyces acidothermus	MH1104	S6	M1	MA2	37	JQ172744	
Acidomyces acidothermus	MH1012	S3	M2	MEA (pH2)	5		
Acidomyces acidothermus	MH855 = CCF 4141	S2	M2	MEA (pH2)	37	JQ172745	
Acidomyces acidothermus	MH892 = CCF 4142	S2	M2	MEA (pH2)	37	JQ172746	
Acidomyces acidothermus	MH112 = CCF 4140	S2	M1	SEA (pH2)	37	JQ172747	

Table 3 List of *Acidomyces acidophilus* (AA) and *Acidomyces acidothermus* (AT) isolates used in phylogenetic analysis and the results of the growth tests at different temperatures.

Species	Original name	Strain No.	GenBank Accession No.			Reference	Growth at 24°C	Growth at 37°C
			SSU	ITS	LSU			
AA	***Acidomyces acidophilus***	MH934	JQ172742	JQ172742	JQ172742		+	-
		MH1085	JQ172741	JQ172741	JQ172741		+	-
	Acidomyces acidophilus	CBS 335.97 $^{\sim T}$	-	AJ244237	-	Gimmler et al. 2001		
	Acidomyces sp.	AK72/03 = CCF 3679	FJ430711	FJ430711	FJ430711	Hujslova et al. 2010		
	“Acidomyces richmondensis“	F1	EU090191	-	-			
		F3	EU090192	-	-			
	Acidomyces sp.	GMABH-Mina	GU951777	-	-			
	Acidomyces sp.		-	HM162852	-			
AT	***Acidomyces acidothermus***	MH855 = CCF 4141	JQ172745	-	JQ172745		+	+
		MH892 = CCF 4142	JQ172746	-	JQ172746		+	-
		MH1112 = CCF 4140	-	-	JQ172747		-	-
		MH1101	-	JQ172743	JQ172743		-	-
		MH1104	JQ172744	JQ172744	JQ172744		+	+
		MH1012	-	-	-		+	-
	Teratosphaeria acidotherma	NBRC 106057 ^{T}	AB537894	AB537894	AB537898	Yamazaki et al. 2010		
		NBRC 106058	AB537896	AB537896	AB537899			
		NBRC 106059	AB537897	AB537897	AB537900			
		NBRC 106060	AB537895	AB537895	AB537901			
	“Acidomyces richmondensis“	B1	AY374298	-	-	Baker et al. 2004		
		A3-7	AY374299	-	-			
		C2	AY374300	-	-			

2.3 Paper III

Hujslová M, Kubátová A, Kostovčík M, Blanchette RA, de Beer ZW, Chudíčková M, Kolařík M (2014) Three new genera of fungi from extremely acidic soils. Mycol Prog 13:819-831

Three new genera of fungi from extreme acidic soils

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Abstract

Extremely acidic soils (pH < 3) harbour poorly diversified mycobiota that are very different from less acidic habitats. During investigations of the mycobiota from several highly acidic soils in the Czech Republic and a coastal site in the Antarctic Peninsula, a group of hyaline fungal isolates was obtained. Based on phenotype and nuclear ribosomal DNA sequences (ITS region, SSU, LSU), the isolates belonged to three phylogenetic lineages within two different classes Sordariomycetes and Leotiomycetes (Pezizomycotina, Ascomycota). The first lineage is described here as a new genus and species *Acidothrix acidophila* gen. nov. et sp. nov. (Amplistromataceae, Sordariomycetes, Ascomycota). The most closely related species to this new clade are wood-inhabiting fungi. The isolates belonging to the second and the third lineages are also described as two new genera and species *Acidea extrema* gen. nov. et sp. nov. and *Soosiella minima* gen. nov. et sp. nov. (Helotiales, Leotiomycetes, Ascomycota). Their position and the relationships within Helotiales are discussed. *Soosiella minima* was acidotolerant, *Acidothrix acidophila* and *Acidea extrema* exhibited both acidotolerant and acidophilic characteristics. All the species were slightly halophilic. The

adaptation of hyaline fungi from mesophilic lineages to highly acidic environments has been revealed. The association between highly acidic and Antarctic habitats is discussed.

Keywords
Amplistromataceae, micromycetes, acidophilic, *Acidomyces*, *Acidiella*, Helotiales

Introduction

Highly acidic habitats (pH < 3) represent some of the most extreme environments for microbial growth. Despite the extreme conditions, these habitats harbour highly diversified microbial communities in which fungi represent an abundant and important component (López-Archilla and Amills 1999; López-Archilla et al. 2001, 2004; Amaral Zettler et al. 2002, 2003, 2013; Baker et al. 2004, 2009). Only fragmentary data are available on fungal diversity and their role within acidophilic microbial communities, but it is apparent that mycobiota of highly acidic substrates are different from less acidic habitats and are dominated by a small number of mainly dematiaceous fungal species (Amaral Zettler et al. 2002, 2003; Baker et al. 2004, 2009; López-Archilla et al. 2004; Hujslová et al. 2010, 2013). To date, only three strictly acidophilic fungi *Acidomyces acidophilus* (Selbmann et al. 2008), *Hortaea acidophila* (Hölker et al. 2004) and *Acidomyces acidothermus* (Yamazaki et al. 2010; Hujslová et al. 2013) have been identified. Taxonomically, all these black meristematic fungal species, together with the acidotolerant fungus *Acidiella bohemica* (Hujslová et al. 2013), another species typical of extremely acidic soils, belong to the family Teratosphaeriaceae (Capnodiales, Dothideomycetes, Ascomycota).

In the present study, we were interested in the comparative analysis of the mycobiota inhabiting extremely acidic soils (pH < 3) primarily in geographically isolated localities of small areas in the Czech Republic. During our investigation a group of hyaline isolates were obtained. Based on phenotype and nuclear ribosomal DNA (ITS region, SSU, LSU) sequences, the isolates were placed in Amplistromataceae (Sordariomycetidae, Sordariomycetes) and in two phylogenetically isolated positions within Helotiales (Leotiomycetes).

The family Amplistromataceae has been established for two genera, *Amplistroma* and *Wallrothiella,* of exclusively wood-inhabiting fungi with similar morphological characteristics and acrodontium-like asexual morphs (Huhndorf et al. 2009). Based on molecular data (LSU rDNA sequences) the family was found to be monophyletic,

nevertheless its position within Sordariomycetidae was not resolved, so it was referred to as *incertae sedis* (Huhndorf et al. 2009).

The order Helotiales represents the largest and the most diverse group in the Leotiomycetes encompassing plant pathogens, endophytes, nematode-trapping fungi, mycorrhizae, ectomycorrhizal parasites, fungal parasites, terrestrial saprobes, aquatic saprobes, root symbionts and wood rot fungi (Wang et al. 2006a,b). Due to the limited knowledge about interconnections between asexual and sexual morphs, the systematics of the Helotiales is complicated (Wang et al. 2006a,b). Based on rDNA sequences some clades were recognized with substantial support within the Helotiales but the monophyly of the Helotiales as well as the most helotialean families has not been (Wang et al. 2006a,b). Thus more data from the rDNA regions and protein-coding genes, wider sampling from all families recognized in the Helotiales and the Leotiomycetes as well as molecular data from environmental samples are needed for a more comprehensive view within the Helotiales (Wang et al. 2006a,b).

In the present paper, one new fungal genus within the family Amplistromataceae and two new genera within the order Helotiales are described and their growth responses to different pH values and salt concentrations are determined.

Materials and methods

Sampling, isolation, morphological and cultural characterization

Sixteen samples of extremely acidic soil (pH < 3) were collected from four sampling sites in the Czech Republic in May and November 2007 (Fig. 1, Table 1). The samples were processed using two methods and three types of isolation media. The methods were direct inoculation of soil (M1) (Fassatiová 1986) and the soil washing technique (M2) (Kreisel and Schauer 1987), and the media were 2% malt agar (MA2), acidified 2% malt extract agar (MEA-pH2) and acidified soil agar with rose Bengal and glucose (SEA-pH2) (Pitt 1980; Fassatiová 1986). The pH of the MEA and SEA was adjusted to 2 with concentrated H_2SO_4. SEA was prepared from the substrata of the respective sampling site. Streptomycin was added to all media (0.1 g/l) to suppress bacterial growth. The plates were incubated at 5°C, 24°C and 37°C. After 7-14 days, the emerging colonies were transferred to identification media.

All measurements and observations were performed using fungal structures grown for 14 days on MEA and incubated in the dark at 24°C. Other media used for colony description were malt extract agar (MEA-pH2) and potato carrot agar (PCA) (Fassatiová 1986). Colour

codes were determined according to the Munsell System (1966). Slides were mounted in water and observed using light microscopy.

Strains examined

Cultures from 84 hyaline fungal isolates obtained from highly acidic soil (pH < 3) outlined above, four strains previously isolated from the same substrate and reported by Hujslová et al. (2010) and a strain SH26-1 isolated from alkaline coastal soil on Snow Hill Island, Antarctica, were studied in the present paper. Culturing procedures for the isolate from Antarctica were previously reported (Arenz and Blanchette 2009). The ex-type and other representative strains have been deposited in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS), Culture Collection of Fungi (CCF), Department of Botany, Faculty of Science, Charles University in Prague, Czech Republic or in the personal collection of the first author (code MH) (Table 2).

DNA and molecular phylogenetic analyses

Genomic DNA was isolated from 14- to 28-day-old cultures using a Microbial DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA USA). RAPD fingerprinting was performed with primers 8F and 10R as described in Hujslová et al. (2010). The ITS1, 5.8S, and ITS2 regions of the rDNA, together with partial LSU rDNA, were amplified using primer pairs ITS1, ITS5 (White et al. 1990) or ITS1F (Gardes and Burns 1993) and NL4 (O´Donnel 1993) or LR6 (White et al. 1990). The SSU rDNA gene was amplified using primers NS1, NS17, NS4 and NS24 (White et al. 1990), NSSU1088R and NSSU1088 (Kauff and Lutzoni 2002). The amplification protocol was the same as in Pažoutová et al. (2012). Custom purification of the PCR products and sequencing of the DNA was performed at Macrogen (Seoul, Korea) using the same primers listed above together with NS2 (White et al. 1990) and NL1 (O´Donnel 1993). GenBank accession numbers are provided in Table 2. Sequence manipulations were done in BioEdit v. 7.0.4.1 software (Hall 1999). A BlastN similarity search (Altschul et al. 1997) was used to find similar sequences in the GenBank database. Two DNA sequence datasets were prepared. The first consisted of LSU rDNA sequences and was used to resolve the placement and phylogenetic relationships of the first group of our isolates within the Sordariomycetes. Taxon selection was patterned on the dataset of Huhndorf et al. (2009). The second dataset consisted of SSU and LSU sequences of Helotiales and other major groups in the Leotiomycetes and was based on the dataset published by Wang et al. (2006a) (TreeBase No. M2570). Both alignments were combined with the closest

matches from GenBank. DNA sequences were aligned using the T-coffee web server, and ambiguous positions were subsequently aligned based on Core analysis of local reliability (Notredame et al. 2000; Poirot et al. 2003). The first LSU dataset consisted of 100 sequences, 1333 positions (567 variable and 404 parsimony-informative sites), and the second dataset consisted of concatenated LSU rDNA (116 sequences) and SSU (107 sequences), with 1520 positions (752 variable, and 487 parsimony-informative sites).

Phylogenetic relationships were inferred from the maximum likelihood and Bayesian methods. *Scutellinia scutellata* and *Neolecta irregularis* were used as outgroups. For both LSU rDNA and a dataset containing concatenated LSU and SSU rDNA, the model of molecular evolution for each separate alignment was assessed using jModelTest (Guindon and Gascuel 2003; Posada 2008). This analysis showed GTR to be the most relevant model, with site-to-site rate variation approximated with a gamma distribution and an estimated proportion of invariable sites. For the likelihood analysis, we used a fast bootstrapping algorithm (Stamatakis 2006) in RAxML (version 7.2.7) conducted on the CIPRES Science gateway Web server (RAxML-HPC2 on TG) (Miller et al. 2010). For the Bayesian analysis, we used MrBayes (version 3.1.2) (Huelsenbeck and Ronquist 2001) run on the same server used above (MrBayes on TG) with 10,000,000 generations, sampling trees every 1000 generations and discarding the first half of the trees as a burn-in. The convergence of two runs with four chains was evaluated by Tracer v. 1.5.0 (Rambaut and Drummond 2003). The resulting files were then combined and a 50% majority-rule consensus tree was computed.

Growth at different pH levels

The effect of pH on the growth of *Acidothrix acidophila* (4 isolates), *Acidea extrema* (5 isolates) and *Soosiella minima* (1 isolate) was determined by measuring colony diameter on MEA (Table 2). Eight different pH values ranging from 1 to 8 were used. To permit polymerisation of the agar at pH 1, twice the amount of agar was added. The pH of the medium was adjusted with concentrated H_2SO_4 or NaOH after sterilisation. The triplicate plates were inoculated with mycelial segments, incubated at 24°C and measured after 14 days.

Growth at different NaCl concentrations

The effect of NaCl on the growth of *Acidothrix acidophila* (2 isolates), *Acidea extrema* (5 isolates) and *Soosiella minima* (1 isolate) was determined by measuring colony diameter on MEA (Table 2). Four NaCl concentrations 0.2M (12g NaCl/l), 0.5M (29g NaCl/l), 2.5M (146g NaCl/l) and 5M (303g NaCl/l) were used according to the scale describe by Kusher

(1978). The triplicate plates were inoculated with mycelial segments, incubated at 24°C and measured after 14 days.

Results

The 89 hyaline fungal isolates studied were divided into three groups using phenotype and RAPD fingerprinting. Selected isolates from each group were characterised by analysis of rDNA sequences (Table 2). Based on the phylogeny data, the isolates were placed in three phylogenetically isolated lineages within two different fungal classes, the Sordariomycetes and Leotiomycetes.

The first lineage represented by the first group (nine isolates) was accommodated within the family Amplistromataceae (Fig. 2). The ITS and LSU rDNA sequences showed the closest similarity to an isolate of *Amplistroma ravum* Huhndorf, A.N. Mill., M. Greif & Samuels (ITS rDNA 95% and LSU rDNA 93% similarity; FJ532378). Based on SSU rDNA sequences our isolates were closely related to *Ascitendus austriacus* (Réblová, Winka & Jaklitsch) J. Campb. & Shearer (GQ996542) and one unidentified sordariomycetaean isolate (EF622536) (96% similarity).

The second and the third lineage (77 and three isolates, respectively) were found to be in two distinct phylogenetic lineages within the order Helotiales (Fig. 3). The second lineage contained ITS rDNA sequences most closely related to helotialean isolate from the Antarctic Peninsula region (99% similarity; FJ235962). LSU rDNA sequences of our isolates were closely related to several isolates of *Articulospora tetracladia* Ingold (96% similarity; EU998922, etc.). Based on SSU rDNA sequences our isolates were similar to two uncultured clones (RT5in6 and RT3n5) from highly acidic river samples in Spain (99% similarity; AY082984, AY082969 Amaral Zettler et al. 2002). The same similarity (99%) was found with isolates of several bryosymbionts and aquatic helotialean fungi - *Hymenoscyphus* sp. (EU940026, EU940027, EU940025), *Discinella schimperi* (Navashin) Redhead & K.W. Spicer (EU940043, EU940054), *Tricladium patulum* Marvanová (AY357285), *Tetrachaetum elegans* Ingold (EU357280), *Anguillospora filiformis* Greath. (AY178825) and several isolates of *Articulospora tetracladia* (EU998927, etc.).

The third lineage represented by three isolates had ITS rDNA sequences most closely related to endophytic leotiomycetaean isolates (98% similarity; JQ759534, HQ207068, HQ207059). Sequences of LSU rDNA show 98% similarity with the same three isolates (JQ759534, HQ207068 and HQ207059) and one unidentified mycorrhizal isolate (AY394892). The SSU rDNA sequences from this lineage were nearly identical to one of the

published *Hyphodiscus hymeniophilus* sequences (99% similarity; DQ227258) and 96% similar to other GenBank entries from this species (GU727555, DQ227263, GU727551).

Both the MB and ML analyses of the first LSU dataset revealed phylogenetic trees that strongly support the placement of the first phylogenetic lineage, described here as a new genus and species *Acidothrix acidophila* Hujslová & M. Kolařík, forming a group sister to *Wallrothiella congregata* (FJ532374, FJ532375) within the family Amplistromataceae (Fig. 2). Among the nearest neighbours were species of *Amplistroma* (Fig. 2).

The second lineage, forming a separated group in the LSU-SSU rDNA phylogenetical trees, here described as a new genus and species *Acidea extrema* Hujslová & M. Kolařík, clustered in a group with two uncultured clones RT5in6 and RT3n5 (AY082984, AY082969). The closest neighbours were *Articulospora tetracladia* (EU998927 EU998922), *Fontanospora fusiramosa* Marvanová, Peter J. Fisher & Descals (GQ411265), *Varicosporium elodeae* W. Kegel (AY425613), *Tricladium patulum* (AY357285) and *Tetrachaetum elegans* (AY357280) (Fig. 3).

Based on the analysis of the same dataset the third lineage formed a separate group (Fig. 3) here described as a new genus and species *Soosiella minima* Hujslová & M. Kolařík. Relationships among *Soosiella minima* and other species in the tree were not well resolved (Fig. 3).

Taxonomy

Acidothrix Hujslová & M. Kolařík, **gen. nov.** MB 805194

Asexual morph, hyphomycetes. Colonies plane, with abundant aerial mycelium forming floccules and funicules, sporulation abundant, white to slightly salmon (5YR8/2); on acidic medium compact, centrally forming funicules, powdery, white. Conidiophores acrodontium – like, semimacronematous or macronematous. Conidia single, globose, or ellipsoidal to lacrimose, with hilum. Sexual morph unknown, phylogenetic placement in Amplistromataceae.

Etymology: from *acidus* (Latin) "acidic" and *thrix* (Greek) "hair", refers to the occurrence in acidic substrata and its morphological resemblance to the genus *Sporothrix*

Type species: ***Acidothrix acidophila*** Hujslová & M. Kolařík, sp. nov.

Acidothrix acidophila Hujslová & M. Kolařík, **sp. nov.** MB 805424 (Fig. 4)

Colonies on MEA (pH 5.5) at 24°C, 21 days reaching a diameter of 76-77 mm; spreading, with abundant aerial mycelium forming floccules and funicules, sporulation abundant, white to slightly salmon (5YR8/2), reverse honey to ochre (5YR5/10). Colonies on acidic medium (MEA pH 2) achieving diameters of 19–33 mm in 21 days at 24°C; compact, centrally forming funicules, with ruffled margin, powdery, coloured white, reverse cream to beige (7.5YR6/10). On PCA at 24°C in 21 days colonies compact, centrally heaped with flat margin, without aerial mycelium, yeast-like; reaching 15-20 mm in diam. Conidiophores semimacronematous consisting of a single phialide only, or macronematous consisting of stipe bearing 2-6 phialides, sometimes in verticilate arrangement (prostrate). Stipe 10-20 × 2.5-3.0 μm. Phialides proliferating sympodially forming a long rachis, 35-100 × 1.0-1.5 μm. The first conidium on the phialide is larger, (3.7-) 4.3 (-6.1), sometimes proliferating by hyphae and bearing other conidia. Subsequent conidia, formed on the proliferating conidiogenous cells are single, globose, or ellipsoidal to lacrimose, with hilum, sometimes budding, giving rise to another conidium (2.8-) 3.5-4.0 (-4.5). Vegetative hyphae 2-3 μm wide, non granular. Sexual morph unknown.

Etymology: from Latin *acidus* "acidic" + Greek *philos* "loving", refers to its physiological abilities

Habitat: highly acidic soil (pH < 3)

Distribution: Czech Republic

Holotype: Czech Republic, Western Bohemia, The Soos National Natural Reserve, 50°08'60" N, 12°24'00" E, alt. 437, from soil (pH 1.8), August 2005, izol. M. Hujslová, holotype PRM 922615 (dried ex-type culture CBS 136259); isotype PRM 922616

Other specimens examined: The above description is based on ex-type strain. Other isolates CCF 3800, CCF 4344, CCF 4565, MH 1036 had the same morphology

Acidea Hujslová & M. Kolařík, **gen. nov.** MB 805195

Asexual morph, hyphomycetes. Colonies compact, in some isolates with ruffled margin, centrally heaped to cerebriiform, wrinkled, funiculose or yeast-like, white to beige (10YR7/4). Mycelium sterile, 2.2-.5.5 µm wide, sparsely branched, fully filled with single line of granules, often fragmenting. Sexual morph unknown, phylogenetic placement in Leotiomycetes.

Etymology: Acidea refers to the occurrence in acidic substrata

Type species: ***Acidea extrema*** Hujslová & M. Kolařík, sp. nov.

Acidea extrema Hujslová & M. Kolařík, **sp. nov.** MB 805425 (Fig. 5)

Colonies on MEA (pH 5.5) at 24°C, 21 days reaching a diameter of 17-36 mm, on acidic medium (MEA pH 2) achieving diameters of 17.5-23 mm in 21 days at 24°C. On both media colonies compact, in some isolates with ruffled margin, centrally heaped to cerebriform, wrinkled, funiculose or yeast-like, white to beige (10YR7/4), reverse honey to ochre (7.5YR5/10). On PCA at 24°C in 21 days colonies compact, centrally heaped with flat margin, without aerial mycelium, yeast-like; 25 mm in diam. Mycelium sterile, 2.2-.5.5 µm wide, sparsely branched, fully filled with single line of granules, often fragmenting. Sexual morph unknown.

Etymology: from the adjective *extremus* (Latin) “extreme”, refers to the extreme character of the substrate of origin

Habitat: highly acidic soil (pH < 3)

Distribution: Czech Republic

Holotype: Czech Republic, Western Bohemia, kaolin quarry Mírová, 50°15'00" N, 12°46'12" E, alt. 414 m, from soil (pH 2), May 2007, izol. M. Hujslová, holotype PRM 922617 (dried ex-type culture CBS 136258); isotype PRM 922618

Other specimens examined: The above description is based on ex-type strain. Other isolates CCF 4346, CCF 4348, MH 1185, MH 1288 had the same morphology

Soosiella Hujslová & M. Kolařík, **gen. nov.** MB 805196

Asexual morph, hyphomycetes. Colonies slow growing, compact, heaped, spiny-like, white, reverse beige (10YR4/4). Mycelium sterile, 2.5-4.0 um wide, sparsely branched, irregularly granular. Sexual morph unknown, phylogenetic placement in Leotiomycetes.

Etymology: Soosiella refers to the locality from which the first isolate was obtained (Soos National Natural Reserve, Czech Republic)

Type species: ***Soosiella minima*** Hujslová & M. Kolařík, sp. nov.

Soosiella minima Hujslová & M. Kolařík, **sp. nov.** MB 805447 (Fig. 5)

Colonies on MEA (pH 5.5) at 24°C, 21 days reaching a diameter of 8 mm; slow growing, compact, heaped, spiny-like, white, reverse beige (10YR4/4). On acidic medium (MEA pH2) at 24°C in 21 days no growth of colonies; on PCA at 24°C colonies yeast-like, achieving diameters of 2 mm. Mycelium sterile, 2.5-4.0 µm wide, sparsely branched, irregularly granular. Sexual morph unknown.

Etymology: from *minimus* (Latin) "extremely small" refers to the poor growth abilities

Habitat: highly acidic soil (pH < 3)

Distribution: Czech Republic

Holotype: Czech Republic, Western Bohemia, Soos National Natural Reserve, 50°08'60" N, 12°24'00" E, alt. 437 m, from soil (pH 2), November 2007, izol. M. Hujslová, holotype PRM 922619 (dried ex-type culture CBS 136257); isotype PRM 922620

Growth at different pH levels

The four isolates of *Acidothrix acidophila* were capable of growing over a pH range from 2 to 8 and two isolates (MH 1205 and MH 1036) showed limited growth at pH 1 (Fig. 6). One isolate (MH 560) showed unimodal growth with optimum at pH 3 and three isolates (MH 1036, MH 1205 and MH 1237) exhibit the bimodal growth response with two distinct peaks at pH 3 and 6 (Fig. 6).

One isolate (SH26-1) of *Acidea extrema* was capable of growing over a pH range from 2 to 7 (Fig. 6). Remaining four *Acidea* isolates were able to grow at pH range from 2 to 8 and three of them (MH 1180, MH 1125 and MH 1288) grew at pH 1 (Fig. 6). Three isolates (MH 1180, MH 1288, SH26-1) showed unimodal growth with optimum at pH 6, pH 3 and pH5 respectively and two isolates exhibited bimodal growth curves with two optima at pH 2 and 6 (MH 1125, MH 1185) (Fig. 6).

The tested isolate of *Soosiella minima* grew over a pH range from 3 to 6 with optimum at pH 4 (Fig. 6). No growth was recorded at pH 1, 2, 7 and 8 (Fig. 6).

Growth at different NaCl concentrations

All tested isolates of all three species were able to grow over a range of NaCl concentration from 0 to 0.5M (Fig. 7). None of the isolates were capable of growing at concentrations of 2.5M and 5M (Fig. 7). *Acidothrix acidophila* isolates showed optimum growth in MEA without salt. The isolates of *Acidea extrema* showed different growth optima, ranging from MEA without salt (MH 1125, MH 1288 and SH26-1) to 0.2M MEA (MH 1180, MH 1185). *Soosiella minima* exhibited optimum growth at 0.5M MEA (Fig. 7).

Discussion

Phylogenetic analysis showed placement of *Acidothrix acidophila* in a group of wood-inhabiting fungi that includes *Wallrothiella congregata* and species of *Amplistroma* (Huhndorf et al. 2009; Checa et al. 2012, 2013) (Fig. 2). Delimitation of both genera is based on the morphology of the sexual stage, the asexual stages are not distinctive. The acrodontium-like asexual morphs occurring in *Amplistroma carolinianum*, *A. erinaceum*, *A. longicollis*, *A. ravum* and *Wallrothiella congregata* (Huhndorf et al. 2009; Checa et al. 2012, 2013) are closely similar to the morphology of *Acidothrix acidophila* (Fig. 4). *A. acidophila* is an acidophilic soil fungus and this characteristic is unique among members of the family. This fact, together with its phylogenetic position outside both mentioned genera, warranted placement in a new genus.

The closest relatives of *Acidea extrema* are two uncultured clones (RT5in6 and RT3n5) isolated from highly acidic samples obtained from the Tinto River in Spain (AY082984, AY082969) (Amaral Zettler et al. 2002) (Fig. 3). Other closely related species belong to aquatic fungi, including *Articulospora tetracladia*, *Fontanospora fusiramosa*, *Varicosporium elodeae*, *Tricladium patulum* and *Tetrachaetum elegans* (Fig. 3). Some aquatic hyphomycetes are able to inhabit various extreme environments such as Arctic and subarctic streams, warm sulphur springs or substrates polluted by heavy metals. Nevertheless more data are needed to elucidate the extent of adaptation of these various aquatic fungi to stress factors (Kraus et al. 2011). Although our fungus is sterile and cannot be morphologically compared to these other related fungi, we decided to place it in a new genus because of its different phylogenetic position, ecology and physiology from other related genera.

Based on the results of the same dataset, the third newly described species *Soosiella minima* occurred as an unsupported sister clade to taxa with various morphologies and ecological preferences (Fig. 3). Placement within the Helotiales remains unclear. In general, the tree was not well resolved, support for the backbone was weak and the monophyly of the Helotiales was not resolved (Fig. 3). These results coincide with Wang et al. (2006a,b) who concluded that more molecular data and wider sampling are needed to elucidate the relationships within the Helotiales as well as Leotiomycetes.

The results of the growth test confirmed high adaptability of all three species to extreme pH. All described species were capable of growth at a pH of 3 or lower (Fig. 6) therefore, they may be classified as acidotolerant (Zak and Wildman 2004). From the studied species, *A. acidophila* and *A. extrema* were capable of growing at pH 2, which coincides with the pH values of the soil from which they were isolated except the *Acidea* isolate SH26-1 originating from alkaline soil (Table 1 and 2). Although this isolate was from a coastal Antarctic site where the soil pH was 8.1 (Arenz and Blanchette 2011), it was not able to grow at pH 8 in the laboratory study (Fig. 6). Since this fungus can grow at pH 2 to about pH 8, it appears that other factor(s) or a combination of factors are involved in the ability of this fungus to grow in non-hyperacid Antarctic soils as well as highly acidic soils. It is unclear which factors are most important to influence fungi inhabiting highly acidic substrates. However, two important factors affecting fungal growth in Antarctic soils are carbon and nitrogen content (Arenz and Blanchette 2011). In Antarctic soils, carbon and nitrogen content is minimal (Arenz and Blanchette 2011) and thus limiting for fungi and it seems that in highly acidic soils, where the lack of vegetation cover results in low organic matter, this factor might also be important. However detailed ecological and physiological studies are needed to confirm this assumption.

Three of four *Acidea* isolates and two of four *Acidothrix* isolates were unique among described taxa by their growth at pH 1 (Fig. 6). Only a few fungal species like *Acidomyces acidophilus*, *A. acidothermus* and *Hortaea acidophila* and two unidentified species *Paecilomyces* sp. and *Penicillium* sp. 4 were previously reported to grow at pH 1 (Gimmler et al. 2001; Hölker et al. 2004; Hujslová et al. 2010; Yamazaki et al. 2010).

Most of the isolates of *A. acidophila* and *A. extrema* exhibited bimodal growth curves which coincide with typical acidotolerant characteristics (Gimmler et al. 2001). Two isolates of each species exhibited a unimodal growth curve where the growth optimum shifted to pH 3 (Fig. 6), hence it should be classified as acidophilic (Cavicchioli and Torsten 2000). This phenomenon was also found in *Acidomyces acidophilus* which is classified as strictly acidophilic (Selbmann et al. 2008), however, the isolate exhibited a bimodal growth curve and thus is considered acidotolerant (Gimmler et al. 2001). The phenomenon of bimodal growth curves as the result of hydrogen ion impact was also recorded in several previous studies (Corum 1941; Mehrotra 1964; Verma 1969; Zabel and Morrell 1992; Griffin et al. 1994).

Concerning the salt tolerance, all species tested were able to grow on salinities from 0 to 0.5 M MEA (Fig. 7), therefore they may be classified as slight halophiles which is how many marine fungi have been classified (Kushner 1978). Despite the isolate SH26-1 originating from soil in Antarctica where high salinity (pH 8.1) represents a significant stress factor, no difference in tolerance to NaCl was found among it and the isolates from acidic soils (Fig. 7). This finding coincides with results from Arenz and Blanchette (2011) who confirmed that salinity affects fungi indirectly through its influence on primary producer presence.

All three newly described species were found in two or more highly acidic locations (Table 1 and 2) and all of them showed high adaptability to extreme conditions of the studied substrate (Figs. 6 and 7), but only two species (*Acidothrix acidophila* and *Soosiella minima*) may be considered exclusive inhabitants of highly acidic environments. The third one, *Acidea extrema,* was also isolated from a non-acidic environment in the Antarctic (Table 2), which indicates this fungus can cope with a wider spectrum of extreme factors than other acidophilic and acidotolerant fungi. Moreover these results show that two seemingly very different environments such as those found in Antarctic soils and extremely acidic soils probably share some factor(s) that allow fungal growth to occur under these unusual conditions. The close connection of these two extreme environments was found also by Hujslová et al. (2013) where an exclusive fungal inhabitant of highly acidic soils, *Acidiella bohemica,* was found to be a close relative of fungi isolated from rocks in Antarctica.

Conclusions

Despite the extreme conditions for life found in highly acidic habitats (pH < 3), these environments harbour a fungal community that is different from less acidic habitats and dominated by a small number of fungal species (Amaral Zettler et al. 2002, 2003; Baker et al. 2004, 2009; López-Archilla et al. 2004; Hujslová et al. 2010, 2013). To date, only four meristematic fungal species were known exclusively from extremely acidic habitats, *Acidomyces acidophilus* (Selbmann et al. 2008), *Hortaea acidophila* (Hölker et al. 2004), *Acidomyces acidothermus* (Yamazaki et al. 2010; Hujslová et al. 2013) and *Acidiella bohemica* (Hujslová et al. 2013). In the present study three new genera and species *Acidothrix acidophila*, *Acidea extrema* and *Soosiella minima* with high adaptability to extreme conditions were described. All of these fungi inhabit extreme acidic habitats in geographically distant sites, and together with the four meristematic fungi a core assemblage of the acidophilic fungal community is being elucidated. All but one species within this community are known exclusively from highly acidic substrates. *Acidea extrema* represents the exception showing ability to also populate non-acidic extreme environments and thus has a wider adaptability to extreme conditions. Previously reported acidophilic fungi are typically dematiaceous and belong to the Teratosphaeriaceae, a family comprising a diverse collection of stress-tolerant fungi. In the present work we have revealed the adaptation of hyaline fungi in mesophilic lineages to highly acidic environments.

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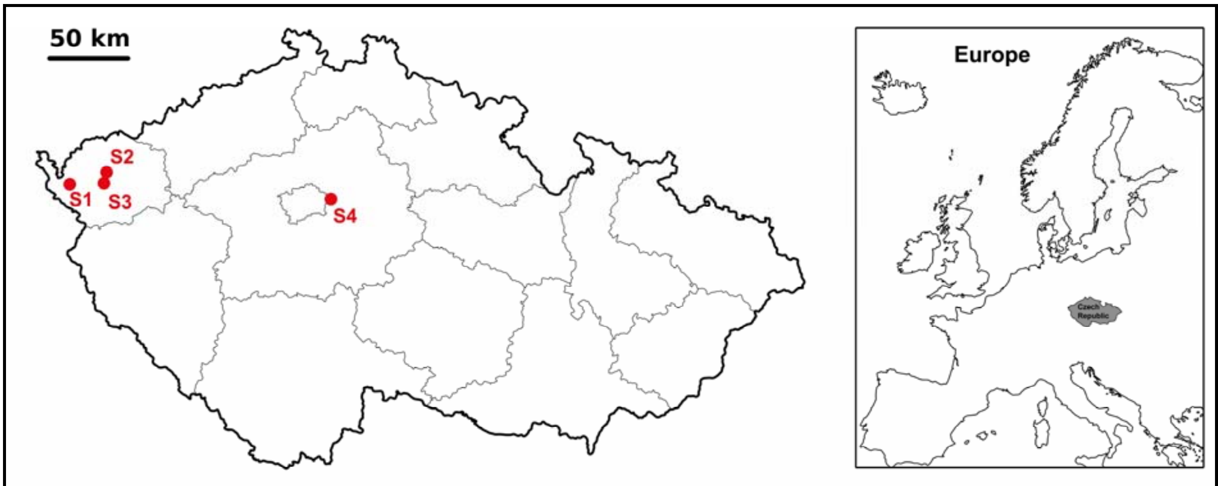

Fig. 1 Map of the Czech Republic showing the four sampling sites: **S1** - 50°08'60" N, 12°24'00" E, **S2** - 50°15'00" N, 12°46'12" E, **S3** - 50°15'00" N, 12°46'48" E, **S4** - 50°06'36" N, 14°31'48" E.

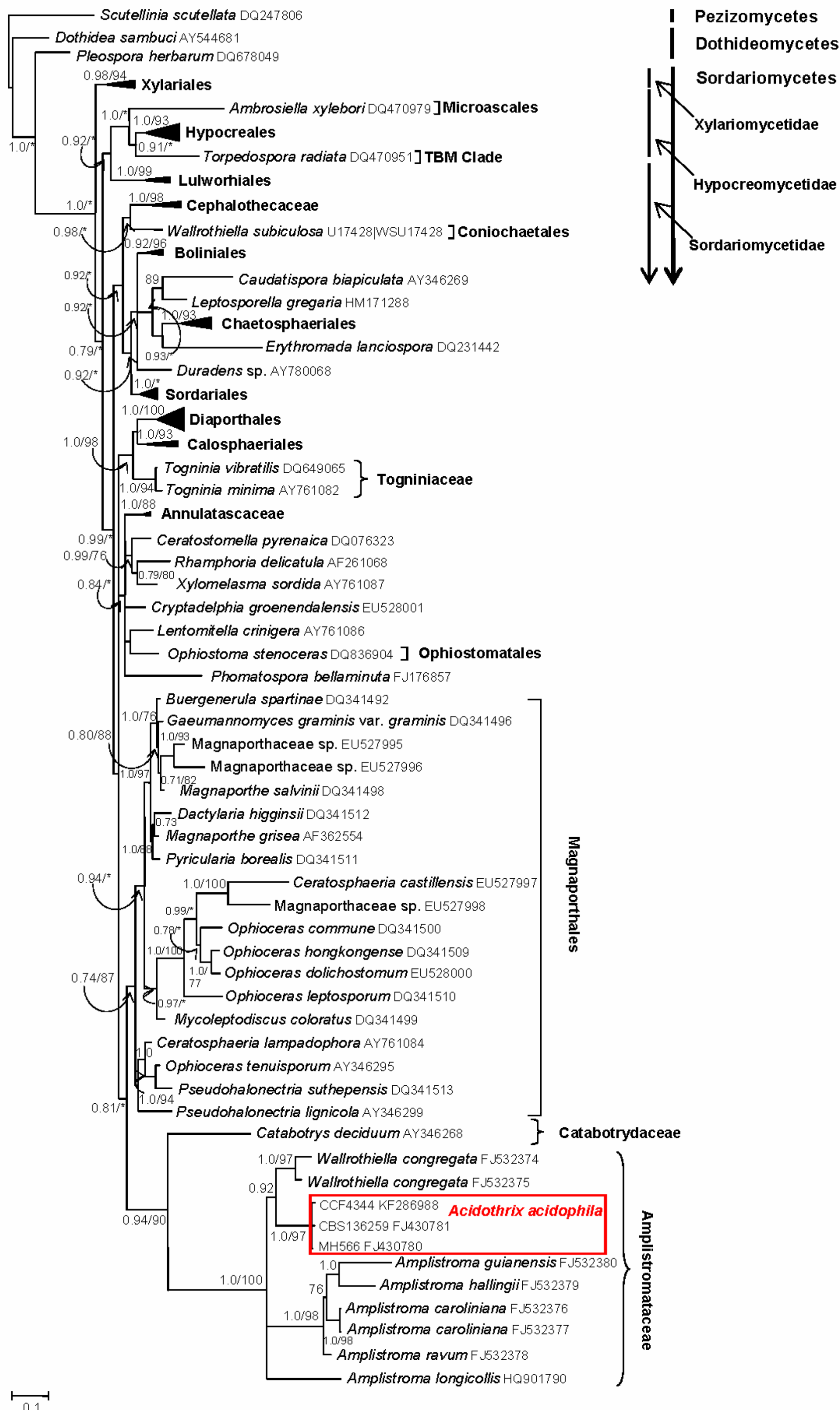

Fig. 2 Phylogenetic tree of Sordariomycetes based on the LSU rDNA sequences. Phylogeny reconstructed using Bayesian inference with Bayesian posterior probabilities (>0.7) noted above individual branches.

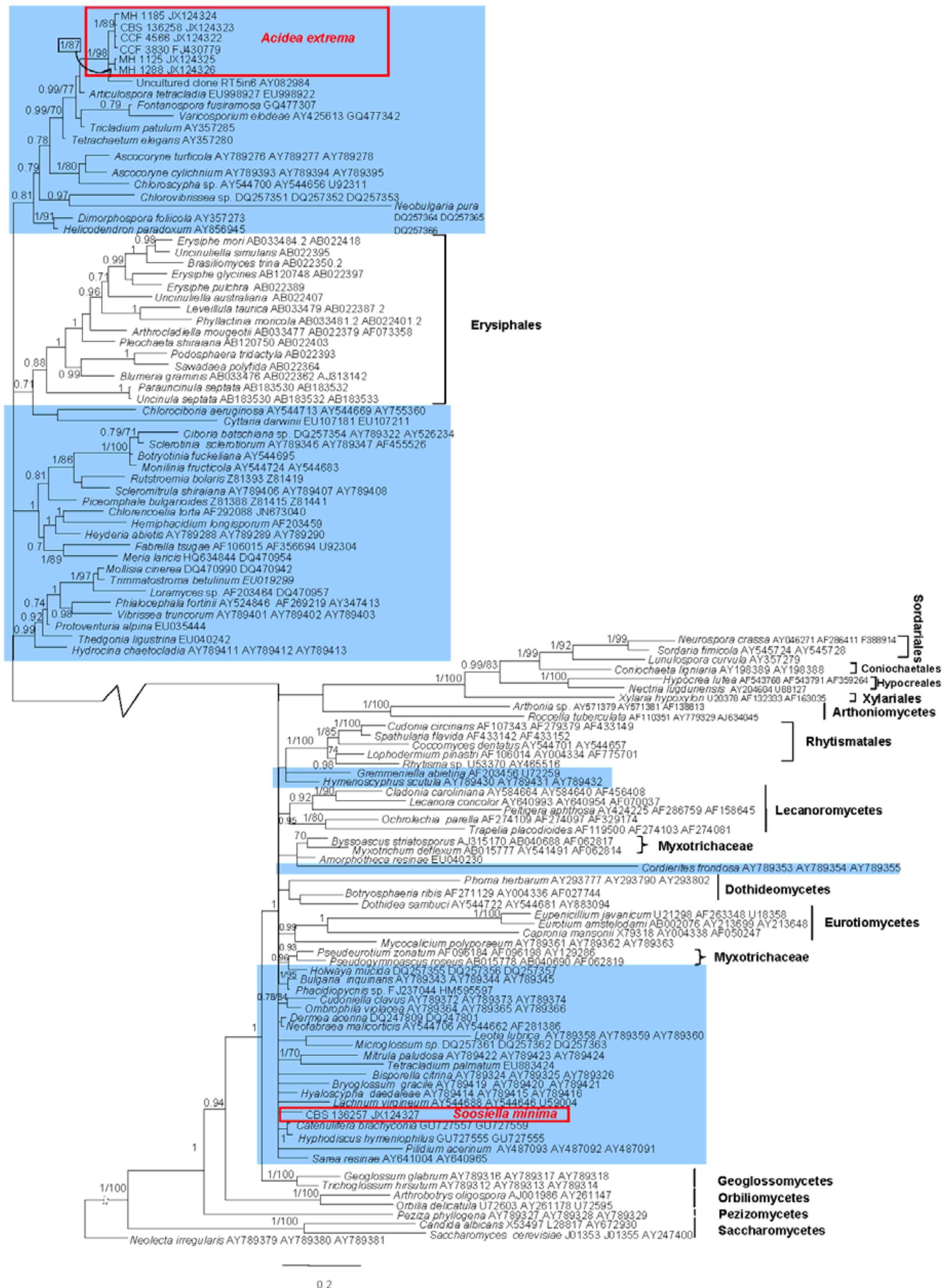

Fig. 3 Phylogenetic tree of Leotiomycetes based on the combined LSU and SSU rDNA sequences. Phylogeny reconstructed using Bayesian inference with Bayesian posterior probabilities (>0.7) noted above individual branches. The blue boxes marked taxa of Helotiales sensu Wang et al. (2006a).

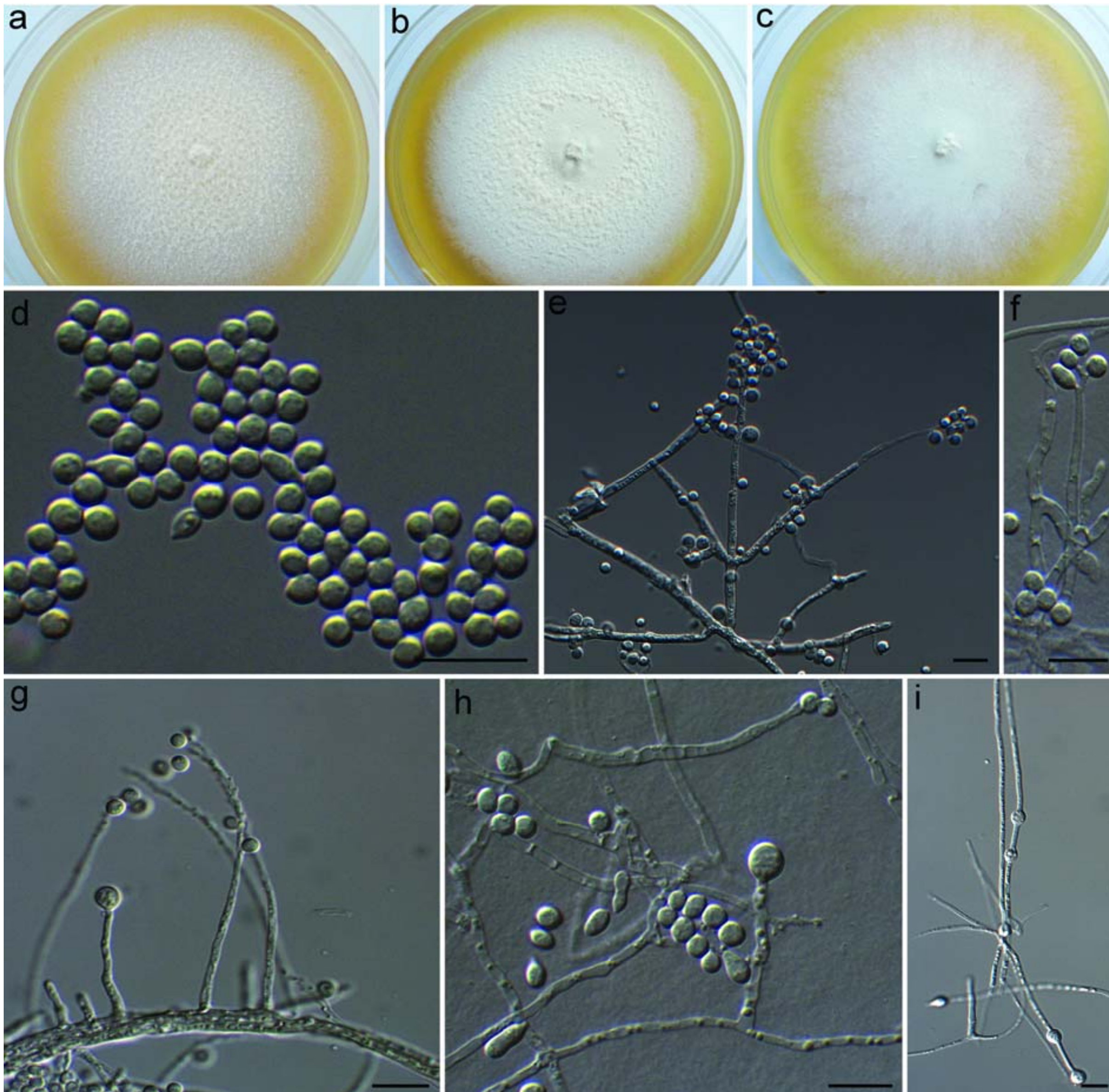

Fig. 4 *Acidothrix acidophila*. **a, b** Colony on MEA pH 5.5 at 24°C, 21 days; **c** Colony on MEA pH 2 at 24°C, 21 days; **d** Conidia globose, elipsoidal or lacrimose with hilum; **e-h** Conidiophores and conidia; **i** Conidia proliferating by hyphae and bearing other conidia. Scale bars = 10 µm.

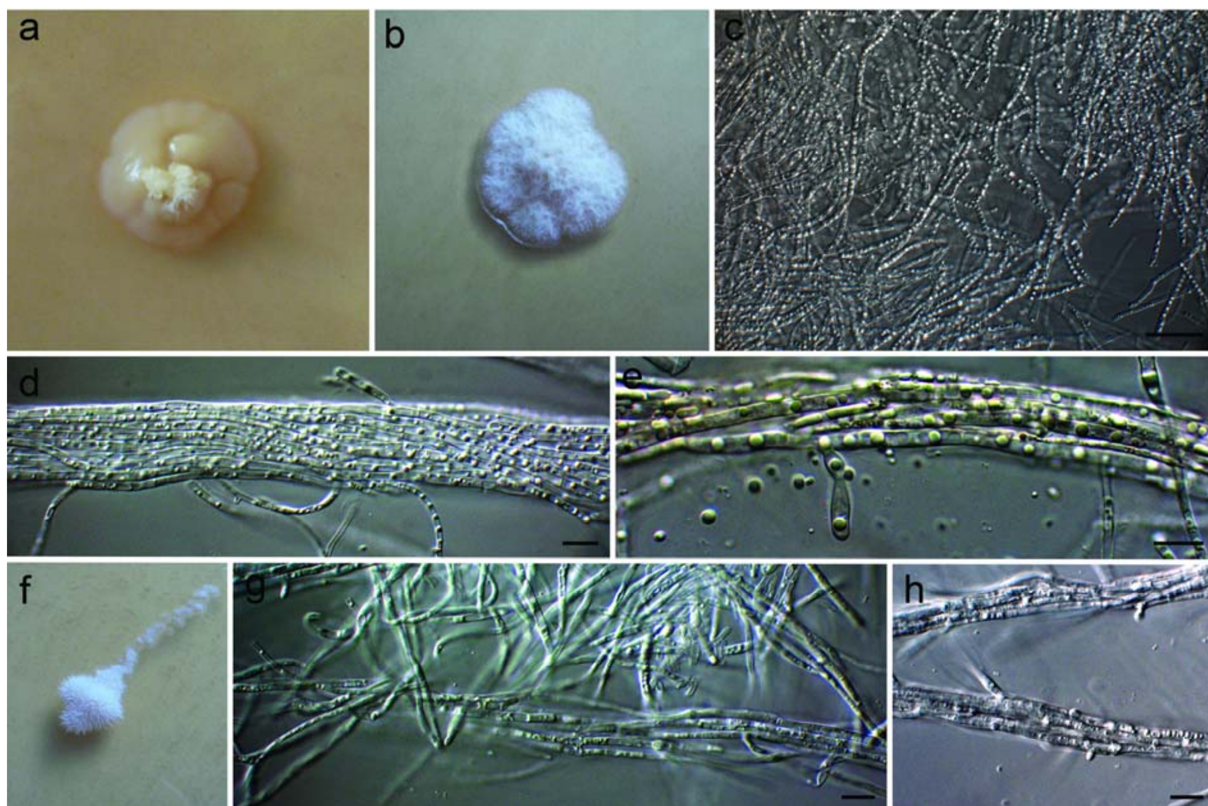

Fig. 5 *Acidea extrema*. a Colony on MEA pH 2 at 24°C, 21 days; b Colony on MEA pH 5.5 at 24°C, 21 days; c, d, e Sterile mycelium fully filled with granules; *Soosiella minima*. f Colony on MEA pH 5.5 at 24°C, 21 days; g, h Sterile mycelium irregularly granular. Scale bars = 10 μm.

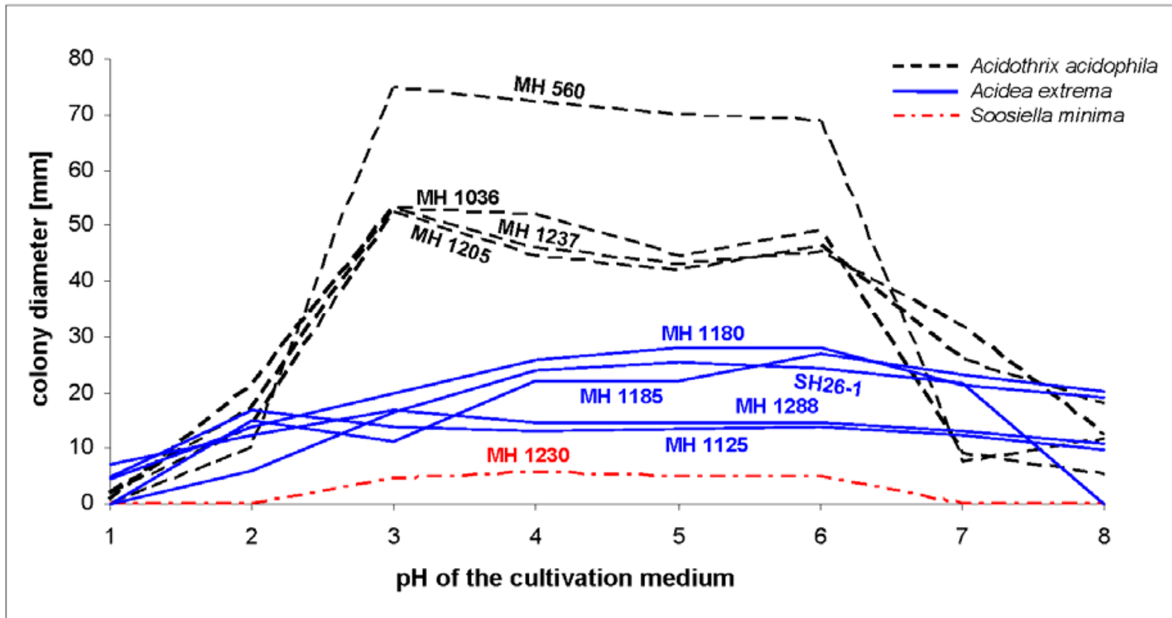

Fig. 6 Growth of the nine strains of Acidothrix acidophila, Acidea extrema and Soosiella minima (Table 2) on MEA at different pH values after 14 days at 24°C.

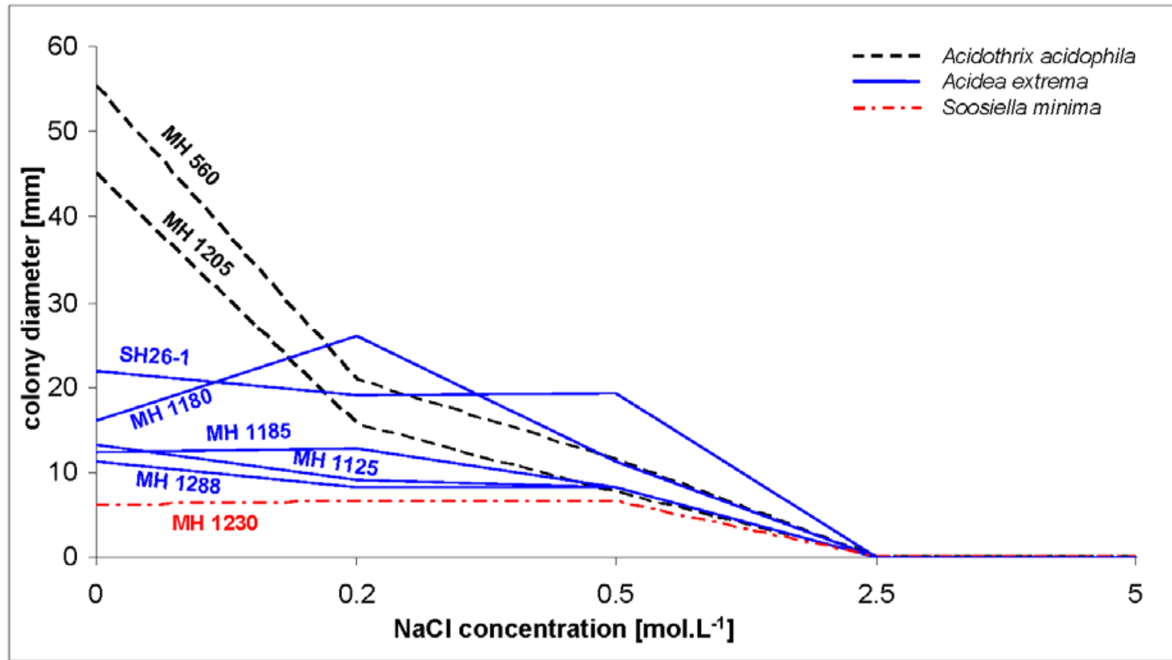

Fig. 7 Growth of the eight strains of *Acidothrix acidophila*, *Acidea extrema* and *Soosiella minima* (Table 2) on MEA with different NaCl concentrations after 14 days at 24°C.

Table 1 Characterization of four sampling sites. Frequency of isolated fungi was calculated as percentage of positive samples. Four samples were analyzed from each site.

Site code	Sampling site	Characterization	Soil pH	Frequency [%]		
				Acidothrix acidophila	***Acidea extrema***	***Soosiella minima***
S1	Soos National Natural Reserve, Czech Republic	Area including peat bogs, mineral fens, salt marshes and highly acidic places with bare soil	1-2	-	25	25
S2	Mírová, Czech Republic	Kaolin quarry with exposed sulfur rich brown coal beds	1.5-2.5	75	100	25
S3	Jimlíkov, Czech Republic	Kaolin quarry with exposed sulfur rich brown coal beds	2-4	-	75	-
S4	Cihelna v Bažantnici National Monument, Czech Republic	Clay quarry with exposed sulfur rich brown coal beds	1-2	-	25	-

Table 2 List of studied isolates and their Genbank accession numbers. Abreviations: S1 – S4, codes of the sampling sites (see Tab. 1); M1 – dirrect inoculation of soil, M2 – soil washing technique; MA2 – 2% malt agar; MEA (pH2) – acidified 2% malt extract agar, SEA (pH2) – acidified soil agar with rose Bengal and glucose; MH - personal culture collection of M. Hujslová; CCF - Culture Collection of Fungi, Prague; ten strains used for the pH growth test are in bold; eight strains used for the salinity growth tests are underlined.

Species	**Strain No. and reference**	**Sampling site**	**Isolation conditions**			**GenBank Accession No. (ITS, LSU, SSU)**
			Method	**Medium**	**t[°C]**	
Acidothrix acidophila	**CBS 136259 (=CCF 3799[1]=MH 560)**	S1	-	-	-	FJ430781
	CCF 4344 (=MH 1205)	S2	M2	SEA (pH2)	24	KF286988
	CCF 3800[1] (=MH 664)	S1	-	-	-	
	CCF 4565 (=MH 1237)	S2	M2	MEA (pH2)	24	
	MH 566[1]	S1	-	-	-	FJ430780
	MH 1036	S2	M2	MEA (pH2)	24	
Acidea extrema	**CBS 136258 (=CCF 4345=MH 1180)**	S2	M1	SEA (pH2)	24	JX124323
	CCF 3830[1] (=MH 72)	S1				FJ430779
	CCF 4346 (=MH 1246)	S3	M2	MA2	5	
	CCF 4348(=MH 1264)	S2	M2	MA2	5	
	MH 1185	S2	M1	MEA (pH2)	24	JX124324
	MH 1125	S2	M2	MA2	24	JX124325

	MH 1288	S2	M2	MEA (pH2)	5	JX124326
	MH 903	S3	M1	MEA (pH2)	5	
	MH 977	S2	M1	MA2	24	
	MH 1277	S3	M2	MEA (pH2)	5	
	MH 1191	S2	M2	MEA (pH2)	24	
	MH 951	S2	M1	MEA (pH2)	5	
	MH 1255	S2	M1	SEA (pH2)	5	
	CCF 4566 (=SH26-1)	Snow Hill Island, Antarctica		Alkaline coastal soils		JX124322
Soosiella minima	**CBS 136257 (=CCF 4350=MH 1230)**	S1	M1	MA2	24	JX124327
	CCF 4575 (=MH 1236)	S2	M1	MEA (pH2)	24	
	MH 1318	S2	M1	SEA (pH2)	5	

2.4 Paper IV

Hujslová M, Bukovská P, Kubátová A, Chudíčková M, Kolařík M. Extremely acidic soils are dominated by species-poor and highly specific fungal communities. Manuscript

Extremely acidic soils are dominated by species-poor and highly specific fungal communities

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Abstract

The diversity of cultivable filamentous microfungi in highly acidic soils (pH < 3) in the Czech Republic was studied. Altogether, sixteen soil samples were collected from four sampling sites and were processed by various approaches. In total, 54 taxa were isolated and identified using classical as well as molecular markers. All dominant species were found both as living mycelia and as resistant stages. Numerous recently described or unknown taxa were isolated. The core of the fungal assemblage under study consisted of phylogenetically unrelated and often globally distributed fungi exclusively inhabiting highly acidic habitats as well as taxa known from less acidic and often extreme environments. The large number of identified specialized species indicates that highly acidic environments provide suitable conditions for the evolution of specialist species. The occurrence of ubiquitous fungi in highly acidic substrates points to the principal role of competition in the colonization of such environments. The detected taxa did not require low pH to survive, because they can grow in a broad range of pH.

Keywords

diversity, Acidomyces, Acidohtrix, Acidea, Acidiella

Introduction

Highly acidic environments (pH < 3) represent some of the most extreme habitats for microbial growth. In addition to high concentrations of hydrogen ions, their inhabitants have to cope with large amounts of heavy metals and dissolved sulfates, low concentrations of dissolved organic carbon, and often extreme temperatures (Brock 1978, Johnson 1998, Zak and Wildman 2004). Despite these inhospitable conditions, extremely acidic habitats harbour highly diversified microbial communities consisting of not only prokaryotes, but also eukaryotes (summarized in Johnson 2012; Amaral-Zettler 2013). Besides algae and protists, fungi have been found to represent an abundant and important component of acidophilic communities (López-Archilla and Amills 1999; López-Archilla et al. 2001, 2004; Amaral Zettler et al. 2002, 2003; Baker et al. 2004, 2009; Gadanho and Sampaio 2006; Bühring et al. 2012; Zirnstein et al. 2012). Fungal hyphae have been found to be the dominant eukaryotic element of acidophilic biofilms. They provide the backbone for these structures, anchor them to sediments and serve as a surface for prokaryotes. In acidophilic food webs, the main role of fungi is decomposition (López-Archilla et al. 2001; Das et al. 2009). Recently, fungi have also been found to play an important role in the metabolism of taurine, one of the key metabolites that likely provides protection from osmotic stress in biofilms (Mosier et al. 2013). However, little is still known about the fungal diversity in extremely acidic environments. Fungi have been studied mostly marginally as a part of microbial or eukaryotic diversity (López-Archilla and Amills 1999; López-Archilla et al. 2001; Amaral Zettler et al. 2002, 2003; Baker et al. 2004, 2009; Gadanho and Sampaio 2006; Zirnstein et al. 2012). Only a few investigations have focused in detail on fungal communities of highly acidic habitats (López-Archilla et al. 2004; Hujslová et al. 2010; Vazquéz-Campos et al. 2014). These studies show that extremely acidic environments harbour low-diversified fungal communities that differ from those inhabiting less acidic habitats. Besides dematiaceous fungal species, which typically inhabit extreme habitats, hyaline fungi have been found to be a frequent component of fungal communities in extremely acidic substrates (López-Archilla et al. 2004; Hujslová et al. 2014). To date, eight fungal species are known to live in extremely acidic habitats: *Acidea extrema*, *Acidiella bohemica* (Hujslová et al. 2014), *Acidiella uranophila* (Vazquéz-Campos et al. 2014, Kolařík et al. 2015), *Acidomyces* acidophilus (Selbmann et al. 2008), *Acidomyces acidothermus* (Yamazaki et al. 2010; Hujslová et al. 2013), *Acidothrix acidophila* (Hujslová et al. 2014), *Coniochaeta fodinicola* (Vazquéz-Campos et al. 2014, *Hortaea acidophila* (Hölker et al. 2004) and *Soosiella minima* (Hujslová et al. 2014). Except for *Acidea extrema,* all of these species inhabit these environments exclusively and have been found to be well

adapted to the extreme conditions of their habitat (Gimmler et al 2001; Hölker et al. 2004; Selbmann et al. 2008; Yamazaki et al. 2010; Hujslová et al. 2013, 2014; Vazquéz-Campos et al. 2014).

Fungi occupying such hostile environments represent a potential source of novel enzymes and other metabolites, and may find uses in bioremediation (e.g. Stierle et al. 2006, 2007, 2012a,b; Selbmann et al. 2013). Four of the acidophilic species mentioned above (*Acidomyces acidophilus*, *Acidomyces acidothermus*, *Coniochaeta fodinicola* and *Hortaea acidophila*) have been studied as possible sources of novel enzymes with enormous biotechnological potential (e.g. Tetsch et al. 2005; Wang et al. 2010; Luo et al. 2010; Zhao et al 2010; Yang et al. 2011; Selbmann et al. 2013; Boonen et al. 2014). *Acidothrix acidophila* has been found to be a suitable organism for genetic transformation, and due to its acidophilic behaviour, it represents a promising candidate for use in biotechnological processes (Hršelová et al. 2015).

In the present study, we focused on the mycobiota inhabiting highly acidic soils (pH < 3) at geographically isolated localities of a small area in the Czech Republic. The main goals of our work were to reveal the diversity of cultivable filamentous fungal species occupying extremely acidic soils and to assess whether there is any similarity among fungal assemblages known from similar substrates worldwide. To cover the broadest range of species, we used two different cultivation methods, three types of isolation media and three incubation temperatures. To maximize the chance of identifying isolated fungal strains, we employed morphological as well as molecular markers. In this paper, we provide an insight into fungal assemblages of highly acidic soils, an environment which might potentially offer new biotechnologically interesting fungi.

Materials and methods

Sampling, isolation, identification

Sixteen samples of extremely acidic soil (pH < 3) were collected from four sampling sites in the Czech Republic in May and November 2007 (Fig. 1, Table 1). The samples were taken from the depth of 5–10 cm, placed into sterile plastic bags and kept at 4°C until processing.

Fungal strains were isolated using two methods and three types of isolation media. The methods were direct inoculation of soil (M1) (Fassatiová 1986) and the soil washing technique (M2) (Kreisel and Schauer 1987). The media were 2% malt agar (MA2), acidified 2% malt extract agar (MEA-pH2), and acidified soil agar with rose Bengal and glucose (SEA-

pH2) (Pitt 1980; Fassatiová 1986). The pH of the MEA and SEA was adjusted to 2 with concentrated H_2SO_4; furthermore, SEA was prepared from the substrata of the respective sampling site. Streptomycin was added to all media (0.1 g/l) to suppress bacterial growth. The plates were incubated at 5°C, 24°C and 37°C. After 7–14 days, emerging colonies were transferred to identification media.

The resulting fungal isolates were taxonomically identified based on both morphological and molecular characteristics. The isolates were divided into groups according to their phenotype and RAPD fingerprinting. Selected isolates from each group were characterized by analysis of rDNA and β-tubulin sequences. Some of the isolated fungi had already been described as new taxa (Hujslová et al. 2013, 2014). Representative fungal strains have been deposited in the Culture Collection of Fungi (code CCF) at the Department of Botany, Faculty of Science, Charles University in Prague, Czech Republic or in the personal collection of the senior author (MH) (Table 2).

DNA analyses

Genomic DNA was isolated from 14- to 28-day-old cultures using a Microbial DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA USA). RAPD was performed with primers 8F and 10R as in Hujslová et al. (2010). Strains with identical RAPD patterns were considered to belong to identical genetic lineages, and representative strains were characterised by their DNA and ß-tubulin sequences. The ITS1, 5.8S and ITS2 regions of rDNA, together with partial LSU rDNA, were amplified using the primer pairs ITS1 and ITS5 (White et al. 1990) or ITS1F (Gardes and Burns 1993) and NL4 (O´Donnel 1993) or LR6 (White et al. 1990). The SSU rDNA gene was amplified using primers NS1, NS17, NS4 and NS24 (White et al. 1990), NSSU1088R, and NSSU1088 (Kauff and Lutzoni 2002). The amplification protocol was the same as in Pažoutová et al. (2012). Partial ß-tubulin gene sequences were amplified and sequenced using primers Bt2a and Bt2b according to the protocol of Glass and Donaldson (1995). Custom purification of the PCR products and sequencing of the DNA was performed at Macrogen (Seoul, Korea) using the same primers as listed above together with NS2 (White et al. 1990) and NL1 (O´Donnel 1993). GenBank accession numbers are provided in Table 2. Sequence manipulations were performed using BioEdit v. 7.0.4.1 (Hall 1999). A Blast similarity search (Altschul et al. 1997) was used to find similar sequences in the GenBank database.

Data analyses

The total frequency of occurrence was calculated as the percentage of all samples (n=16), and the frequency of occurrence at an individual site was calculated as the percentage of samples from an individual site (n=4) in which a particular species was identified (Table 2).The frequency of occurrence was calculated as the percentage of plates processed for an individual sample (n=36) and an individual site (n=144) at which the particular species was identified. These quantitative data were used in all analyses. The analysis of diversity was based on the Shannon-Weaver index (H). To compare the fungal assemblages from the four sampling sites, the Bray and Curtis similarity index (C) was used (Bray and Curtis 1957). The C index ranged from 0 (no overlap between assemblages) to 1 (total overlap). Both indices were calculated using PAST (Hammer et al. 2001). Species accumulation curves and associated 95% confidence intervals were generated for each sampling site using EstimateS (Colwell 2006). Multivariate statistical analyses were conducted using CANOCO 4.5 for Windows (Ter Braak and Šmilauer 1998). Two modified datasets, which were restricted to consider only those species that were recovered at frequencies higher than 6%, were used. The first dataset consisting of presence/absence data was used to estimate the influence of the methodological approaches on the obtained species composition by redundancy analysis (RDA). The second one consisting of abundance data was used to determine the influence of the site on the species composition using distance based redundancy analysis (dbRDA). Principal coordinates analysis (PCoA) was performed to obtain information on the inner structure of the data. Significance levels of the indicator values were calculated by applying a Monte-Carlo permutation procedure (1000 permutations).

Results

Species composition and identification

Out of the 16 soil samples, two samples from the Soos National Natural Reserve did not yield any fungal colony. Out of the remaining 14 samples, 395 isolates were obtained, from which altogether 54 taxa representing 26 genera and three morphological groups of undetermined mycelia were identified. The most frequently isolated genus was *Penicillium* (16 species). Only three taxa, *Acidea extrema* (56%), *Penicillium simplicissimum* s.l. (56%) and *Acidiella bohemica* (50%) were found with the frequency of 50% or more of all samples. Half of all taxa (27) were recorded at frequencies of 6% (one sample) and most of them (24) only as one isolate. In total, 221 plates (39%) of all the 576 plates processed yielded no fungal colony. The highest frequency of plates with no fungal colonies was obtained from the Soos

National Natural Reserve (106) (74%), compared to the (32) 22% plates without fungal colonies from the Mírová quarry (Table 2).

Except for 14 taxa (marked with asterisks in Table 2), taxonomic identification was based on both morphological and molecular characteristics. Out of these 43 taxa, 23 were linked with known species, four of them were described as new genera and species (Hujslová et al. 2013, 2014), but 16 remained unidentified and probably represent undescribed species (Table 2).

Fungal communities at the four sampling sites

Comparing fungal communities among the four sampling sites, site 1 differed from the rest in that it hosted an extremely poor fungal assemblage (Table 2). This finding was confirmed by the result of statistical analysis showing that the sampling site significantly influenced species composition ($p = 0.001$). However, communities from other sites were noticeably similar (Fig 3). The similarity index among the fungal communities of the different sites ranged from 0.1 (site 1 vs. site 3) to 0.5 (site 2 vs. site 3) (Table 3). Two species (*Acidea extrema* and *Acidomyces acidophilus*) were obtained from all sampling sites. Three species (Helotiales sp. 1, *Penicillium simplicissimum* s.l. and *P. spinulosum*) were isolated from three sites, and ten taxa were obtained from two sites. More than half of all species (35 species) were isolated from only one sampling site (Table 2). The large number of rarely recorded taxa at sites 2, 3 and 4 resulted in rarefaction curves that displayed an almost linear slope, indicating no saturation of species richness (Fig 2). The rarefaction curve of site 1 reached the asymptote, showing that our sampling was sufficient (Fig 2). The overlap of 95% confidence intervals (not shown to improve figure clarity) of the rarefaction curves indicate no statistically significant differences in fungal richness observed among the sampling sites. Species richness was highest at site 2 (31 taxa), followed by site 3 (29 taxa), site 4 (21 taxa) and site 1 (6 taxa) (Table 2). The diversity of the fungal species recovered from the sampling sites increased from site 1 ($H = 1.5$) to site 4 ($H = 2.3$), site 3 ($H = 2.5$) and site 2 ($H = 2.6$).

Methodological approaches

The observed fungal spectrum was affected by the incubation temperature and the isolation medium (Table 2, Fig 4, 5). The influence of both factors was statistically significant (temperature: $p = 0.001$; medium: $p = 0.011$). The isolation procedures helped to distinguish fungi present in the soil as resting stages (isolated by M1) and as actively growing stages (obtained by M2) (Table 2).

The most abundant species were isolated using both procedures (M1, M2). A majority of species was obtained using all isolation media (MA2, MEA-pH2, SEA-pH2). None of the taxa were detected at all incubation temperatures (5, 24, 37°C). Frequently isolated species grew successfully at a minimum of two of the incubation temperatures. Species isolated exclusively from a single isolation procedure, medium or incubation temperature belonged to less abundant species (with a maximum frequency of 19 % of all samples) (Table 2).

Discussion

Species composition at four sampling sites

The fungal communities that we encountered consisted of several dominant taxa accompanied by species that had low frequencies of spatial and temporal occurrence. In total, we found 54 taxa. The detected assemblage consists of fungi exclusively inhabiting highly acidic habitats, i.e. *Acidiella bohemica* (50%), *Acidomyces acidophilus* (44%), Helotiales sp. 1 (31%), *Penicillium* sp. 4 (25%), *Penicillium* sp. 7 (19%), *Acidomyces acidothermus* (19%), *Acidothrix acidophila* (19%), *Coniochaeta fodinicola* (13%) and *Soosiella minima* (13%) as well as those which are known from both acidic and more or less extreme environments, i.e. *Acidea extrema* (56%), *Penicillium simplicissimum* s.l. (56%), *P. spinulosum* (31%), *Trichoderma harzianum* (19%), *Talaromyces helicus* var. *major* (13%), *Aspergillus fumigatus* (13%), *Penicillium* sp. 1 (6%) and *Hypholoma fasciculare* (6%) (Table 4).

The first group of exclusively acidophilic/tolerant inhabitants includes three unidentified representatives. The closest known relatives of these species are acidotolerant. The helotialean fungus (Helotiales sp. 1) exhibited 99% similarity to the LSU rDNA sequence of *Acidomelania panicicola*, a fungus isolated from switchgrass roots in acidic and oligotrophic pine barrens (Walsh et al. 2014), and two unidentified fungi *Penicillium* sp. 4 (25%) and *Penicillium* sp. 7 (19%) show 99% similarity to rDNA and Bt-tub sequences of *Penicillium* strain CCF 3828 isolated from highly acidic soil by Hujslová et al. (2010) (Table 2). It seems that these three morphologically closely similar but genetically different penicillia form a well-defined group sharing ecological preferences for extremely acidic soils. Only a detailed analysis can elucidate their taxonomic position and relationships.

The second group mentioned above is composed of species that prefer stressful environments. The species *Acidea extrema* is known from two different extremes, highly acidic habitats (Table 4) and alkaline soils (Hujslová et al 2014). In the present study, the name *Penicillium simplicissimum* s.l. is used in its broadest circumscription for a complex of species comprising *P. brasilianum*, *P. janthinellum*, *P. piscarium*, *P. pulvillorum*, *P.*

ochrochloron and *P. simplicissimum* (for a review, see Tuthill et al. 2001). Species of this complex are known from sites with highly stressful conditions such as low decomposition rates or high content of heavy metals (Kubátová et al. 2002). *Penicillium spinulosum* has been isolated quite frequently from substrates containing heavy metals, acids and tannins, and is known to be acidotolerant (Pitt 1979; Gross and Robbins 2000). Both taxa have also been reported from highly acidic environments (Table 4). Two species, *Talaromyces helicus* var. *major* (13%) and *Penicillium* sp. 1 (6%), identical to unidentified *Penicillium* isolates CCF 3782 and CCF 2941, previously showed a preference for saline (Kubátová et al. 2002) as well as acidic soils (Tab 4). *Trichoderma harzianum* (19%), *Aspergillus fumigatus* (13%) and *Hypholoma fasciculare* (6%) are able to tolerate acidic conditions (Gross and Robbins 2000). The second species has also been previously detected in highly acidic substrates (Tab 4).

Global pattern of fungal community composition in highly acidic habitats

As concerns species richness and composition across sites, site 1 was extremely poor and the most different from the rest (Table 2, 3; Fig 2). Nevertheless, five of the six taxa recorded at this site were also isolated from other sites (Table 2). There is an apparent general similarity between the fungal assemblages of our sites and those reported from highly acidic environments elsewhere (Table 4). In addition, the core of this assemblage consists of phylogenetically unrelated fungi, most of which are exclusive inhabitants of highly acidic habitats (Table 2, 4). This corroborates data from hypersaline or alkaline habitats (e.g. Gunde-Cimerman and Zalar 2014; Grum-Grzhimaylo et al. 2015). For a long time it has been assumed that eukaryotic organisms are unable to adapt to extreme habitats. It is apparent, however, that extreme environments, including highly acidic ones, provide suitable conditions for the development of specialist species, as postulated by Gostinčar et al. (2010). Besides specialists we obtained fungi known from various extremes like *Acidea extrema* and species detected from moderate habitats as well as from extremes, for example, *Penicillium simplicissimum* s.l. or *P. spinulosum*. These findings agree with those reported from other extremes such as hypersaline or alkaline environments (Gunde-Cimerman and Zalar 2014; Grum-Grzhimaylo et al. 2015). It seems that these species represent so-called generalists as defined by Gostinčar et al. (2010), for which extreme environments represent a vacant niche with limited competition, so they take their chance and colonize them. The authors suggest that generalist species serve as a genetic reservoir of potential candidates for the evolution of taxa specialized for living in extremes, because they are able to persist across a range of environments due to their robust genotypes.

The geographic distribution of acidophilic/tolerant fungi is generally consistent with findings made for hypersaline habitats, as is the composition of the detected fungal assemblage (Gunde-Cimerman and Zalar 2014). These results suggest that these extremophiles have cosmopolitan distributions, which is in agreement with the general hypothesis that most fungal taxa are distributed world-wide (Tedersoo et al. 2014). In addition, it is apparent that competition plays a principal role in the colonization of highly acidic environments. That competition plays an important role is also seen from the behaviour of certain species that prefer cultivation conditions which decrease competition. One example is *Acidea extrema*, for which the cultivation temperature of 5°C provides conditions that decrease the growth rate of fast-growing mesophilic species (Fig 5). López-Archilla et al. (2004) mention a similar pattern concerning the importance of competition.

To sum up, we detected numerous recently described or still undescribed species. This fact could be explained by the scarcity of studies on fungi inhabiting highly acidic habitats and insufficiently accurate identification of fungal isolates, such as in López-Archilla et al. (2004) or Amaral Zettler et al. (2002, 2003).

Methodological approaches

Since each of the isolation methods favours certain groups of fungi, we applied two methods, direct inoculation of soil (M1) and the soil washing technique (M2). The first method favours fast-growing fungi and fungi producing large numbers of spores (Kirk et al. 2004). The second method allows to isolate fungi actively growing at the time of sampling (Zak et Wildman 2004). Direct inoculation is commonly used in studies on the diversity of soil fungal communities (Bills et al. 2004) and has previously also been applied in investigations of fungi inhabiting various extreme soils (Kubátová et al. 2002; Hujslová et al. 2010). The washing technique is also considered suitable for isolating fungi that inhabit stressful environments (Zak and Wildman 2004). All most commonly isolated species were obtained by both isolation procedures (Table 2), indicating that these fungi are present in the soils under study not only as spores but also in actively growing form. This was confirmed by the results of statistical analysis showing no significant differences between the procedures used.

The isolation media used were designed to cover the demands of different fungi, from those preferring soil with moderate pH to acidotolerant/acidophilic ones. Agar with 2% of malt extract (MA2) represents a medium with a high content of sugar, which favours species with rapid germination and robust and rapid radial growth (Gams 1992; Bills et al. 2004). It is

widely used in studies of soil fungi (Bills et al. 2004, Gams 1992), and in various modifications it has been used in most studies dealing with extreme habitats. For example, Gunde-Cimerman et al. (2000) prepared MEA (malt extract agar) by mixing distilled water and water from the Dead Sea, aiming to detect halophilic species. In the present study, we used an acidified modification of MEA, 2% malt extract agar (MEA-pH2) and acidified soil agar with rose Bengal and glucose (SEA-pH2) prepared from the sampled substrate. Due to the low content of nutrients, the SEA substrate simulated the conditions of oligotrophic soils, and the content of rose Bengal as a fungitoxic agent should have suppressed rapidly growing fungi (Bills et al. 2004). Except for one species, Helotiales sp. 1, isolated exclusively from sugary media, all of the most abundant taxa were obtained using all isolation media (Table 2, Fig 4). It is nevertheless apparent that individual species prefer particular media. It is, for instance, evident that *Acidiella bohemica* prefers non-acidic media. *Penicillium spinulosum* and *Acidea extrema*, by contrast, were mostly isolated from sugary media. The same tendency was observed in the less abundant species *Aspergillus fumigatus* or *Trichoderma harzianum*, which entirely preferred sugary media regardless of pH (Table 2; Fig 4). Conversely, *Acidomyces acidothermus* was isolated exclusively from highly acidic media (Table 2; Fig 4). This fungus could not be considered an obligate acidophile, because it is able to grow also on less acidic media (Yamazaki et al. 2010; Hujslová et al. 2013; Gostinčar et al. 2010). That using different isolation media broadens the spectrum of obtained fungi has also been confirmed by a statistical analysis.

Incubation temperature affects growth, so it can affect the kind of fungi developing on isolation plates (Bills et al. 2004). The plates were thus incubated at three cultivation temperatures (5, 24 and 37°C). Incubation at cool temperatures reduces the colony expansion rate of mesophilic species, thereby allowing more time for slowly growing taxa (Bills et al. 2004). The second temperature is commonly used in soil studies (Bills et al. 2004). The third temperature, which was successfully applied in Hujslová et al. (2010), was used to broaden the fungal spectrum. A majority of the most abundant species were isolated from plates incubated at 5°C and 24°C (Fig 5). It is apparent, however, that individual species prefer different temperatures. *Acidea extrema*, for example, was predominantly isolated from plates incubated at 5°C whereas two other species, *Acidomyces acidothermus* and *Aspergillus fumigatus,* were mostly obtained under the temperature of 37°C (Table 2; Fig 5). That using different incubation temperatures broadens the spectrum of detected fungi has also been confirmed by the results of a statistical analysis, and the findings agree with those reported by Kochkina et al. (2012).

Conclusions

In this study, we described the core fungal assemblage typical of extremely acidic habitats and provided a baseline for comparisons with similar localities worldwide. It is apparent that highly acidic environments provide suitable conditions for the evolution of specialist species and that these specialists tend to have cosmopolitan distributions. In addition, competition seems to be the principal process taking place during the colonization of highly acidic environments. In the present study, numerous recently described or still undescribed species were detected, which suggests that fungal communities of extremely acidic habitats are highly diversified, making them a good target for bioprospecting.

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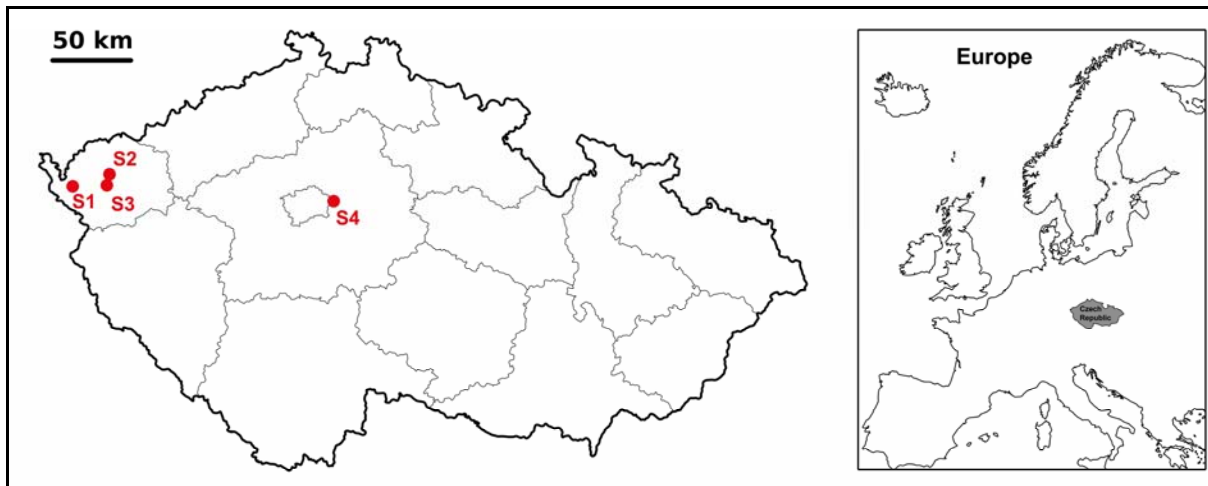

Fig. 1 Map of the Czech Republic showing the four sampling sites: **S1** - 50°08'60" N, 12°24'00" E, **S2** - 50°15'00" N, 12°46'12" E, **S3** - 50°15'00" N, 12°46'48" E, **S4** - 50°06'36" N, 14°31'48" E.

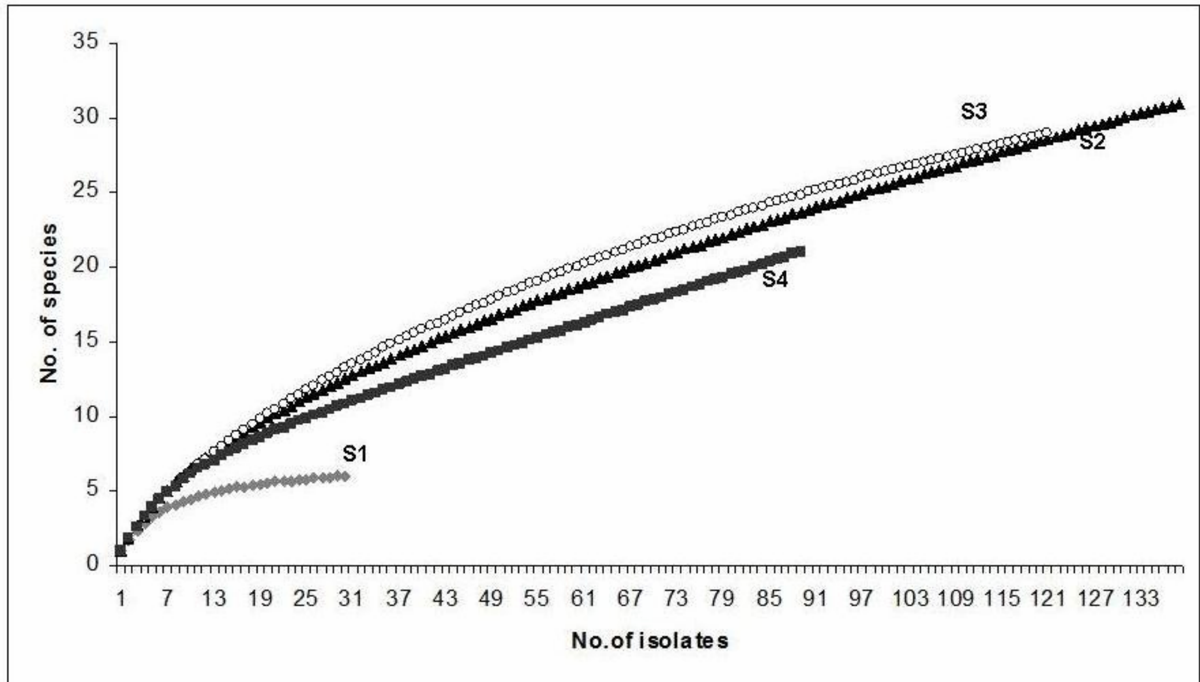

Fig. 2 Rarefaction curves describing the species richness detected at the four sampling sites. For clarity, the 95% confidence intervals are not shown.

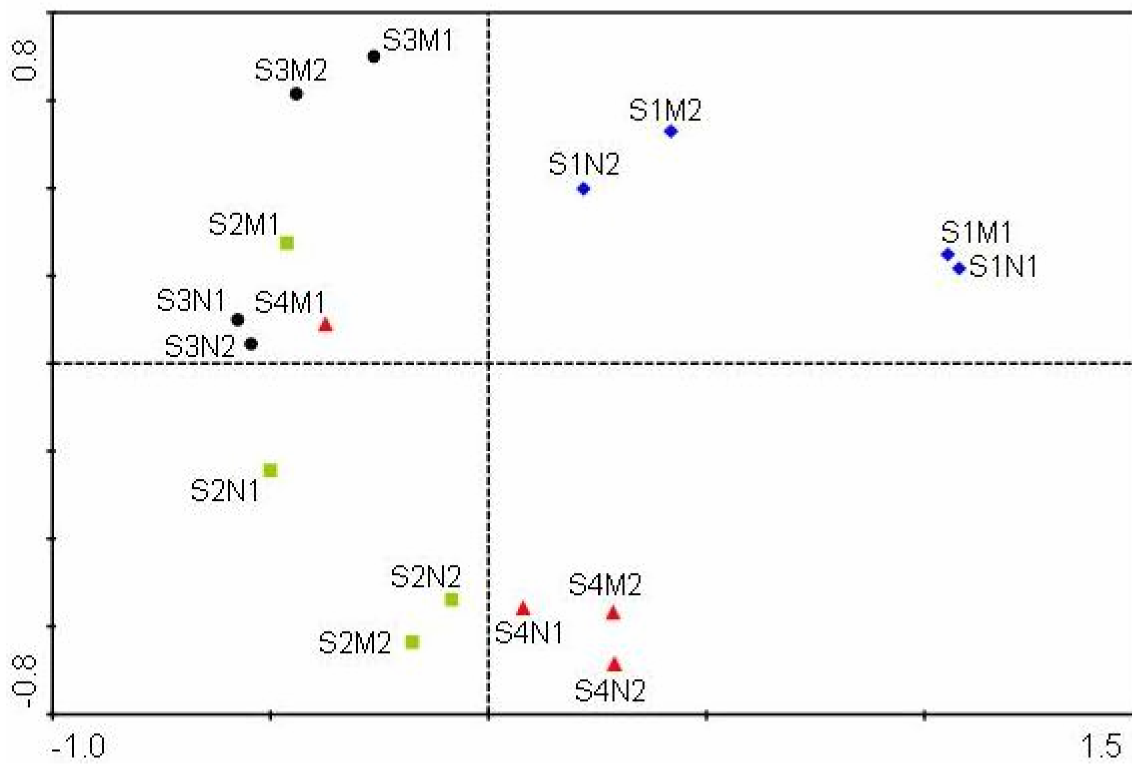

Fig. 3 Ordination diagram of PCoA showing the position of samples on the plane formed by the first two axes. The figure shows a tendency to split the samples into groups regardless of the sampling site. Besides the dissimilarity of samples taken from the site 1 is apparent (S1 site1, S2 site2, S3 site3, S4 site4; M May, N November; 1 sample 1, 2 sample 2).

Fig. 4 Redundancy analysis (RDA) showing the relationships between abundantly isolated species (triangles) and isolation media (squares) on the plane formed by the first two axes. Most of the species were obtained using all isolation media. Some species preferred non-acidic media (*Acidiella bohemica*), some grew better on acidic ones (*Acidomyces acidothermus*) and some of them were mostly isolated from sugary media (*Acidea extrema*, Helotiales sp. 1).

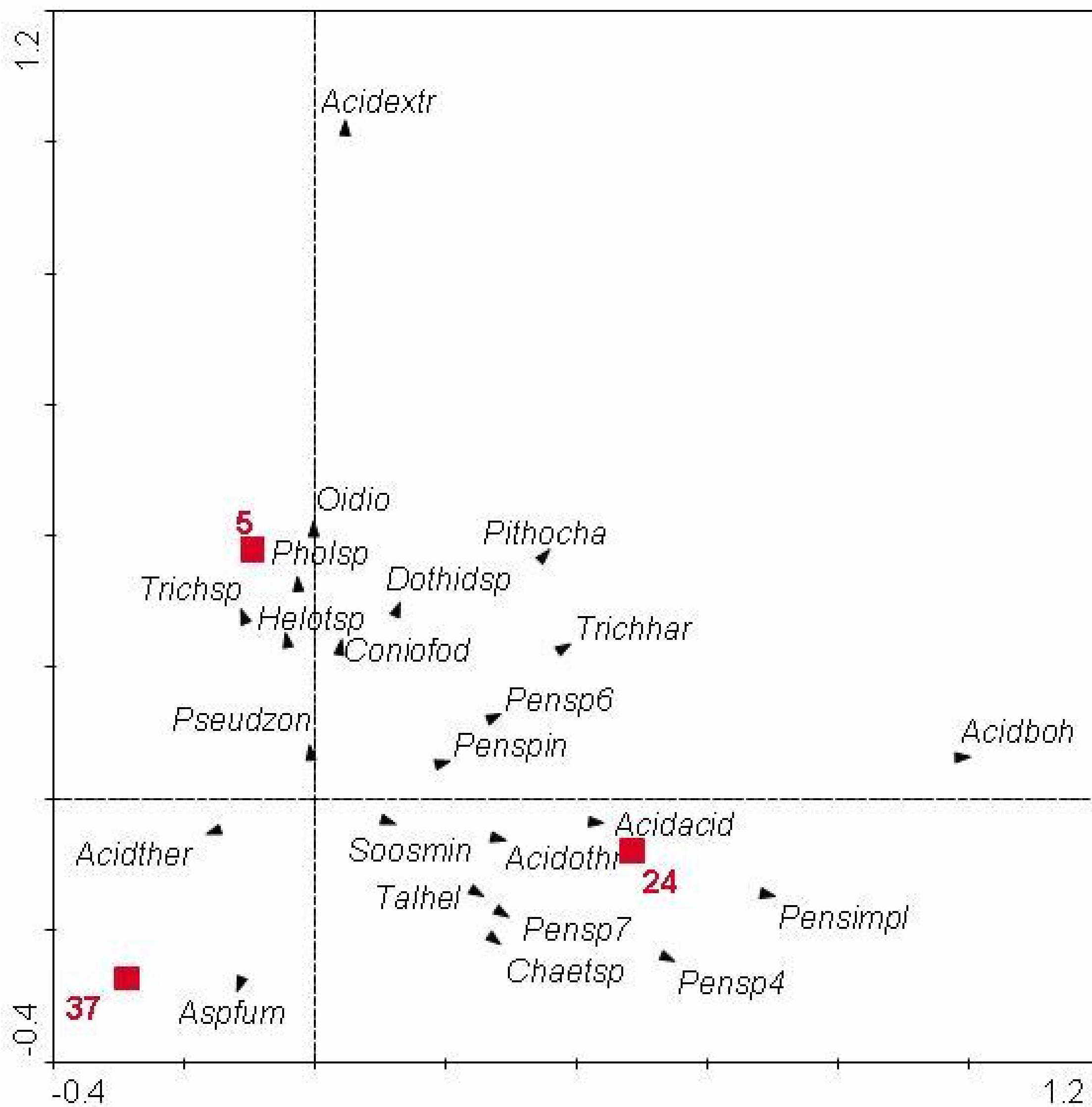

Fig. 5 Redundancy analysis (RDA) showing the relationships between most frequently isolated species (triangles) and incubation temperature (squares) on the plane formed by the first two axes. A majority of the most abundant species were isolated from 5°C and 24°C. Some species preferred 5°C (*Acidea extrema*) and some of them grew better at 37°C (*Acidomyces acidothermus* and *Aspergillus fumigatus*).

Table 1 Description of the four sampling sites.

Sampling Site Code	Locality	Latitude	Longitude	Characterisation	Soil pH	Sampling Date/No of samples
S1	Soos National Natural Reserve, Czech Republic	50°08'60" N	12°24'00" E	Bare area of acid sulfate soils	1-2	May, November 2007/4
S2	Mírová, Czech Republic	50°15'00" N	12°46'12" E	Bare area of kaolin quarry with sulfur and humic acids rich brown coal layers	1.5-2.5	May, November 2007/4
S3	Jimlíkov, Czech Republic	50°15'00" N	12°46'48" E	Bare area of kaolin quarry with sulfur-rich brown coal layers	2-4	May, November 2007/4
S4	Cihelna v Bažantnici National Monument, Czech Republic	50°06'36" N	14°31'48" E	Bare area of exposed clay sediments and sulfur-rich brown coal layers	1-2	May, November 2007/4

Table 2 List of the isolated taxa with frequencies of their occurrence, the methodological approaches used, sequence accession Nos. and the closest matches from the GenBank database. Final identification was done using a combination of morphological and molecular data.

Taxon	Sampling sites frequency (%) (total No. of isolates)					Methodological approach			Accession No.	Blast similarty search in NCBI Genbank
	S1[b]	S2[b]	S3[b]	S4[b]	Total [a]	Method	Medium	T(°C)		
Acidea extrema Hujslová & M. Kolařík CBS 136258	25 (3)	100 (48)	75 (21)	25 (2)	**56**	M1 M2	MA2 MEApH2 SEApH2	5 24	JX124323	discussed in Hujslová et al. (2014)
Acidiella bohemica Hujslová & M. Kolařík CBS 132721	-	100 (13)	-	100 (23)	**50**	M1 M2	MA2 MEApH2 SEApH2	5 24	JQ172752	discussed in Hujslová et al. (2013)
Acidomyces acidophilus (Sigler & J.W. Carmich.) Selbmann, de Hoog & De Leo MH 934	50 (16)	25 (1)	50 (5)	50 (2)	**44**	M1 M2	MA2 MEApH2 SEApH2	5 24	JQ172742	discussed in Hujslová et al. (2013)
Acidomyces acidothermus A. Yamaz., K. Toyama & Nakagiri CCF 4140	-	50 (3)	25 (1)	-	**19**	M1 M2	MEApH2 SEApH2	5 37	JQ172747	discussed in Hujslová et al. (2013)
Acidothrix acidophila Hujslová & M. Kolařík CCF 4344	-	75 (7)	-	-	**19**	M1 M2	MA2 MEApH2 SEApH2	5 24	KF286988	discussed in Hujslová et al. (2014)
Arthrinium sacchari (Speg.) M.B.Ellis MH823	-	25 (1)	-	-	**6**	M2	MA2	5		HQ115662 *Arthrinium sacchari*, 710/710, 100%, LSU AB220188 *Arthrinium sacchari*, 1024/1024; 577/577, 100%, SSU
Aspergillus fumigatus Fresen[c]. MH 837, MH 866, MH 867, MH 887, MH 888	-	25 (3)	25 (2)	-	**13**	M1 M2	MA2 MEApH2	24 37		KJ775281 *Aspergillus fumigatus*, 526/526, 100%, cal

Aspergillus sp. 1 * MH 1225	-	-	25 (1)	-	**6**	M2	MA2	37		
Aspergillus sp. 2 * MH 1166	-	-	-	25 (1)	**6**	M2	MA2	37		
Chaetomium sp. MH 935, MH 1228, MH 1229, MH 1231	50 (4)	-	-	-	**13**	M1	MA2 MEApH2 SEApH2	24		AJ271592 *Thielavia tortuosa*, 438/501, 87%, ITS JN209913 *Chaetomium globosum*, 555/566, 98%, LSU JQ964323 *Chaetomium globosum*, 997/1002 99%, SSU
Coniochaeta fodinicola X. Vázquez-Campos MH 1062, MH 1232, MH 1234, MH 1290, MH 1303, MH1315	-	-	-	50 (11)	**13**	M1 M2	MA2 MEApH2 SEApH2	5 24		JQ904607 *Coniochaeta fodinicola*, 511/512, 99%, ITS KF857178 *Coniochaeta fodinicola*, 555/555, 100%, LSU KF857177 *Coniochaeta fodinicola*, 1610/1610, 100%, SSU
Dothideomycetes sp. MH 960, MH 1332	-	-	25 (1)	25 (1)	**13**	M1	MA2 MEApH2	5 24		JQ759882 Dothideomycetes sp., 503/558, 90%, ITS JQ759882 Dothideomycetes sp., 453/464, 98%, LSU
Epicoccum nigrum Link * MH 1245	-	-	25 (1)	-	**6**	M2	MA2	5		
Fusarium spp. *	-	-	50	-	**13**	M1 M2	MA2	24		
Helotiales sp. 1. MH 842, MH 905, MH 916, MH 918, MH 956, MH 1150, MH 1248, MH 1292	-	25 (3)	75 (3)	25 (2)	**31**	M1 M2	MA2 MEApH2	5 24		AY259135 *Mollisia cinerea*, 506/521, 97%, ITS KF874624 *Acidomelania panicicola*, 531/536, 99%, LSU DQ471005 *Loramyces macrosporus*, 1639/1650, 99%,
Helotiales sp. 2 MH 991	**-**	**-**	25 (1)	**-**	**6**	M2	MA2	5		DQ257357 *Holwaya mucida*, 478/494, 97%, ITS DQ257357 *Holwaya mucida* 498/499, 99%, LSU AF292091 *Holwaya mucida*, 1664/1672, 99%, SSU
Hypholoma fasciculare (Huds.) P.Kumm. MH 1296	-	-	-	25 (1)	**6**	M1	MA2	5		FJ430716 *Hypholoma fasciculare*, 518/518, 100%, ITS FJ430716 *Hypholoma fasciculare*, 690/690, 100%, LSU
Nigrospora oryzae (Berk. & Broome) Petch MH 827	-	25 (1)	-	-	**6**	M2	MA2	5		GQ428201 *Nigrospora oryzae*, 667/667, 100%, LSU
Oidiodendron cf. *rhodogenum* * MH 1174, MH 1320, MH 1322, MH 1323, MH 1324	-	-	50 (5)	-	**13**	M1 M2	MA2 MEApH2	5 24		

Phaeosphaeriaceae sp. MH 898	-	25 (1)	-	-	**6**	M1	SEApH2	24		KJ188706 *Phaeosphaeria* sp., 512/515, 99%, ITS JQ238625 *Diederichomyces cladoniicola*, 558/559, LSU KC841079 *Ophiosphaerella korrae*, 1660/1666, 99%, SSU
Orbiliaceae sp. MH 1011	-	-	25 (1)	-	**6**	M2	MEApH2	5		DQ317334 Orbiliaceae sp. BC5, 521/521, 100%, ITS JX507669 *Mollisia* sp., 523/529, 99%, LSU DQ471005 *Loramyces macrosporus*, 1639/1652, 99%, SSU
Paraphaeosphaeria sporulosa (W. Gams & Domsch) Verkley & Stielow MH 815, MH 816, MH 923, MH 958	-	-	25 (4)	-	**6**	M1 M2	MA2	5 24		JX496114 *Paraphaeosphaeria sporulosa*, 550/550, 100%, ITS JX496114 *Paraphaeosphaeria sporulosa*, 581/581, 100%, LSU
Penicillium bialowiezense Zaleski MH 828, MH 957	-	25 (1) 25 (1)	-	-	**6** **6**	M1 M2	MA2	5		HM469395 *Penicillium bialowiezense*, 656/656, 100%, ITS DQ486640 *Penicillium bialowiezense*, 503/504, 99%, Bt-tub
Penicillium bilaiae Chalab. MH	-	-	-	25	**6**	M2	MA2	24		AF033402 *Penicillium bilaiae*, 726/726, 100%, ITS
Penicillium glabrum (Wehmer) Westling MH 1293	-	-	-	25 (1)	**6**	M2	MA2	5		AY373915 *Penicillium glabrum*, 507/507, 100%, ITS AF033407 *Penicillium glabrum*, 596/596, 100%, LSU EU128574 *Penicillium glabrum*, 390/391, 99%, Bt-tub
Penicillium montanense M. Chr. & Backus MH 813	-	-	25 (1)	-	**6**	M1	MEApH2	24		AF527058 *Penicillium montanense*, 548/548, 100%, ITS AF527058 *Penicillium montanense*, 476/476, 100%, LSU
Penicillium purpurogenum var. *rubrisclerotium* Thom MH 915	-	25 (1)	-	-	**6**	M1	MEApH2	24		KC992261 *Penicillium purpurogenum* var. *rubrisclerotium*, 380/380, 100%, Bt-tub
Penicillium roseopurpureum Dierckx MH 1298	-	-	-	25 (1)	**6**	M1	MA2	5		AF034462 *Penicillium roseopurpureum*, 549/549, 100%, ITS AF034462 *Penicillium roseopurpureum*, 549/549, 100%, LSU
Penicillium simplicissimum s.l. (Oudem.) Thom[*d]	-	100 (24)	100 (30)	25 (5)	**56**	M1 M2	MA2 MEApH2 SEApH2	5 24 37		
Penicillium sp. 1 MH 848	-	25 (1)	-	-	**6**	M1	MA2	37		FJ430758 *Penicillium* sp. CCF 2941, 499/499, 100%, ITS

Penicillium sp. 2 MH 902	-	-	25 (1)	-	**6**	M1	MEApH2	5		AF033442 *Penicillium daleae*, 529/534, 99%, ITS AF033442 *Penicillium daleae*, 593/595, 99%, LSU GU981649 *Penicillium daleae*, 386/405, 96%, Bt-tub
Penicillium sp. 3 MH 939, MH 943	-	25 (2)	-	-	**6**	M1	SEApH2	24		HQ406811 *Penicillium spinulosum*, 497/498, 99%, ITS AF527058 *Penicillium montanense*, 273/273, 100%, LSU FJ004436 *Penicillium spinulosum*, 380/386, 98%, Bt-tub
Penicillium sp. 4 MH 840, MH 1177, MH 1190	-	75 (14)	-	25 (1)	**25**	M1 M2	MA2 MEApH2 SEApH2	24 37		FJ430753 *Penicillium* sp. CCF 3828, 527/531, 99%, ITS FJ430753 *Penicillium* sp. CCF 3828, 587/587, 99%, LSU FM865814 *Penicillium* sp. CCF 3828, 412/415, 99%, Bt-tub
Penicillium sp. 5 MH 819	-	-	25 (1)	-	**6**	M1	MA2	37		EF422848 *Penicillium griseolum*, 788/790, 99%, ITS EF506213 *Penicillium griseolum*, 383/393, 97%, Bt-tub
Penicillium sp. 6 MH 1173, MH 1212	-	25 (1)	25 (1)	-	**13**	M1	MA2 MEApH2	24		KJ775633 *Penicillium glabrum*, 495/500, 99%, ITS JF922034 *Penicillium glabrum*, 503/504, 99%, LSU KM089009 *Penicillium armarii*, 369/370, 99%, Bt-tub
Penicillium sp. 7 MH 1132, MH 1209, MH 1222, MH 830	-	50 (2)	-	25 (2)	**19**	M1 M2	MA2 MEApH2 SEApH2	24		FJ430753 *Penicillium* sp. CCF 3828, 526/532, 99%, ITS FJ430753 *Penicillium* sp. CCF 3828, 584/585, 99%, LSU FM865814 *Penicillium* sp. CCF 3828, 412/415, 99%, Bt-tub
Penicillium spinulosum Thom MH 831, MH839, MH 894, MH 1065, MH1213, MH 1240, MH 1267, MH 1268, MH 1272	50 (7)	50 (4)	-	25 (1)	**31**	M1 M2	MA2 MEApH2 SEApH2	5 24		AF033410 *Penicillium spinulosum*, 751/751, 100%, ITS
Penicillium spp.*	-	50	50	25	**31**	M1 M2	MA2 MEApH2	5 24		
Phaeoacremonium scolyti L. Mostert, Summerb. & Crous MH 950	-	25 (1)	-	-	**6**	M1	MEApH2	5		KF467599 *Phaeoacremonium scolyti*, 679/679, 100%, Bt-tub

Pholiota sp. MH 912, MH 922, MH 925, MH 927, MH 959)	-	-	50 (5)	-	**13**	M1 M2	MA2	5 24		JF908580 *Pholiota gummosa*, 547/548, 99%, ITS AF195605 *Pholiota gummosa*, 653/656, 99%, LSU
Pithomyces chartarum (Berk. & M.A. Curtis) M.B. Ellis MH 1123, MH 1133	-	-	25 (1)	25 (1)	**13**	M1	MA2	24		HG518061 *Pithomyces chartarum*, 500/500, 100%, ITS DQ384571 *Pithomyces chartarum*, 594/595, 99%, LSU
Pleospora herbarum P. Karst. MH 955	-	25 (1)	-	-	**6**	M2	MA2	5		KJ790251 *Pleospora herbarum*, 409/409, 100%, LSU PHU43458 *Pleospora herbarum*, 936/937, 99% SSU
Pochonia bulbillosa (W. Gams & Malla) Zare & W. Gams MH 1302, MH 1326	-	-	25 (2)	-	**6**	M1 M2	MA2	5		KC180702 *Pochonia bulbillosa*, 521/521, 100%, ITS AB378555 *Pochonia bulbillosa*, 560/560, 100%, LSU
Pseudeurotium zonatum J.F.H. Beyma MH 817, MH 910, MH 926, MH 928	-	-	50 (4)	-	**13**	M1 M2	MA2	5 24		DQ470988 *Pseudeurotium zonatum*, 437/437, 100%, LSU
Purpureocillium lilacinum (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson MH 836 *	-	25 (1)	-	-	**6**	M2	MA2	24		
Soosiella minima Hujslová & M. Kolařík CBS 136257	25 (1)	25 (2)	-	-	**13**	M1	MA2 MEApH2 SEApH2	5 24	JX124327	discussed in Hujslová et al. (2014)
Talaromyces flavus (Klöcker) Stolk & Samson MH911 *	-	-	25 (1)	-	**6**	M1	MA2	5		
Talaromyces helicus var. *major* Stolk & Samson MH814, MH822, MH900	-	-	50 (3)	-	**13**	M1	MEApH2 SEApH2	24		FJ430764 *Talaromyces helicus* var. *major*, 456/456, 100%, ITS
Trichoderma atroviride P. Karst. MH843	-	25 (1)	-	-	**6**	M2	MEApH2	5		EU715667 *Trichoderma atroviride*, 547/547, 100%, ITS HQ115671 *Trichoderma atroviride*, 543/543, 100%, LSU
Trichoderma sp. 1 MH938, MH 940, MH 941	-	25 (1)	50 (10)	-	**19**	M1 M2	MA2 MEApH2	5 24		KP316434 *Trichoderma harzianum*, 457/457, 100%, ITS KC330218 *Trichoderma harzianum*, 556/556, 100%, LSU
Trichoderma sp. 2 MH907, MH 919, MH920	-	-	50 (3)	-	**13**	M1 M2	MEApH2	5		KP009372 *Trichoderma viride*, 496/500, 99%, ITS HQ115671 *Trichoderma atroviride*, 597/598, 99%, LSU
Trichoderma spp. *	-	25	-	-	**25**	M1	MA2	24		

Zygomycetes spp. *	-	25 (2)	-	25 (1)	**13**	M1 M2	MA2	5 24		
Unisolated/unidentified dark mycelia *	50	100	75	100	**81**	M1 M2	MA2 MEApH2 SEApH2	5 24 37		
Yellow sterile mycelium *	-	-	-	50	**13**	M1 M2	MA2 MEApH2 SEApH2	24 37		
Unisolated/unidentified white mycelia *	-	100	100	75	**69**	M1 M2	MA2 MEApH2 SEApH2	5 24 37		
Total No. of taxa (including unidentified mycelia)	**6**	**31**	**29**	**21**	**54**					
No. of plates without fungal colony (F%)[b]	**74**	**22**	**23**	**35**	**39**					

S1 = NPR Soos, S2 = DP Mírová, S3 = DP Jimlíkov, S4 = PP Cihelna v Bažantnici

[a] Frequency was calculated as percent of total number of 16 samples in which particular taxon was identified

[b] Frequency was calculated as percent of 4 samples processed from individual site in which particular taxon was identified

[c] The identification of *Aspergillus fumigatus* was based on calmodulin gene

[d] The name *Penicillium simplicissimum* was used in its broadest circumscriptions including *P. brasilianum*, *P. janthinellum*, *P. piscarium*, *P. pulvillorum*, *P. ochrochloron* and *P. simplicissimum* (for review see Tuthill et al. 2001)

* Taxa which were identified only by morphology (the viability of the isolates was lost)

Table 3 Similarity of fungal communities among study sites.

	Site 1	Site 2	Site 3	Site 4
Site 1	1	0.18	0.13	0.19
Site 2	0.18	1	0.51	0.41
Site 3	0.13	0.51	1	0.23
Site 4	0.19	0.41	0.23	1

Table 4 Core assemblage of fungi from highly acidic soils detected in the present study at frequencies higher than 6% and also reported by other authors. The pH of all substrates from which the fungi were isolated was less than 3.

Species	Isolated from	Reference
Acidea extrema	highly acidic soil (Czech Republic) highly acidic river water (Spain)	Hujslová et al. (2014)
Acidiella uranophila[*]	highly acidic water from uranium mine (Australia)	Vazquéz-Campos et al. (2014)
Acidiella bohemica	highly acidic soil (Czech Republic) highly acidic river water (Spain)	Hujslová et al. (2013)
Acidomyces acidophilus	acidophilic algae, acid drainage (Germany) soil near sulfur pile (Canada) sulphuric acid (Danmark) volcanic soil (Iceland) acidic industrial water (The Netherlands)	Selbmann et al. (2008)
	highly acidic soil (Czech Republic)	Hujslová et al. (2013)
Acidomyces acidothermus	highly acidic hot springs (Japan)	Yamazaki et al. (2010)
	highly acidic water from uranium mine (Australia)	Vazquéz-Campos et al. (2014)
	highly acidic soil (Czech Republic) highly acidic soil (Island) acid mine drainage water (USA)	Hujslová et al. (2013)
Acidothrix acidophila	highly acidic soil (Czech Republic)	Hujslová et al. (2014)
Coniochaeta fodinicola	highly acidic water from uranium mine (Australia) acidic waste water from uranium mine (China)	Vazquéz-Campos et al. (2014)
	highly acidic soil (Czech Republic)	Table 2
Helotiales sp. 1	highly acidic soil (Czech Republic)	Table 2
Hypholoma fasciculare	highly acidic soils (Czech Republic)	Hujslová et al. (2010)

	highly acidic soils (Czech Republic)	Table 2
Penicillium simplicissimum s.l.	highly acidic soils (Czech Republic)	Hujslová et al. (2010)
	highly acidic soils (Czech Republic)	Table 2
Penicillium spinulosum	highly acidic soils (Czech Republic)	Table 2
	highly acidic river water (Spain)	López-Archilla et al. (2004)
Penicillium sp. 1	highly acidic soil (Czech Republic)	Table 2
	highly acidic soil (Czech Republic)	Hujslová et al. (2010)
Penicillium sp. 4	highly acidic soil (Czech Republic)	Table 2
Penicillium sp. 7	highly acidic soil (Czech Republic)	Table 2
Soosiella minima	highly acidic soil (Czech Republic)	Hujslová et al. (2014)
Talaromyces helicus var. *major*	highly acidic soil (Czech Republic)	Table 2
	highly acidic soil (Czech Republic)	Hujslová et al. (2010)

*sister or identical to *Acidiella bohemica* (Kolařík et al. 2015)

3 CONCLUSIONS

In summary, the present thesis provides new insight into the fungal communities inhabiting highly acidic environments which seem to be closely similar worldwide and different from the ones known from less acidic habitats. The core of the fungal assemblage under study consisted of phylogenetically unrelated and often globally distributed fungi exclusively inhabiting highly acidic habitats as well as taxa known from less acidic and often extreme environments. The dominant representatives of the community include different dematiaceous species of Dothideomycetes, several penicillia as well as different hyaline species belonging to Leotiomycetes and Sordariomycetes. The large number of identified specialized species indicates that highly acidic environments provide suitable conditions for the evolution of specialist species. The occurrence of ubiquitous fungi in highly acidic substrates points to the principal role of competition in colonization of highly acidic environments. Numerous recently described and unknown taxa has been detected and some of them are described here as new genera and species which suggests that fungal community of highly acidic habitats is more diversified than it has been supposed so far. The detected taxa did not require low pH to survive, because they can grow in a broad range of pH. No obligate acidophile has been detected. To sum up, the present work primarily advances our understanding of fungi inhabiting highly acidic habitats. In addition, it provides a great deal of isolates of specialized fungi from which several are known for their enormous biotechnological potential which indicate that acidophilic/tolerant fungi represent good target for bioprospecting.

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5 CURRICULUM VITAE

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