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HETEROBASIDION ANNOSUM SENSU LATO IN THE CZECH REPUBLIC: BIOLOGY AND ECOLOGY

DOCTORAL THESIS

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ABSTRACT

The aim of this thesis is to investigate the distribution of *H. annosum* s.l. in the Czech Republic. Sampling was undertaken either in natural forests, in stands with former presence of fir and spruce and in forests on former agricultural lands. The identification and phylogenetic relationship of the species complex was studied by analysis of DNA sequences of three gene regions: glyceraldehyde 3-phosphate dehydrogenase (G3P), translation elongation factor 1-alfa (EFA) and transcription factor (TF). The distribution of each species is demonstrated. *Heterobasidion annosum* s.s. was found on thirteen genera (*Pinus, Picea, Fraxinus, Betula, Corylus, Alnus, Abies, Acer, Salix, Ligustrum, Quercus, Larix and Prunus*). *Heterobasidion parviporum* was found on spruce (*Picea abies*), fir (*Abies alba*), apple tree (*Malus sylvestris*), *Vitis vinifera* and *Fagus sylvatica* and *Heterobasidion abietinum* was observed on fir (*Abies*), spruce (*Picea*), pine (*Pinus*). Sequences from different genes yielded conflicting results for 6 specimens, which were interpreted as belonging to interspecific hybrids. Furthermore, all the three European species were found during mycological explorations in Spain, Romania, Czech Republic and Slovakia in extraordinary habitats. Isolates of anamorph of *H. annosum s.l.* were obtained from moonmilk, air, underground gallery wall, isopod faeces and bat guano. The origin and ways of fungal dispersal are discussed. The forest stands surrounding caves and tunnels are hypothesized as the potential source of inoculum to inhabit these unusual spaces, although the real paths of the pathogen to the habitats remain unclear. The occurrence of *Heterobasidion* sp. selected wood decay fungi was investigated inside the stem of living Norway spruce (*Picea abies* (L.) Karsten) using methods of rapid detecting by multiplex PCR. Occurrence was the main purpose of the study and results showed a high number of species occurred inside the stem.

Keywords: *Heterobasidion annosum*, Polymerase chain reaction, root rot, butt rot, PCR, DNA sequencing

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Dedication

To everyone who hungers for knowledge.

CONTENT

ABBREVIATIONS

BP – bootstrap branch support values

Bp – base pair

C - carbon

DNA – deoxyribonucleic acid

EFA – elongation factor 1-alfa

F_{ST} – Wright's F-statistic

GPD – glyceraldehyde 3-phosphate dehydrogenase

GenBank – open access sequence database produced and maintained by National Center for Biotechnology Information (NCBI)

H – number of haplotypes

H_d – haplotype diversity

IS-group – intersterility group

K – average number of nucleotide differences

NP – National Park

Mya – million year ago

NIF – national inventory of forest

PCR – polymerase chain reaction

PG – *Phlebiopsis gigantea*

PG IBL – biological control agent, Poland

S – number of polymorphic/indel/missing sites

s.l. – sensu lato

s.s. – sensu stricto

TBE – Tris-borate buffer

TF – transcription factor

FOR READERS

This doctoral thesis associates different investigations in the field of root rot and butt rot. It is a composition of two original papers, published in Forest Pathology, and it contents overview from research that has been aimed to investigation of presence of Heterobasidion sp. and other wood inhabiting fungi in living Norway spruces.

PREFACE

No forest - no water, no water - no life.

The temperate forest is one the most complex and most vital biome and it has always been very crucial and directly connected to the human activities since the dawn of humankind. However, some of these activities have had negative impact on the stability of the forest, such as clear-cuts well known from Southern Europe from the time of Fenics or Roman Empire (1 000 B.C.) or in much bigger scale from the time of the industrial revolution (early 18th century). Some of the consequences of changed environment have been faced until today, like desertification in Mediterranean area. Since the time, no complete remedy of the forest conditions has ever been done despite many attempts. The composition of forest has been completely changed and therefore it has become more vulnerable. Decreased stability and unfavorable conditions tend to increase the dispersal and severity of - among others - *Heterobasidion annosum* s.l.

After the fall of the Iron Courtain, vast afforestation activites had started in the Czech Republic. These actions were done on the former agricultural lands because of decreasing demands for pasture for cattle. Former arable land was afforested generally with Norway spruce and Scots pine. It has already been two decades since the change of political regime and the area of forest land in the Czech Republic is still increasing. Notwithstanding, root rot is a well known issue among foresters, no precautions have been made on bigger scale. Due to the large areas of these new plantations, we will surely be facing the consequences of *Heterobasidon* root rot in great extent in very near future.

1 INTRODUCTION

1.1 THE PATHOGEN

Figure 1. Basidiocarp of *Heterobasidion abietinum*. Photo by P. Sedlák.

Heterobasidion annosum s.l. (Fr.) Bref. is regarded as one of the most serious and important forest pathogens. It causes a butt and root rot of numerous coniferous and less broad-leaved trees. In economical terms, it is the most important disease of conifers in the forests of northern temperate regions. Although, other fungal pathogens may be of greater local importance, but *H. annosum* s.l. occurs in the most of the managed coniferous forests of the Northern Hemisphere from central Finland in the north and Northern Africa and Central America in the south (Korhonen et al. 1998). Economic losses attributable to *Heterobasidion* infection in Europe are estimated at 800 million euro annually (Asiegbu et al. 2005). This estimated losses include decay as well as reduction in the diameter growth of infected trees (Seifert 2007, Oliva et al. 2012), but it exclude windthrows and reduction of resistance of stands to storm damages caused by fungus (Gonthier and Garbelloto 2013). Severity of the situation was concluded during intensive study in Latvia, where 63.1$m^3.ha^{-1}$ at 100 years stand volume of wood had been degraded by *H. annosum* infection, which comprises 6-16% of total standing volume (Arhipova et al. 2011).

Heterobasidion sp. was regarded as a single species until mating experiments among different individuals revealed the occurrence of intersterile groups (ISGs) (Korhonen 1978). The genus *Heterobasidion* (Basidiomycota, Russulales, *Bondarzewiaceae*) included, until recently, five taxonomic species: *Heterobasidion annosum* (Fr.) Bref., *Heterobasidion araucariae* P. K. Buchanan, *Heterobasidion insulare* (Murrill) Ryvarden, *Heterobasidion pahangense* Corner, and *Heterobasidion rutilantiforme* (Murrill) Stalpers. However, *H. rutilantiforme* and *H. pahangense* was rejected by Hattori (2001) and on the other hand, Tokuda et al. (2009) accept nine species of *Heterobasidion* in Euroasia: *H. abietinum, H. annosum, H. araucariae, H. arbitrarium*, *H. ecructosum*, *H. insulare*, *H. linzhiense*, *H. orientale* and *H. parviporum*. Recently, two new species of *Heterobasidion insulare* complex – *H. amyloideum* and *H. tibeticum* – have been added to the list of *Heterobasidion* species (Chen et al. 2014).

Although most *Heterobasidion* species are likely to be strict saprotrophs or display uncertain pathogenicity (Dai and Korhonen 2009; Oliva et al. 2010), the *H. annosum* species complex, (*H. annosum* s.l.*)*, comprises necrotrophic parasites regarded as the most destructive disease originator of conifers. Currently, three species of *Heterobasidion annosum* s.l. are recognized in Eurasia and two in North America; all are now formally described as species. Eurasian groups were described as *H. annosum sensu stricto* (s.s.), *Heterobasidion abietinum* Niemelä & Korhonen, and *Heterobasidion parviporum* Niemelä & Korhonen (Niemelä and Korhonen 1998), whereas North American groups were named *Heterobasidion irregulare* (Underw.) Garbel. & Otrosina and *Heterobasidion occidentale* Otrosina & Garbel. (Otrosina and Garbelotto 2010). However, North American *H. irregulare* has been introduced into central Italy during World War II (Gonthier et al. 2004) and has since become invasive by spreading in Italian stone pine (*Pinus pinea* L.) stands (Gonthier et al. 2007). The current distribution area of *H. irregulare* includes an area of approximately 100 km along the Tyrrhenian coast west of Rome (D´Amico et al. 2007; Gonthier et al. 2007, Gonthier et al., 2015).

Heterobasidion spp. is a white rot fungus, causing selective delignification. In forestry the rot is well known as red rot. It occurs in both, conifers and broad-leaved trees. During selective delignification, at

the early stage of decay, lignin is broken down more than hemicelluloses and cellulose. There is a ductile fracture at the initial stages followed by a slight increase in impact bending strength (Schwarze et al., 2000). Initial growth in wood causes a stain that varies in colour depending on host tree species. The fungus enters the tree via root-to-root contacts with neighbouring infected trees or stumps, and spreads through the heartwood forming decay columns of up to 10 m (Stenlid and Redfern 1998). Oliva et al. (2012) observed that spruce trees at initial stages of decay do not show a reduction in radial growth when the decay developed in the heartwood. Furthermore, growth losses occurred on trees at early stages of decay, when the first reaction zone was formed. This fact is connected to poor crown forming and viability of first symptoms.

Root rot, butt rot, suppressed growth, and tree mortality are significant loss factors. Nevertheless, the symptoms of the *Heterobasidion* root rot are not diagnostic – the undoubt identification of *Heterobasion* infection is due to recognition of the respective basidiocarps (Fig. 1). These are perennial, resupinate to pileate, widely effused and tough; pilear surface at first light brown and finely tomentose, soon darker and smooth with a distinct thin black cuticule; pore surface light cream, pores regular, round to angular, mostly small, glancing, usually irregulary shaped, 3.5 (–7) cm thick and up to 40 cm in diameter. The asexual stage, described as *Spiniger meineckellus* (A.J. Olson) Stalpers, produces conidia. Conidia are 3.8–6.6 × 2.8–5.0 µm in size (Černý 1989, Ryvarden and Gilbertson 1994). Disease symptoms, e.g. exhudation of resin, crown deterioration due to *Heterobasidion* root rot in living trees are not particularly characteristic and in many cases cannot be distinguished from those caused by other root pathogens, *Armillaria* spp. (Asiegbu et al. 2005).

In the past 40 years, *H. annosum* s.l. has been the object of more than 1,700 scientific papers, which makes it one of the most intensively studied forest fungi. The complete genome sequence of the fungus is now available, making *H. irregular* the first sequenced plant pathogenic homobasidiomycete (Garbelotto and Gonthier 2013).

Heterobasidion annosum is able to infect a tree by airborne spores which colonize freshly cut stumps creating an effective platform for the fungus to spread to living trees over the root system (Stenlid and Redfern 1998, Blanchard and Tattar 1997). Nevertheless, pathogen is not able to grow freely in the soil and hence direct root contact among tress is vital for

secondary infection (Garbelotto and Gonthier 2013). In contrast, *H. annosum* is often outcompeted by other species in the colonization of stem wounds (Isomaki and Kallio 1974). The root rot intensifies in stand after the second and subsequent thinnings because of local production of spores although Gunulf et al. (2013) proved that infection takes a significant position already in precommercial thinning if the small stumps are not treated. Infection risk is critical in warmer seasons when a higher spore load is present in the air. Success of airborne infection can be reduced by applying chemical or biological treatments on the freshly cut stumps (Holdenrieder and Grieg 1998). Therefore several methods preventing the spore dispersal has been developed. Silvicultural methods (e.g. stump removal), chemicals (urea, borates) and biological control agent (*Phlebiopsis gigantea*, marketed as PG Suspension in the UK, PG IBL in Poland and Rotstop in Fennoscandia) are commonly used approaches for minimizing the disease spread (Asiegbu et al. 2005).

Heterobasidion is an important risk factor on spruce or pine stands in first rotation placed on former agricultural lands (Oliva et al. 2008, Sierota 2013). The problem is caused by high fertility (high nitrogen concentration) and drainage of such soils or by variable soil-moisture content during the year. The sandy loam containing little organic matter is also sensitive to *Heterobasidion* infection. The extensive problems of *Heterobasidion* infections are frequent in plantations and monocultures of conifers in Scandinavia, Baltic countries, Spain and Poland (Łakomy and Werner 2003; Lygis et al. 2004; Oliva et al. 2008; Vasiliauskas and Stenlid 1998; Vasaitis et al. 2008; Arhipova et al. 2011; Mesanza 2012; Oliva et al. 2013, Sierota 2013). In Europe, the problem of *Heterobasidion* rot has been studied in many countries. The substantial attention to the disease is paid in Fennoscandia, Italy, UK, Lithuania, Poland, Switzerland (eg. Asiegbu et al. 2005; Bendel et al. 2006; Gonthier et al. 2001, 2003) and minor data has been published also from other countries – eg. Bulgaria, Byelorussia, Estonia, Slovenia (Korhonen et al. 1992; La Porta et al. 1998, Munda 1994, Kadunc 2007).

1.2 CONTROL OF DISEASE

Basidiospores of *H. annosum* s.l. often colonise freshly cut stumps in managed forest stands. The stump surfaces of *Picea abies* and *Pinus sylvestris* are highly susceptible to infection during approximately the first 3-4 weeks after cutting (Rishbeth 1951, Schönhar 1979). After germination, *H. annosum* s.l. is able to spread to neighbouring trees via connecting root systems (Rishbeth 1951, Redfern & Stenlid 1998). The initial stage of colonization is therefore a crucial stage of the pathogen life cycle where a control method can be applied to minimize the damages to the managed forest stand.

I. **Stump removal** – radical method to clean a site from possible inoculum. It has to be carried out precisely as the fungus can survive and carry over disease to the subsequent stand even in 1 cm thick root (Greig 1984, Korhonen and Stenlid 1998). Long term stump removal experiments done in Canada, Sweden and Denmark by Cleary et al. (2013) show stump removal efficacy 20-87%.

II. **Chemical control** – sodium tetraborate decahydrate (borax), disodium octoborate tetrahydrate (DOT) and urea have proved to be effective (Korhonen et al. 1994, Oliva et al. 2010, Woodward et al. 1998) and are widely used in practical forestry within manual or mechanical stump treatment programs. Mode of actions include direct effect of borates on fungal metabolism; a temporary increase in the pH of the stump surface, resulting in inhibition of spore germination in case of the urea treatment.

III. **Biological control** – a number of fungal species (*Phlebiopsis gigantea*, *Bjerkandera adusta, Fomitopsis pinicola, Resinicium bicolor*, *Hypholoma* spp., *Trichoderma* spp., *Scytalidium* spp.) have been tested on stumps as competitors and antagonists against *H. annosum* (Holdenrieder and Greig 1998, Holmer et al. 1997, Kallio and Hallaksella 1979, Nicolotti and Varese 1996, Nicolotti et al. 1999). Among these, *P. gigantea* has been shown the best antagonistic species currently used as disease control agent. *P. gigantea* is currently available in three different commercial

products (PG Suspension® in the UK, PG IBL® in Poland, Rotstop® in Fennoscandia) across Europe (Pratt et al. 2000), however these products are not registered in all countries and consequently their use is limited.

Thor and Stenlid (2005) showed that the treatment reduced *H. annosum* s.l. colonisation on stump surfaces by 89-99% compared to untreated stumps. However, the efficacy of different *P. gigantea* strains against *H. annosum* s.l. spore infections of stumps varies with local environmental conditions (especially humidity) and fluctuating spore dispersal of *H. annosum* s.l. (Rönnberg et al. 2006). There are also reports that there is variation between different strains of *P. gigantea* in the ability to prevent *H. annosum* s.l. infections of stumps (Berglund et al. 2005).

IV. **Integrated management** – combining different approaches to fight Heterobasidion root rot and butt rot are generally more effective and even cheaper than single control methods (Gonthier and Thor 2013). Different strategies based on silvicultural methods have been recommended:

(*a*) As widest as possible spacing among planted trees to lower the probability of root contacts.

(*b*) The delay of thinning and selecting the suitable season for thinning, when the spores are absent in the ecosystem (winter cuts, temperatures around 0-5°C).

(*c*) Reduction of rotation length. Pratt and Greig (1988) reduced felling ages by 20 and 25 years and it resulted in loses of potential discounted revenues of 53% and 74%, respectively.

(*d*) Admixing more broadleaved trees into the stand (Woodward et al. 1998). This strategy may not completely eliminate the pathogen after a single rotation (Lygis et al. 2004) but should significantly and progressively reduce disease incidence (Garbelotto and Gonthier 2013). On the other hand, Huse (1983) and Arhipova (2011) found that the level of mixture with other tree species in the composition of *Picea abies* stand had no influence on incidence of the rot.

1.3 HOSTS

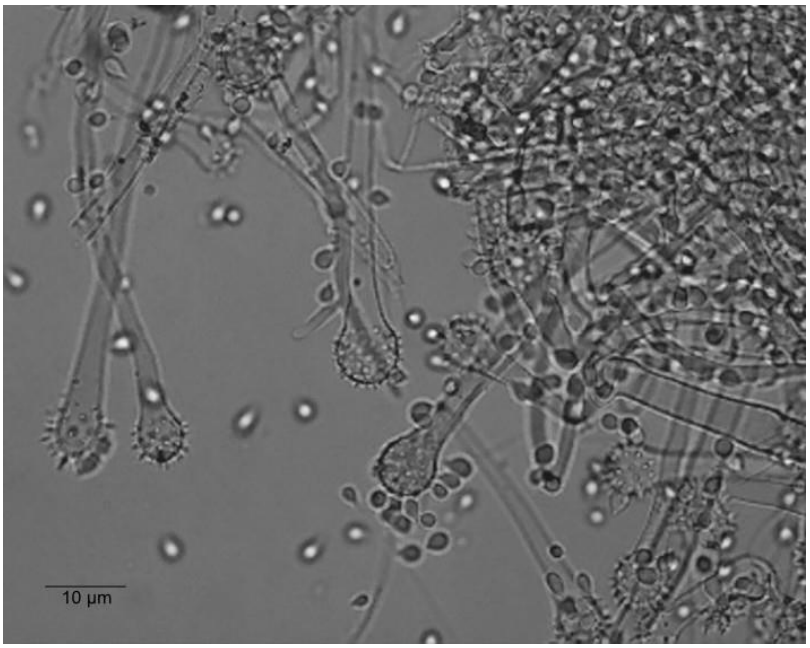

Figure 2. Conidiophores with conidia of the anamorph of *H. annosum* s.s. scale bar-10 um. (Photo by Petr Sedlák)

Since the first description of *H. annosum* as a destructive root and butt rot of European conifers (Hartig 1874), the recorded host range has broadened considerably to include many other gymnosperms as numerous hardwood, woody shrubs, and herbaceous hosts. *Heterobasidion* rot has been reported from more than 200 species of woody plant including ca. 45 species of pine 25 species of fir and 10 species of spruce (Pilát 1936, Reid 1978, Kotlaba 1984, Webb and Alexander 1985). Many of these records come from introduced tree species in areas outside original distribution. Reid (1978) suspected occurrence of pathogen on deciduous tree species, eg. *Acer pseudoplatanus, Betula* spp., *Crataegus* spp., *Fagus sylvatica, Fraxinus excelsior, Salix caprea,* and *Quercus* spp., due to spreading of pathogen from adjacent conifers. In Europe, *Heterobasidion* sp. is present in almost all coniferous forests. Among native tree species it attacks especially *Picea abies* (mainly *H. parviporum*) and *Pinus* spp. *(H. annosum* s.s.*)*, *Abies* spp. (*H. abietinum*), *Larix* spp. and *Juniperus communis* (both, *H. annosum* s.l.). Deciduous trees are much less susceptible to *Heterobasidion* infections, but *Betula pendula*, the introduced *Quercus rubra*, *Populus tremula* and other species, can be attacked when growing

in mixtures with conifers or on unsuitable sites, or when suffering from heavy industrial pollution (Korhonen and Stenlid 1998).

The distribution of *Heterobasidion annosum* s.l. at species level has not been sufficiently studied in the Czech Republic. The prevailing hosts are *Picea abies* or *Picea* spp. (Černý 1989) but the pathogen is reported on other conifers and hardwoods – eg. *Alnus glutinosa*, *Acer pseudoplatanus, Betula pendula, Fraxinus* spp., *Populus tremula* and *Salix caprea* (Kotlaba, 1984).

Nevertheless, there have been few records from other habitats – underground spaces. These records of *Heterobasidion* from underground spaces as caves and tunnels are rather rare. Detailed studies of microscopic fungi in remote sediments of West Virginia caves, USA (Rutherford and Huang 1994), in various cave substrates including cave air in NP Slovak Karst, Slovakia (Nováková 2009, Nováková 2012), and the Doña de Trinidad Cave, Spain (Hermosín et al. 2010) as well as in cave air and bat guano in Gruta Lapa Nova, Brasil (Taylor et al. 2013) or in the Nazi military complex Osówka in Poland (Pusz et al. 2013) did not report this fungus. Vanderwolf et al. (2013) in their extensive worldwide review cited 8 records of *Heterobasidion* sp. from underground spaces, but only from decayed wood (Great Britain, Poland, Austria, Slovenia, Croatia, Bosnia, Czech Republic, and Romania). The record of *Heterobasidion annosum* basidiocarps on spruce timber in mines of the Kladno coal district (Czech Republic,) was mentioned by Příhoda (1965). The report of *Spiniger meineckellus* from the air of underground tunnel under Bedřichov, Czech Republic was published by Kubátová et al. (2005).

1.4 WOOD INHABITING FUNGI IN LIVING NORWAY SPRUCE

Forest pathologists have been interested in wood inhabiting fungi since Hartig published his first study on forest pathogens in the late 1800s (Hartig 1894). Wood inhabiting fungi play a significant role in the forest ecosystem. They are involved in various nutrient cycles, decomposition of biomass, forest regeneration and improvement of the soil quality

(Gonthier et al. 2013). In contrast, several wood decay fungi are threats for commercial forests due to their pathogenic life strategy. The conditions in plantations or forest management under changed tree composition favor the development of fungal infection and conduction of serious economic timber losses. Additionally, urban trees are often susceptible to wood decay fungi where the tree or limb failures can lead to property damage and/or injury (Schmidt et al. 2012). Different management strategies had been introduced to minimize such damages caused by the various fungi species and its pathogenic activity (Leach 1939, Rishbeth 1951, Etheridge 1969, Černý 1989, Shaw et al. 1991, Holdenrieder and Greig 1998, Berglund et al. 2005,). Although, the successful management strategy can be implemented only in case of accurate and timely diagnosis to significant reduction of the damages on forest stands in great scale or tree or limb failure in the individual scale (Lonsdale 1999, Schwarze et al. 2004). Techniques based on fungal DNA detection are rapid, sensitive and accurate alternative for routine diagnosis directly from wood samples. Methods of amplification taxon-specific primers have been already used for identification and rapid detection of fungal decay directly from the wood (Johannesson and Stenlid 1998, Jasalavich et al., 2000, Moreth and Schmidt 2001, Gonthier et al., 2003, Guglielmo et al. 2007, Guglielmo et al., 2008, Nicolotti et al., 2009). The identification of decay fungi associated with suspect trees provides important information for assessing structure stability and predicting the probability of failure or decline (Glaeser and Lindner 2010). The identification of potentially infected trees is mainly based on visual tree assessment, an approach based on visual inspection of signs and symptoms associated with anomalies in the structure of trees. However, these symptoms rarely allow the detection of incipient decay or the identification of the rotting fungi involved (Morrison 1981, Černý 1989, Shaw et al., 1991, Schwartze 2000, Kolařík 2005). This study reviews a molecular diagnostic approach for the detection and identification of some of the most abundant wood decay fungi in the living spruce under the conditions in the Czech Republic, including not only *Heterobasidion annosum* sensu lato, but also *Armillaria* spp., *Porodaedalea chrysoloma*, *Fomitopsis pinicola*, *Stereum sanquinolentum* and *Onnia* spp.

2 OBJECTIVES

I. Investigate the species distribution of necrotrophic wood decaying basidiomycete, *Heterobasidion annosum* s.l., in the Czech Republic (**paper I**).

II. Inspect the host spectrum and evaluate the most diseased forest stands and localities/regions caused by this pathogen (**paper I).**

III. Investigate the distribution of *Heterobasidion annosum sensu lato* from extraordinary habitats, unusual hosts and substrates and evaluate the real paths of the pathogen into the habitats (**paper II**).

IV. Investigate the occurence of *Heterobasidon* sp. and other selected wood decaying fungi in living trees (*Picea abies* (L.) Karsten) using multiplex PCR.

3 MATERIAL AND METHODS

3.1 SPECIES DISTRIBUTION, HOST AFFINITY AND GENETIC VARIABILITY OF *HETEROBASIDION ANNOSUM* SENSU LATO IN THE CZECH REPUBLIC (PAPER I)

In **paper I**, basidiocarps of *Heterobasidion* spp. were collected by random sampling in either, natural forest stands and artificial forests in the Czech Republic. Attention was paid to explore the majority of different biotopes presented in the Czech Republic from south to north and from east to west. Wind throws, symptomatic trees and decayed stumps were checked for the presence of fruit bodies at each site visited. Cultures were isolated from some field specimens on malt extract agar (HiMedia, India). We also examined *Heterobasidion* isolates held in the Czech culture collections CCF (Charles University, Prague) and CPPF (Crop Research Institute, Prague), some of which were obtained from unusual hosts: grape wine (*Vitis vinifera*), maize (*Zea mays*) and apple tree (*Malus sylvestris*). The fungal isolates, their origins, substrates and identifications according to partuclar genes are summarised in Table 1. In addition, four basidiocarp collections were borrowed from the herbaria BRNM (the Moravian Museum, Brno), PRM (the National Museum, Prague) and MJ (The Museum of the Bohemian-Moravian Highlands, Jihlava). A total of 110 specimens were used for this study.

After collection, each sample was dried for approximately 24 hours at 54 °C. The influence of altitude on the distribution of the three species was tested using a one-way analysis of variance (ANOVA). The null hypothesis of no relation between altitude and distribution of the species was not rejected. DNA was isolated from pure cultures and dried basidiocarps using the PowerSoil™ DNA Isolation Kit (Mo-Bio, Carlsbad, USA) and the QIAGEN DNeasy Plant Mini Kit, according to the manufacturers' instructions.

Table 1. List of specimens included in the study*.

SPECIMEN	LOCATION	SUBSTRATE	ALTITUDE	IDENTIFICATION		
				EFA	TF	G3P
CZ134	Rotava	*Picea abies*	613	*a*	*a*	*a*
CZ135	Nejdek	*Picea abies*	835	*p*	*p*	*p*
CZ133	Nejdek	*Picea abies*	772	*p*	*p*	*p*
CZ136	PLA Slavkovský forest, Nová Ves	*Picea abies*	840	*p*	*p*	*p*
CZ139	Stříbro	*Pinus sylvestris*	498	*a*	*a*	*a*
CZ137	Pluhův bor NNR, Louka	*Picea abies*	784	*a*	*a*	*a*
CZ70	Louny	*Picea abies*	420	*a*	*a*	*a*
CZ79	NP Bohemian forest, Stožec	*Picea abies*	800	*p*	*p*	*p*
CZ44	NP Bohemian forest, Trojmezná	*Picea abies*	1220	*p*	*p*	*p*
CZ119	NP Bohemian forest, Černý Kríž	*Picea abies*	740	*p*	*p*	*p*
CZ110	Řepešín	*Picea abies*	750	*a*	*a*	*a*
CZ145	Peruc	*Picea abies*	320	*a*	*a*	*a*
CZ130	Peruc	*Larix decidua*	320	*a*	*a*	*a*
CZ129	Peruc	*Pinus sylvestris*	325	*a*	*a*	*a*
CZ126	Kačice	*Picea abies*	425	*a*	*a*	*a*
CZ107	Husinec	*Pinus sylvestris*	540	*a*	*a*	*a*
CZ3	Český Krumlov	*Picea abies*	680	*a*	*a*	*a*
CZ143A	Prague, Ruzyně	*Zea mays*	345	*b*	*b*	*b*
CZ3368	Prague	indoor air	-	*p*	*p*	*b*
CZ345	Karlštejn	*Vitis vinifera*	-	*p*	*p*	*b*
CZ111	Hluboká nad Vltavou	*Picea abies*	385	*p*	*p*	*p*
CZ112	Hluboká nad Vltavou	*Pinus sylvestris*	390	*a*	*a*	*a*
CZ148	NP Bohemian Switzerland	*Pinus sylvestris*	425	*a*	*a*	*a*
CZ81	NR Baba, Dobřejovice	*Picea abies*	300	*p*	*a*	*p*
CZ115	Libníč	*Picea abies*	505	*p*	*p*	*p*
CZ95	PLA Třeboňsko, Chlum u Třeboně	*Picea abies*	475	*a*	*a*	*a*
CZ57	Žofínský prales NNR	*Picea abies*	799	*p*	*p*	*p*
CZ59	Žofínský prales NNR	*Abies alba*	852	*b*	*b*	*b*
CZ58	Žofínský prales NNR	*Picea abies*	826	*p*	*p*	*p*

CZ31	Žofínský prales NNR	*Abies alba*	750	*p*	*a*	*a*
CZ92	Žofínský prales NNR	*Picea abies*	750	*p*	*p*	*p*
CZ93	Žofínský prales NNR	*Abies alba*	750	*p*	*p*	*p*
CZ94	Žofínský prales NNR	*Picea abies*	750	*p*	*p*	*a*
CZ96	Žofínský prales NNR	*Picea abies*	750	*p*	*p*	*p*
CZ98	Žofínský prales NNR	*Picea abies*	750	*p*	*a*	*a*
CZ108	Klec	*Pinus sylvestris*	445	*a*	*a*	*a*
CZ125	Vestec	*Pinus sylvestris*	415	*a*	*a*	*a*
CZ123	Vestec	*Picea abies*	460	*a*	*a*	*a*
CZ25	Rataje nad Sázavou	*Abies alba*	400	*b*	*b*	*b*
CZ40	Černousy	*Picea abies*	350	*a*	*a*	*a*
CZ38	Oldřichov v Hájích	*Picea abies*	550	*p*	*p*	*p*
CZ51	Slavonice	*Picea abies*	610	*a*	*a*	*a*
CZ50	Slavonice	*Picea abies*	605	*p*	*p*	*p*
CZ257	Bítouchov u Semil	*Malus sylvestris*	430	*p*	*p*	*p*
CZ64	Cejle	*Picea abies*	808	*a*	*a*	*a*
CZ63	Stonařov	*Picea abies*	705	*p*	*p*	*p*
CZ69	Želetava	*Picea abies*	717	*p*	*p*	*p*
CZ5	Třebelovice	*Picea abies*	545	*a*	*a*	*a*
CZ48	Jackov	*Picea abies*	490	*a*	*a*	*a*
CZ72	Moravské Budějovice	*Picea abies*	560	*a*	*a*	*a*
CZ07H	NNR Ransko	*Picea abies*	560	*p*	*p*	*b*
CZ117	NP Podyjí, Čížov	*Pinus sylvestris*	405	*a*	*a*	*a*
CZ19	Slavice	*Picea abies*	590	*a*	*a*	*a*
CZ118	NP Podyjí, Hnanice	*Pinus sylvestris*	340	*a*	*a*	*a*
CZ03	Křižánky	*Picea abies*	640	*p*	*p*	*p*
CZ02	Křižánky	*Picea abies*	630	*p*	*a/ p*	*p*
CZ7	NNR Adršpach rocks, Zdoňov	*Betula pendula*	600	*a*	*a*	*a*
CZ122	Jinošov	*Picea abies*	520	*a*	*a*	*a*
CZ149	PLA Orlicke Mts., Lukavice	*Picea abies*	360	*p*	*p*	*p*
CZ16	Vysoké Popovice	*Pinus sylvestris*	513	*a*	*a*	*a*
CZ1078	Lískovec, Štěpánov nad Svratkou	*Salix caprea*	470	*a*	*a*	*a*

CZ1194	PLA Orlické Mts., Uhřínov	*Picea abies*	450	*b*	*b*	*b*
CZ75	Březová nad Svitavou	*Picea abies*	470	*p*	*p*	*p*
CZ55	Brno, Kamenný vrch	*Picea abies*	300	*b*	*b*	*b*
CZ6	Kuřim, Podlesí	*Picea abies*	470	*b*	*b*	*b*
CZ78	Kuřim, Podlesí	*Picea abies*	330	*b*	*b*	*b*
CZ76	Letovice	*Picea abies*	370	*p*	*p*	*p*
CZ1810	Brno, Mahenova stráň	*Picea abies*	300	*p*	*p*	*p*
CZ2409	Brno, Wilsons forest	*Pinus sylvestris*	250	*a*	*a*	*a*
CZ2309	Brno, Wilsons forest	*Picea abies*	252	*p*	*p*	*p*
CZ91	Brmo, Ořešín	*Picea abies*	345	*b*	*b*	*b*
CZ1709	Brno, Zaječí hora	*Picea abies*	290	*p*	*p*	*p*
CZ1910	Brno, Čertova rokle	*Picea abies*	280	*p*	*a*	*p*
CZ35	Brno, NR Coufavá	*Pinus sylvestris*	514	*b*	*b*	*b*
CZ80	Brno, NR Coufavá	*Fagus sylvatica*	510	*p*	*a*	*a*
CZ36	Brno, NR Coufavá	*Abies alba*	485	*b*	*b*	*b*
CZ77	Řícmanice	*Larix decidua*	380	*a*	*a*	*a*
CZ1	Kanice	*Picea abies*	315	*a*	*a*	*a*
CZ54	Řícmanice	*Larix decidua*	390	*a*	*a*	*a*
CZ1909	Brno, Říčka valley	*Picea abies*	300	*a*	*a*	*a*
CZ74	Lanškroun	*Picea abies*	335	*p*	*p*	*p*
CZ73	Babice	*Picea abies*	430	*a*	*a*	*a*
CZ1210	Brno, Říčka valley	*Picea abies*	350	*a*	*a*	*a*
CZ86	Lednice	*Pinus sylvestris*	165	*a*	*a*	*a*
CZ84	Lednice	*Pinus sylvestris*	160	*a*	*a*	*a*
CZ85	Lednice	*Pinus sylvestris*	185	*a*	*a*	*a*
CZ82	Lednice	*Pinus sylvestris*	180	*a*	*a*	*a*
CZ83	Lednice	*Pinus sylvestris*	180	*a*	*a*	*a*
CZ2710	Lednice	*Corylus avellana*	169	*a*	*a*	*a*
CZ100	Drahany	*Picea abies*	669	*b*	*b*	*b*
CZ87	Lednice	*Betula pendula*	160	*a*	*a*	*a*
CZ88	Lednice	*Chameacyparis law.*	160	*p*	*a*	*a*
CZ103	Ruprechtov	*Picea abies*	520	*b*	*b*	*b*

CZ104	Husinec	*Pinus sylvestris*	620	*a*	*a*	*a*
CZ46	Bučovice	*Picea abies*	370	*a*	*a*	*a*
CZ11	Bzenec	*Pinus sylvestris*	307	*a*	*a*	*a*
CZ14	Uherské Hradiště	*Picea abies*	295	*a*	*a*	*a*
CZ141	PLA White Carpathians, Suchov	*Quercus robur*	430	*a*	*a*	*a*
CZ04	Budišov nad Budišovkou	*Picea abies*	565	*a*	*a*	*a*
CZ144	PLA White Carpathians, Vápenky	*Picea abies*	440	*p*	*a*	*p*
CZ142	PLA White Carpathians, Vápenky	*Picea abies*	450	*a*	*a*	*a*
CZ143	PLA White Carpathians, Vápenky	*Larix decidua*	440	*a*	*a*	*a*
CZ89	NR Tesák, Vičanov	*Picea abies*	655	*a*	*a*	*a*
CZ90	NR Tesák, Vičanov	*Picea abies*	630	*p*	*a*	*a*
CZ01	Hradec nad Moravicí	*Fraxinus excelsior*	380	*a*	*a*	*a*
CZ21	Oznice	*Picea abies*	570	*a*	*a*	*a*
CZ131	Rožnov pod Radhoštěm	*Picea abies*	510	*p*	*p*	*p*
CZ42	Ostrava, Bělský forest	*Picea abies*	350	*a*	*a*	*a*
CZ43	Ostrava, Bělský forest	*Picea abies*	355	*p*	*p*	*p*
CZ30	PLA Razula, Javorníky	*Abies alba*	760	*b*	*a*	*b*

*The abbreviations *a*, *b* and *p* refer to *Heterobasidion annosum* s.s., *H. abietinum* and *H. parviporum*; NP, NNR, NR and PLA refer to National Park, National Nature Reserve, Nature Reserve and Protected Landscape area.

To identify the samples at the species level and assess their genetic diversity, we sequenced three nuclear gene regions: glyceraldehyde 3-phosphate dehydrogenase (G3P), translation elongation factor 1-alfa (EFA) and transcription factor (TF) (Dalman et al. 2010; Linzer et al. 2008). PCR conditions are mentioned in **paper I**. The minimum-evolution phylogenetic trees were constructed using MEGA software version 5 (Tamura et al. 2011) for EFA, G3P and TF separately. The sequence data obtained were analysed using two different approaches, Bayesian analysis and haplotype network construction. The haplotype networks were constructed using Network 4.6.1.1 (Fluxus Technology Ltd., Germany), including only sequences without heterozygous positions.

3.2 UNDERGROUND SPACES AS NEGLECTED NICHE FOR OCCURRENCE OF *HETEROBASIDION ANNOSUM* COMPLEX (PAPER II)

Figure 3. a) *Trachelipus troglobius* (photo by Petr Zajíček) b) Faecal pellets (faeces) of *Trachelipus troglobius* (photo by A. Nováková).

Seventeen *Heterobasidion* isolates (**paper II**) were obtained from 14 different caves and underground tunnels in the Czech Republic, Slovakia, Romania, and Spain during 2008-2012, see Tab. 2. Samples from caves and tunnels (cave sediment, bat droppings and guano, moonmilk, isopods faeces, wall swabs) were collected aseptically into sterile plastic bags, vessels or microtubes and they were kept cold during the transport to the laboratory. Air-borne microfungi were isolated using the gravity settling culture plate method (Buttner and Stetzenbach 1991) and SGA or DRBC agar (Atlas 2010) as the isolation media. Petri dishes were incubated in the dark at 10 and 25°C for 7-28 days. DNA was isolated from pure cultures using the PowerSoil™ DNA Isolation Kit (Mo-Bio, Carlsbad, USA) and the QIAGEN DNeasy Plant Mini Kit, according to the manufacturers' instructions.

To identify the samples at the species level and assess their genetic diversity, we sequenced three nuclear gene regions: glyceraldehyde 3-phosphate dehydrogenase (G3P), translation elongation factor 1-alfa (EFA) and transcription factor (TF) (Dalman et al. 2010; Linzer et al. 2008). PCR conditions are mentioned in **paper II**. The sequences were aligned manually using the freeware BioEdit (Hall 1997). Sequences published by Dalman et al. (2010) and Gonthier et al. (2007) from Genbank of EFA, G3P and TF gene were added to the alignment to ensure identification of the *Heterobasidion* species. The maximum likelihood (ML) analyses of the combined dataset were performed using the online

version PhyML 3.0 at Phylogeny.fr (Dereeper et al. 2008) using "A la Carte" module. The GTR substitution model was selected for the dataset and bootstrap branch support values (BP) were estimated in PHYML under the maximum likelihood criterion using default 100 replicates.

Table 2. List of examined specimens of *Heterobasidion* spp.

Isolate	Identification	Locality	Substrate	Year of isolation
CMF 1808	*H. parviporum*	Spain, The Castañar de Ibor Cave, Laberinto Norte	moonmilk	2009
CMF 1829	*H. annosum*	Czech Rep., Moravian Karst, The New Amateur Cave, Písečný dome	cave air	2009
CMF 1952	*H. parviporum*	Czech Rep., The Javoříčko Caves, passage to emergency exit	cave air	2010
CMF 1956	*H. parviporum*	Czech Rep., Moravian Karst, The Výpustek Cave, German Boiler Room	cave air	2010
CMF 1957	*H. parviporum*	Czech Rep., Moravian Karst, The Balcarka Cave, Gallery	cave air	2010
CMF 2084	*H. abietinum*	Slovakia, The Demänovská Piece Cave	outdoor air	2011
CMF 2143	*H. abietinum*	Slovakia, The Bobačka Cave, near the site "Whale"	bat guano	2011
CMF 2369	*H. annosum*	Romania, The Movile Cave, Lake Room	isopod faeces	2011
CMF 2377	*H. annosum*	Romania, The Movile Cave, Lake Room	isopod faeces	2011
CMF 2519	*H. parviporum*	Czech Rep., Moravian Karst, The Výpustek Cave, Low Passage	cave air	2012
CMF 2520	*H. parviporum*	Czech Rep., The Bozkov Dolomite Caves	outdoor air	2012
CMF 1953	*H. annosum*	Czech Rep., Moravian Karst, The Chýnov Cave, passage to Dragon´s Head.	cave air	2010
CMF 2522	*H. parviporum*	Czech Rep., Moravian Karst, The Kateřina´s Cave, entrance corridor	cave air	2012
CMF 2521	*H. parviporum*	Czech Rep., Moravian Karst, The Výpustek Cave, Henry´s Hall	cave air	2012
CMF 1740	*H. annosum*	Spain, The Altamira Cave, Walls Hall	cave sediment	2008
CCF 4741	*H. parviporum*	Czech Rep., Solenice, abandoned tunnel	wall	2010
CCF 4742	*H. annosum*	Czech Rep., Nový Knín, abandoned tunnel	wall	2010

3.3 OCCURENCE OF *HETEROBASIDON* SP. AND OTHER WOOD DECAYING FUNGI IN LIVING NORWAY SPRUCE TREES

In November 2013, the experimental site located in south Moravia, 20 km northeast of the city of Brno in the region of the Proklest ranger district, Forest District Křtiny, TFE Křtiny Masarykův les (49° 18' 48.9" N, 16° 46' 22.08" E) was evaluated for presence of some wood inhabiting fungi in living Norway Spruce (*Picea abies* (L) Karst.) trees using multiplex PCR method. The Proklest ranger district belongs to the 4th forest vegetation zone (FVZ) (93% of the stand area) and to the 3rd FVZ (7% of the stand area). Spruce covers about 60% of the stand area. The investigated *Picea abies* stand was 120 years old. In the stand, 11 trees were randomly selected for sampling. Some of the trees were damaged during previous selective cuttings and by big game.

The scheme used by Guglielmo et al. (2010) was used during the experiment. In total, 11 trees were sampled by drilling a hole at base of the stem and also in breast height (contrary to Guglielmo et al., 2010). For each tree, eight holes were bored, holes were always placed in main cardinal directions (N, S, W, E) using a magnetic compass. A 6-mm-diameter (commercially available), 40cm long wood auger was used to drill stem base, c. 5 cm aboveground, and in breast height. The marked spot was debarked before drilling with clean and sterile knife (cleaned by hypochlorite solution and 70% ethanol – see further). Sawdust generated during the drilling process was collected into sterile 15ml Falcon Conical Centrifuge Tube. Collected sawdust was further used as material for DNA extraction. After each drilling, wood auger was carefully cleaned by 10% sodium hypochlorite solution, Termi-DNA-tor (Biotools B&M Labs, S.A.) rinse with sterile water and wiped with 70% ethanol. Samples were then frozen in -20°C for a day before being placed in lyophilizer for a period of

3 days. After a 3-days of lyophilization, DNA from sawdust was extracted. Approximately 100 mg of frozen and dried sawdust was placed into sterile screw-top grinding jars with a 10mm steel ball a processed in a mixer mill Retsch MM 400 (Haan, Germany) by shaking for 90s at maximum vibration frequency (30Hz ~ 1800 min^{-1}). DNA from homogenized samples was extracted using PowerSoil™ DNA Isolation Kit (Mo-Bio, Carlsbad, USA), according to the manufacturers' instructions. DNA concentration was assessed with NanoDrop 1000 Spectrofotometer (Thermo Scientific, Waltham, MA, USA).

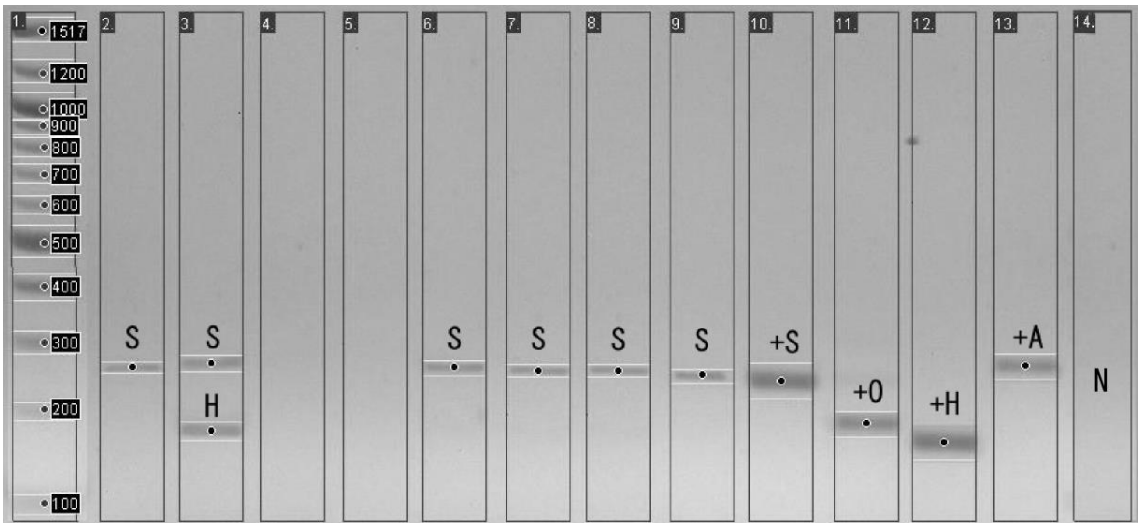

Figure 4. Multiplex (MC2) PCR products vizualized on a UV-gel documentation system after 2h electrophoresis. Graphics added in GenAnalyzer 2010.

Lane 1 - molecular weight marker 100bp; lane 4, 5 – no detection; lane 2, 6, 7, 8, 9 – detection of *Stereum* sp.; lane 3 – *Stereum* sp. and *Heterobasidion* sp.; +S, +O, +H, +A - positive controls; N - negative control.

The following 6 fungal taxa were selected as targets of multiplex PCR assay: *Armillaria* spp., *Fomitopsis pinicola*, *Heterobasidion annosum* sensu lato (*Heterobasidon annosum* s.s., *H. parviporum*, *H. abietinum*), *Onnia* spp. (*Onnia leporina*, *Onnia tomentosa*), *Porodaedalea* spp (*P. chrysoloma*, *P. pini*, *P. laricis*), and *Stereum sanquinolentum*.

Three taxon-specific reverse primers (Gonthier et al. 2014) of the ITS1 region were used to amplify, in combination with the forward primer ITS1f, rDNA fragments of *Fomitopsis pinicola* and *Porodaedalea* spp. (Table 3). These primers were combinated in a multiplex PCR, MC1. It also includes the universal reverse primer ITS4 as a positive control for positive fungal DNA extraction. Similarly, PCR for detection *Armillaria* spp., *Heterobasidion* spp., and *Onnia* spp. was drafted. The forward primer ITS3 and fragments of ITS II region were amplified. The multiplex

PCR, named MC2, in our study also included primer Ste2R designed for the detection of *Stereum* spp. (Guglielmo et al. 2007). These primer combinations allowed amplifying taxon-specific DNA fragment easily detectable after gel electrophoresis. PCR conditions were for MC1 as follows: 5 min denaturation at 95°C; 35 cycles of: 30 s at 95°C, 45 s at 55°C, 45 s at 72°C; 10 min final extension at 72°C; for MC2: 5 min denaturation at 95°C; 35 cycles of: 30 s at 95°C, 45 s at 62°C, 45 s at 72°C; 10 min final extension at 72°C. Except for *Stereum* and *Armillaria* spp., where the amplicons ranged 234 to 240bp and from 253bp, respectively. Therefore, when ambiguous fragments appeared, primers AR1 and AR2 designed by Lochman et al. (2004), were used for confirmation of identification the fragment. The PCR products were electrophoresed in 2.5% agarose gel in TBE buffer at 3V/cm for approximately 2h. MC1 and MC2 product were visualized on a UV-gel documentation system. GelAnalyzer 2010 (See Fig. 4) was used to determine lengths of fragments. To confirm some of identifications we sequenced amplified fragment of ITS region in Macrogen (Seoul, S Korea).

Table 3. Multiplex PCR primers combination and diagnostic purpose.

Multiplex PCR name	Primer combination		Taxon-specific amplicon size (bp)[3]	Diagnostic purpose
	Forward	Reverse[2]		
MC-1	ITS1f[1]	ITS4[1]	-	Fungi
		Fpi1r[4] (GAAGACCCCACTCCAATC)	193	*Fomitopsis pinicola*
		Por1r[4] (CACTACTAACAAAGTCAACC)	220-222	*Porodaedalea* spp.
MC-2	ITS3[1]	Het1r[4] (GCGCTTTCACAAGAAAAGC)	162	*Heterobasidion* spp.
		Onn2r[4] (AAGTCCCTTTTCCTTTCAGT)	183	*Onnia leporina*, *O. tomentosa*
		Armi3r[4] (GCCTAGCAGCCARAGTCA)	253	*Armillaria* spp.
		Ste2R[5] (GTCGCAACAAGACGCACTAA)	231-236	*Stereum* spp.

[1]Nucleotidic sequences of primers ITS1f, ITS3 and ITS4 are reported in Gardes and Bruns (1993) and White et al. (1990).
[2]Nucleotidic sequences (5'→3') of reverse primers are reported in brackets.
[3]Amplicon sizes are indicated in base pairs (bp); no amplicon size is reported for ITS4 since this primer is not specific to a taxon but allows amplifying, in combination with the primer ITS1f, the fungal rDNA.
[4]Nucleotidic sequences of primers are reported in Gonthier et al. (2014)
[5] Nucleotidic sequences of primers are reported in Guglielmo et al. (2007)

4 RESULTS AND DISCUSSION

4.1 SPECIES DISTRIBUTION, HOST AFFINITY AND GENETIC VARIABILITY OF *HETEROBASIDION ANNOSUM* SENSU LATO IN THE CZECH REPUBLIC (PAPER I)

For **paper I,** total of 110 samples, comprising 330 sequences, were used. All loci were successfully amplified, with the exception of the TF and G3P regions from 12 herbarium specimens, due to the lower quality of their DNA. Our data recognised the occurrence of all three European *Heterobasidon* species in the Czech Republic. Some specimens included either sequences with heterozygous nucleotides at species-specific positions or genotypes of different genes corresponding to different species. Such specimens were treated as interspecific hybrids. The hybrid specimens were well separated in both the phylogenetic tree and the median-network diagrams (Fig. 5). The geographic distribution of the samples used in this study is shown (See paper I, Fig. 3). Most of the

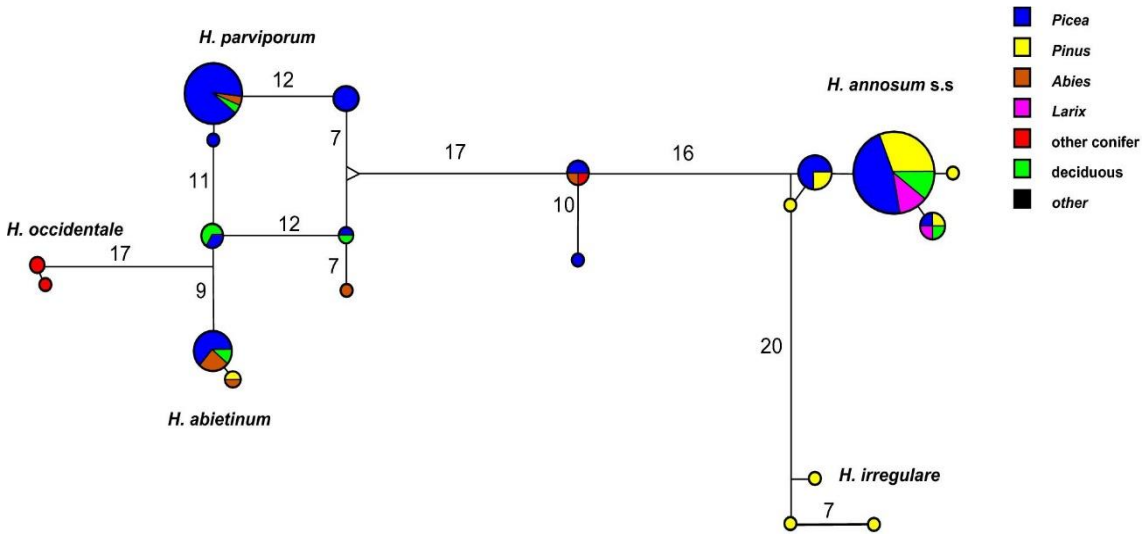

Figure 5. Unrooted median-joining (MJ) haplotype networks for connected alignement of TF, G3P and EFA was constructed using Network 4.6.1.1. (Fluxus Technology Ltd, Kiel, Germany). The sizes of the nodes are proportional to the number of isolates in each haplotype. Numbers indicate the numbers of mutations between haplotypes and values above one are shown.

specimens analysed (56 specimens, 51 % of the total of 110) were identified as *H. annosum* s.s., while 36 specimens (33 %) fell into the *H. parviporum* clade, 12 specimens (11 %) fell into *H. abietinum* and 6 specimens (5 %) were interspecific hybrids.

H. annosum s.s. was collected from the widest range of hosts: *Picea abies, Pinus sylvestris, Larix decidua*, *Betula pendula*, *Alnus glutinosa*, *Corylus avelana*, *Fraxinus excelsior* and *Quercus robur*. Some herbarium specimens, which we were able to amplify only with EFA primers and whose sequences were identified as *H. annosum* s.s., were collected from the following hosts: *Abies alba, Ligustrum vulgare, Acer spp.*, *Prunus domestica*, *Pinus nigra* and *Prunus avium*. These sequences were not included in our Bayesian and haplotype network analyses. We recorded *H. parviporum* on *Picea abies*, *Abies alba, Fagus sylvatica, Vitis vinifera* and *Malus sylvestris*. *H. abietinum* was collected on *Picea abies*, *Abies alba* and *Pinus sylvestris* and was documented on *Zea mays*. The interspecific hybrids were found on the following hosts: *Abies alba, Picea abies,* and *Chamaecyparis lawsoniana*.

In the Czech Republic, *H. annosum* s.l. has been found at a wide range of altitudes (160–1,220 m) and in various types of soil. The median altitudes at which *H. annosum*, *H. parviporum* and *H. abietinum* has been found are 463.5 msl, 560 msl and 477.5 msl, respectively. Furthermore, there is a statistically significant difference in the vertical distribution only within *H. annosum* and *H. parviporum* (one-way ANOVA; F=8.7061, p=0.0037).

Our results confirm the occurrence of hybrids among the three *Heterobasidion* species, although the F_{ST} values of nucleotide data indicate only rare gene flow. In Europe, 4-10% interfertility between *H. parviporum* and *H. annosum* has been reported and even higher value of 37% has been recorder when analysing *H. parviporum* and *H. abietinum* (Korhonen and Stenlid, 1998). The possibility of hybridisation between *H. abietinum* and *H. parviporum* due to partial compatibility of interspecific mating in vitro was repeatedly confirmed (Dai et al. 2003, Oliva et al. 2011). We also revealed sympatrical co-occurrence of both species in some localities, which enables inter-specific mating to occur.

ECOLOGICAL PATTERNS (NOT PUBLISHED)

Investigation of ecological patterns in the distribution of *Heterobasidon* infection in the Czech Republic was not primary target of the study. Therefore, it is mentioned here as by-product and it would certainly require detailed survey. Nevertheless, some results can be interpret.

The occurrence of *Heterobasidion* infection in forest stand depends on many factors. Stand type, management history, tree species composition, soil properties, elevation, slope and other factors take a part in incidence of the pathogen (Woodward et al. 1998). Soil fertility correlates with the occurrence of *H. annosum* very well. High calcium content and high pH is favourable to *H. annosum* s.l. infection, mainly due to the lack of antagonistic fungi (Korhonen and Stenlid 1998). Incidence is also reported to be higher in mineral soils with a fluctuating water regime (Thor 2005). On the other hand, the risk of infection is lower in peat soils (low pH and activity of antagonistic fungi) and in high altitudes (climatic factors and shorter growing season) (Korhonen and Stelind 1998, Thor and Stenlid 2005).

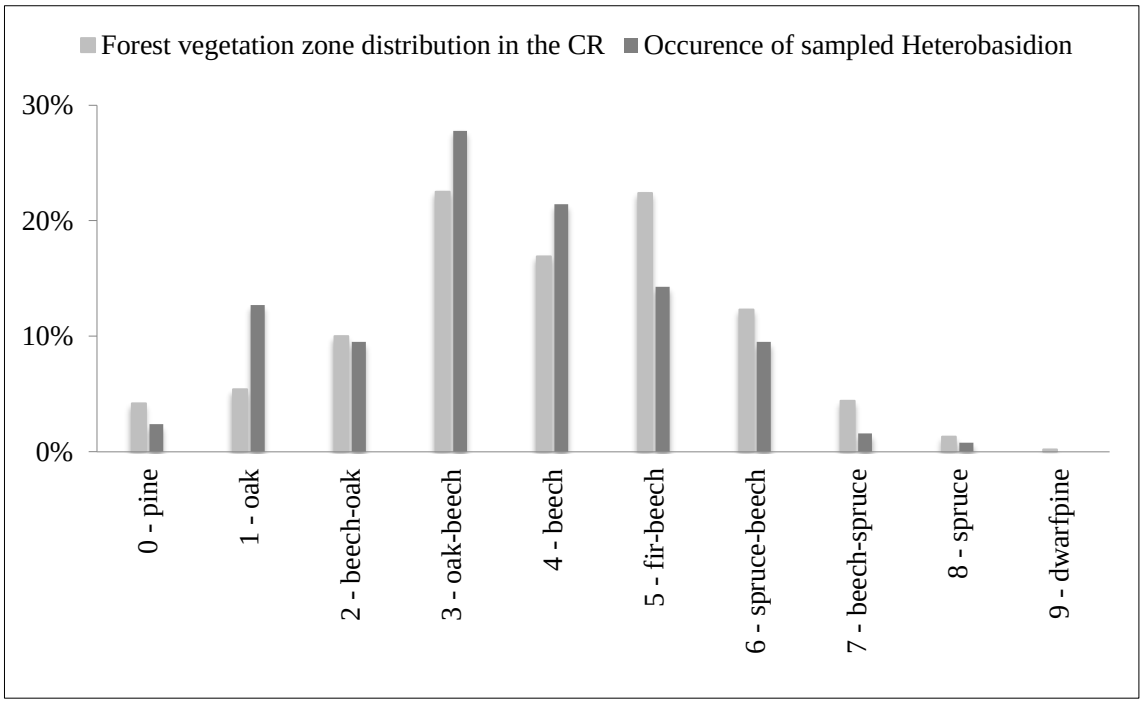

Figure 6. Comparison of distribution of Forest vegetation zones and occurrence of *Heterobasidion* samples.

Groups of forest types (GFT) were used for ecological classification of pathogen distribution. Ecological data were collected during sampling and ecological pattern of distribution of *Heterobasidion* was investigated.

Collected and analyzed data based on investigated layers revealed no ecological predisposition in various ecological conditions which corresponds to previous research (Woodward et al. 1998, Thor 2005). The occurrence of infection in different forest vegetation zones is shown in Fig. 6. Distribution of *Heterobasidion* copies the distribution of forest vegetation zones in the area of the Czech Republic. There has been no statistically significant difference found in this pattern (one-way ANOVA; F=0.025, p=0.992).

On the other hand, frequency of pathogen incidence in forest vegetation zones refers to certain model. The abundance has been highest in lower forest vegetation zones (See Fig. 7) with low natural (potential) distribution of Norway spruce. Occurrence of *H. parviporum* was shifted into higher altitudes contrasting to *H. annosum* and *H. abietinum*, as was already shown previously (**paper I**).

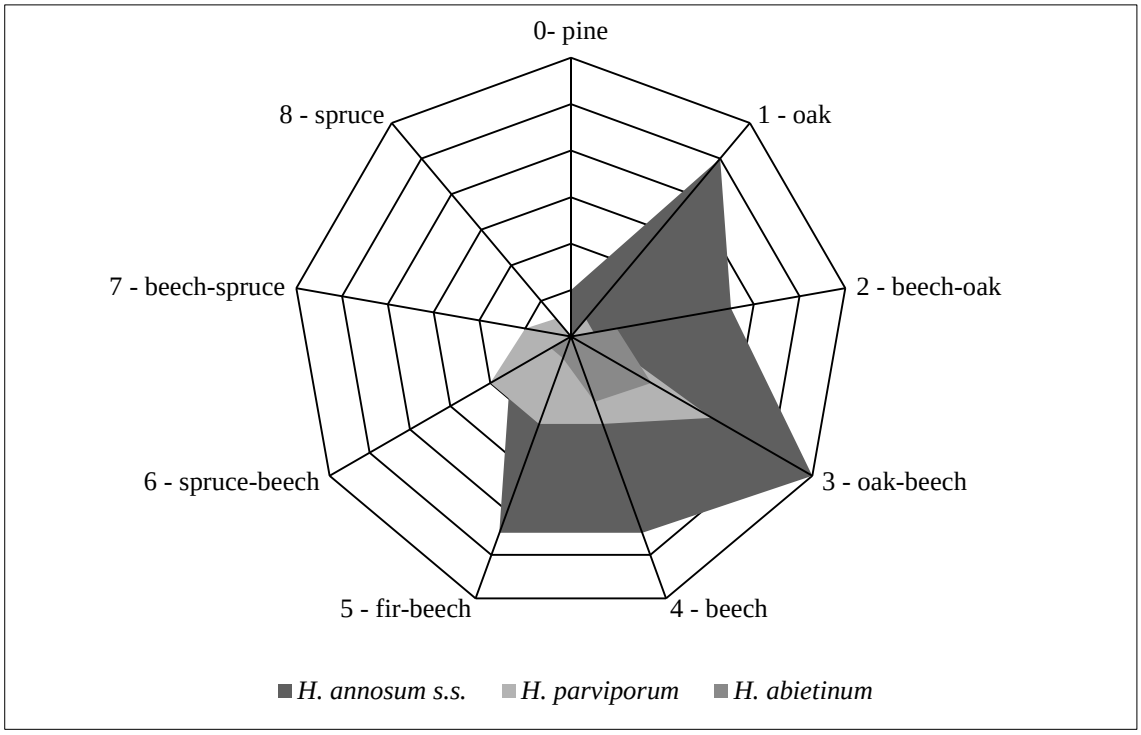

Figure 7. Abundance of *H. annosum* s.l. in forest vegetation zones in the Czech Republic.

Shaw et al. (1994) mentioned that *H. annosum* is less pathogenic and plays a more subordinate role in natural forest ecosystems than in intensively managed forests, characterized by monocultures and plantations.

Distribution of *Heterobasidon* species in different ecological series (See Fig. 8) has shown the same pattern as distribution of the pathogen in forest vegetation zones. The distribution of the pathogen correlates with the distribution of different ecological series. No statistical significant difference has been found in this pattern (one-way ANOVA; F=0.36, p=0.862).

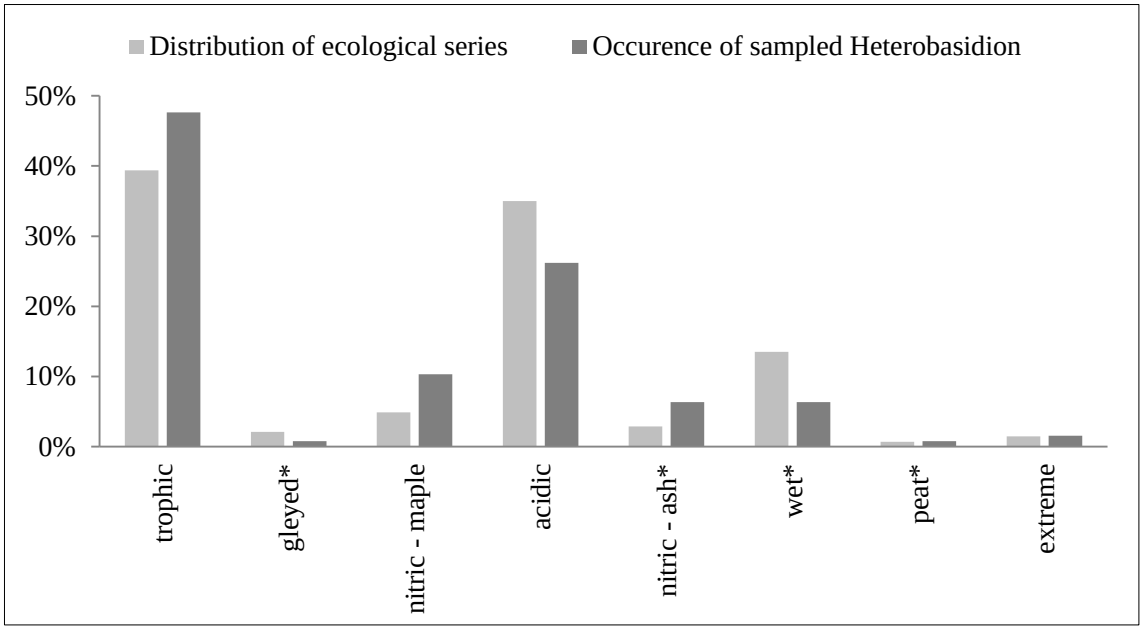

Figure 8. Comparison of distribution of ecological series and occurrence of *Heterobasidion* samples. Values with asterisk indicate influence of water.

Presented data indicate how difficult it is to interpret the site predisposition to the presence of *Heterobasidion*. This fact very well corresponds with other authors (Korhonen and Stenlid 1998, Thor 2005, Kadunc 2007) and it only displays the complexity of the problem. On the other hand, water distribution in soil plays a significant role and trees became highly susceptible to Heterobasidion infection especially during summer droughts (Korhonen and Stenlid 1998, Puddu et al. 2003).

RAPID IDENTIFICATION BY PCR – HAF/HAR PRIMERS (NOT PUBLISHED)

For rapid identification of *Heterobasidion annosum s.l.* we used taxon-specific competitive-priming (TSCP-PCR) method modified according to (Gonthier et al. 2003). However, according to ambiguous results of TSCP-PCR in discrimination of *H. abietinum* and *H. parviporum* new pair of specific primers HAF/HAR (see Tab. 4) was designed. These primers were selected from a heterogenic part of translation elongation factor 1-alpha gene sequence and were designed for the differentation of the *H. abietinum* and *H. parviporum*. PCR was performed in the following 25 ul reaction mixture: approximately 50 ng of template DNA, 0,5 umol of each primer (HAF/HAR), 1 mM of dNTP, 2 mM $MgCL_2$, 1× NH_4 Buffer and 1 U Diamond DNA Polymerase (Bioline, UK). The PCR program used was: 3 min at 95 °C, followed by 35 cycles of 40 s at 95 °C, 20 s at 64 °C, and 20 s at 72 °C with a final extension of 7 min at 72 °C. PCR products were electrophoresed in 1 % agarose gel in TBE buffer at 5 V/cm for approximately 0.5 hour. Final product is 134 bp long and is specific for *H. abietinum*.

Table 4. HAF, HAR PCR primers.

Primer	Sequence (5´- 3´)	region
HAF	TATCGACAAGCGTACCATCG	Elongation factor 1 – alpha
HAR	CTGTATCACCACATGGATCAGAA	Elongation factor 1 – alpha

Combination of TSCP-PCR and HAF/HAR specific primers has shown satisfying results for rapid identification of the three European species of *Heterobasidion annosum* s.l.

4.2 UNDERGROUND SPACES AS NEGLECTED NICHE FOR OCCURRENCE OF *HETEROBASIDION ANNOSUM* COMPLEX (PAPER II)

In this study, 17 isolates originated from caves or underground galleries were examined. Most of isolates analyzed (9) were identified as *H. parviporum*, with isolates obtained from moonmilk, cave and outside air, and wall from underground gallery. Six isolates were identified as *H. annosum*. These isolates were obtained from cave air and sediment, isopod faeces and underground gallery wall. *Heterobasidion abietinum* was obtained from Slovakian caves and was isolated from bat guano and outdoor air.

The origin and main ways that *Heterobasidion* isolates spread in underground spaces are not clear. The fungal basidiospores or conidia may persist in underground environment in air, on sediment, roots of trees or wood substrates as mycelium with conidiophores. The most of inspected caves are surrounded by woods where conifers are present, so short-distance dispersal of fungal spores from the wood to underground spaces is probable. But the identified isolates from the Výpustek Cave (Czech Rep.) do not confirm this theory. While isolates from caves were identified as *H. parviporum*, isolates collected from roots and stumps of *Picea abies* in the neighbourhood of the Výpustek Cave were identified as *H. annosum* (Sedlák and Tomšovský 2014). However, the Výpustek Cave has exceptional history due to its utilization for military purposes in 1936-2001 (Cave Administration of the Czech Republic 2014). During this time, spruce wood for human activities infected by *H. parviporum* may have been transferred to the cave.

Also the record of *H. parviporum* from Castañar de Ibor Cave worths a detailed discussion. On the contrary to Oliva et al. (2010), who report known distribution of *H. parviporum* in Spain as co-occurrence with its preferential host – *Picea abies*, this locality is several hundred kilometers apart from natural localities of *Picea abies* in Spain (Skrøppa 2003).

The Movile Cave in Romania is among mentioned caves the most unique (Chen et al., 2009) characterized by another food chain based on chemolithotrophic and chemoautotrophic bacteria and by completely different type of food chain known in aboveground ecosystems. The cave became isolated from surface (thick layer of loess had isolated the cave fauna from the surface forms) at the end of the Miocene (5.5 to 5.2. Mya)

therefore a unique ecosystem in the world had evolved. Isolates of *S. meineckellus* come from *Trachelipus troglobius* faeces (see paper II, Figure 2c) from more than 25m underground level of The Movile Cave. *Trachelipus troglobius* (paper II, Figure 9a) *(Isopoda, Oniscidea)* is an endemic troglobiont (cave-dwelling animal) inhabiting permanently the subterranean environment of the Movile Cave where the base of the trophic chain appears to be chemoautotrophic microbiota that uses H_2S from the thermomineral stream in the cave as a source of energy (Brustur 1999, Sarbu 1991). Organic compounds such as cellulose, lignin, and other organic compounds typical for plant tissues absent in this cave system which may affect enzyme production in *S. meineckellus* isolates. This may be a unique, relict population of Tertiary origin or a result of contamination by a recent inoculum. Although conditions of access to the cave are strict, therefore human-mediated transfer is less probable.

4.3 OCCURENCE OF *HETEROBASIDION* SP. AND OTHER WOOD DECAYING FUNGI IN LIVING NORWAY SPRUCE TREES (NOT PUBLISHED)

In total, 11 Norway spruce trees were examined for presence of *Heterobasidon* sp. and other wood inhabiting fungi. *Stereum* spp. was the most abundant species detected during investigation. Out of 88 holes, it was present in 59 cases (26 in base/33 in breast hight). *Fomitopsis pinicola* was present in 43 samples of wood dust (18/25), *Porodaedalea* spp. we detected in 34 samples (18/19), *Heterobasidion* spp. in 34 (16/18) wood dust samples, *Armillaria* spp. in 14 (9/5) samples and *Onnia* spp. was not detected. Fungal rDNA was amplified in 57 samples (29/28) and no fungal DNA was detected in 10 samples. Multiplex PCRs allowed identification of fungal taxa in wood samples (see Tab. 5). We confirmed practical usage of the multiplex PCR method for rapid identification of pathogens inside the living Norway spruce trees. Furthermore, there is no statistically significant differences in pathogen's distribution inside the trees (within base and breast height) (one-way ANOVA; F=4.0981, p=0.3408). Results obtained from two multiplex PCRs were confirmed with single PCR reactions and in some cases BLASTn analysis of the sequence of taxon-specific amplicons confirmed the multiplex PCR identification. Species of *Armillaria ostoyae, Heterobasidion annosum*, *Porodaedalea chrysoloma* and *Stereum sanguinolentum* were identified using sequencing of ITS region of some obtained amplicons. The presence of targeted wood inhabiting fungi in its particular distribution in trees is shown in Table 5.

Study has shown that modified multiplex PCR is an effective tool which can be used for rapid identification and detection of considered most destructive wood inhabiting fungi in the Czech Republic promptly in the field. The most often detected fungus was *Stereum* sp. *Stereum sanguinolentum* is a common causal agent of wound decay in Norway

spruce (Čermák et al. 2004, Arhipova 2011). In the stand, the base wounds were commonly observed (See Fig. 9), therefore *Stereum* sp. and other organisms had opportunity to infect the trees. According to our study, the largest volume of decay was cused by *Stereum* sp., which is in conflict with others (Hallaksela 1984, Stenlid 1984), where the volume of decay was studied and *Heterobasidion* sp. has the largest volume of decay. On the contrary, Vasiliauskas and Stenlid (1997) during investigation of fungi inhabiting stems of *Picea abies* found *Stereum sanguinolentum* as the most common species infecting butt wounds. *Armillaria* sp. was found in seven trees out of eleven. This is in agreement to an older study by Čermák et al. (2004) held on the same area that evaluated the stand as high risk from the probability of *Armillaria* infection. *Armillaria* sp. was found mostly on the base of the trunk (64%), but it was seldom detected also in breast height part of the stem (27%). The fact only confirms previous studies of occurrence of decay columns inside the trees, when *Armillaria*, as primary root fungus, infect the base and is able to grow in stem up to 2m height (Hallaksela 1984, Shaw et al. 1991, Solheim 2006). Interestingly, *Porodaedalea* sp. and *Fomitopsis pinicola* had higher occurrence than *Heterobasidion* sp. and *Armillaria* sp. Presence of *Porodaedalea chrysoloma* was confirmed in the field after felling sampled trees according to rot (characteristic white, rather large pockets). Nevertheless, the basidiomes of *Porodaedalea* were absent in the stand. The aim of this study was to verify the methodology by Gonthier et al. (2014) in the field, therefore the particular overaged Norway spruce stand was chosen for testing the method and thus high number of fungi was found. However, this result worths a detailed discussion.

Figure 9. One of the sampled Norway spruce (#3). Photo by P. Sedlák.

The technique of multiplex PCR confirmed presence of DNA belonging to wood inhabiting fungi inside the living tree. Vainio and Hantula (2000)

published data, where some decayed wood samples showed a completely different fungal community according to isolated mycelia pure cultures or by direct extraction of DNA from wood. This confirmed the selectivity of the culture conditions preferring the growth of certain fungal species, while selecting against other species that could only be detected using direct extraction of DNA or by new generation sequencing methods (Kubartová et al. 2012, Lindner et al. 2011).

Furthermore, more work is planned in detecting wood inhabiting fungi from living Norway spruce trees. More and younger stands will be selected for the experiment of detecting wood inhabiting fungi from drilling wood chips and inside. The collaboration with ongoing project detecting decay using Complex resistivity tomography (CRT) is planned on forest stands in Training Forest Enterprise Masaryk Forest Křtiny (TFE). Additionally, occurrence of selected wood inhabiting fungi through the gradient in wood disc (sapwood – heartwood) worths another study. Furter work has the potential for better understanding of decay process and colonization of the living stems by selected wood inhabiting fungi.

Table 5. Occurrence of targeted wood- inhabiting fungi in particular samples.

TREE	BASE				BREST HEIGHT			
Cardinal directions where drills were taken from	**N**	**S**	**W**	**E**	**N**	**S**	**W**	**E**
14	P H Fu	P S Fu	P A Fu	S	H S	S	S A	H
2	P F	-	P F H S Fu	P F H S A Fu	S P F Fu	S P F Fu	S P F H Fu	S P F. Fu
3	S H P F Fu	S H P F Fu	S H P F Fu	S F	S H	S H P F Fu	S H P F Fu	S P F Fu
10	-	S F Fu	-	S F P Fu	-	Fu	-	P F Fu
5	A S F Fu	A P F Fu	S F Fu	P F Fu	S F Fu	S F Fu	S P F Fu	S P F Fu
6	S H A P Fu	-	S H Fu	S A Fu	S H P F	S H P F Fu	S H F Fu	S H F Fu
7	-	P F Fu	A F	F Fu	P F	S P F Fu	A F	S P F Fu
8	S F Fu	-	S F	Fu	Fu	S H F Fu	-	S P F H Fu
9	S H Fu	S H A Fu	S Fu	S Fu	S F P Fu	S H Fu P F	S H Fu P F	S H Fu
11	H	H A	H S	H	H S Fu	H S	-	H S
12	S P Fu	S H P Fu	S P Fu	S H P Fu	S	S H Fu	S Fu A	A

A – *Armillaria* sp.
H – *Heterobasidion* sp.
F – *Fomitopsis pinicola*
Fu – unspecified fungal DNA
P – *Porodaedalea* chrysoloma
S – *Stereum sanguinolentum*

5 CONCLUSIONS

Figure 10. Consequences of root rot and butt rot infection on logs. Photo by Petr Sedlák, Ljunby, SW of Sweden.

The **paper I** confirmed distribution of three European species of *Heterobasidion annosum* s.l. in the Czech Republic. Most of the specimens were identified as *H. annosum s.s.* which has also the widest host spectrum. It has been documented on following host tree species: *Picea abies, Pinus sylvestris, Larix decidua*, *Betula pendula*, *Alnus glutinosa*, *Corylus avelana, Fraxinus excelsior, Quercus robur*, *Abies alba, Ligustrum vulgare, Acer spp.*, *Prunus domestica*, *Pinus nigra* and *Prunus avium*. We recorded *H. parviporum* on *Picea abies*, *Abies alba, Fagus sylvatica, Vitis vinifera* and *Malus sylvestris*. *H. abietinum* was collected on *Picea abies*, *Abies alba* and *Pinus sylvestris* and was documented on *Zea mays*. The interspecific hybrids were found on the following hosts: *Abies alba, Picea abies,* and *Chamaecyparis lawsoniana*. In the Czech Republic, *H. annosum* s.l. has been found at a wide range of altitudes (160–1.220 msl) and in various types of soil. The median altitudes at which *H. annosum*, *H. parviporum* and *H. abietinum* has been found are

463.5 msl, 560 msl and 477.5 msl, respectively. Furthermore, there is a statistically significant difference in the vertical distribution only within *H. annosum* and *H. parviporum*. Interestingly, it has been shown that *H. abietinum* can survive in localities with a changed tree composition (*Abies alba* → *Picea abies*) for decades.

Secondary stands, stands on former agriculture lands and conifer plantations and monocultures of Norway spruce and Scots pine in altitude ca 600 msl. has been considered as the most endangered stands with root rot and butt rot caused by *Heterobasidion annosum* (**paper I**). No significant relationship has been found within soil type and *Heterobasidion annosum* occurrence. However, various stress factors (drought, frost, drainage, mechanical disruptions etc) can play major role in *Heterobasidon* attack.

Seventeen isolates of *Heterobasidion annosum* s.l. were obtained in extraordinary environment in the Czech Republic, Slovakia, Romania, and Spain (**paper II**). The three species, *H. abietinum, H. annosum sensu stricto,* and *H. parviporum* were identified. Isolates of *H. parviporum* were obtained from moonmilk, air, and underground gallery wall. *H. parviporum* recorded from Spain, Castañar de Ibor Cave, was obtained far from the natural distribution of its main host, *Picea abies*. *H. annosum* was obtained from cave air and sediment, isopod faeces and underground gallery wall. *Heterobasidion abietinum* was isolated from bat guano and outdoor air. The origin and ways of fungal dispersal remain unclear however the forest stands surrounding caves and tunnels are hypothesized as the potential source of inoculum to inhabit these uncommon spaces.

We confirmed applicability of the multiplex PCR method (Gonthier et al. 2014) for rapid identification of pathogens inside the overmatured living Norway spruce trees in the Czech Republic. We detected *Stereum sanguinolentum* as the most common fungus, followed by *Fomitopsis pinicola*, *Porodaedalea chrysoloma*, *Heterobasidion annosum s.l.*, and *Armillaria* spp. *Onnia* spp. were not detected in our study. No statistically significant differences in pathogen's distribution between tree base and breast height was recorded (one-way ANOVA; F=4.0981, p=0.3408).

Occurrence of *H. annosum* s.l. depends on many factors as previous study had confirmed (Woodward et al. 1998). We documented the lowest parts of the Czech Republic, with artificial stands of Norway spruce, as the most vulnarable ones. The severity of abundance of *H. annosum* s.l.

rely more on indigenity of tree composition in forest stands than on ecological patterns.

The HAF/HAR specific primers has shown satisfying results for rapid identification of the two European species of *Heterobasidion annosum* s.l. – *H. parviporum* and *H. abietinum*.

As the recent data confirm distribution of *H. annosum* s.l. in various forests type and various altitudes it would be appropriate to implement control of *Heterobasidion* root rot to forest management in the Czech Republic. *Heterobasidion* has been mentioned as very serious pathogen agent in forest management books since as early as 50's and no precautions have been made yet. Root rot caused by *H. annosum* s.l. is one of the top problems in conifer plantations in certain parts of the Czech Republic, still not enough attention is paid to the mitigation of the spread of this agent by forest managers and stakeholders (small forest owners, big forest corporations).

The problems associated with H. infections in the CR are mainly caused by the lack of information accesible to both the professional foresters as the wide public. There are few basic rules how to reduce the pathogen in conifer plantations and they do not directly include any biological or chemical control. The key lies within a more nature friendly integral forest management with the emphasis on natural stand composition.

Control of *Heterobasidon* infection has been applied into many European countries decades ago, but very little or nothing has been done in the Czech forestry despite availability of alarming data (Baláš 2008, Jankovský 2000, 2002, Mrkva 2000). –

Stump removal seems to be effective and economically convenient only on light and sandy soils which is appropriate only in some areas in the Czech Republic. More interesting would be establishment of biological control and introducing the silvicultural methods to reduce *Heterobasidion* infection in Czech forests. Experiment based on efficacy of admixing conifer plantations with broadleaved trees under the conditions of the Czech Republic could give and clear answer as there has been debates on the true effect of this treatment. Thor (2005) and Korhonen and Stenlid, (1998) confirmed the positive effect of admixture of broadleaved trees into stands as the number of root contacts between

susceptible trees is decrased. Seemingly, it has been matter of different environmental conditions and the approach cannot be identical for forestry in Scandinavia and Central Europe. Future prospect of this study would be carry on in the practical pathology to add the control aspect into the Czech forestry praxis.

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7 APPENDIX

LIST OF PUBLICATIONS

Paper I

Sedlák, P., Tomšovský, M., 2014: Species distribution, host affinity and genetic variability of *Heterobasidion annosum* sensu lato in the Czech Republic. Forest Pathology 44(4): 310-319. doi:10.1111/efp.12102

Paper II

Nováková, A., Sedlák, P., Kubátová, A., Tomšovský, M. 2014: Underground spaces as neglected niche for occurrence of *Heterobasidion annosum* complex. Forest Pathology. doi: 10.1111/efp.12177.

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Paper I

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PAPER I

Forest Pathology WILEY-BLACKWELL

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Species distribution, host affinity and genetic variability of *Heterobasidion annosum sensu lato* in the Czech Republic

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Summary

The distribution and host affinity of *Heterobasidon annosum* (Fr.) Bref. *sensu lato* (Basidiomycota, *Bondarzewiaceae*) in the Czech Republic was investigated. Sampling was undertaken in natural forests, in stands with former presence of fir and spruce and in forests on formerly agricultural lands. The identification and phylogenetic relationship of the species complex was studied by comparing DNA sequences of three gene regions: glyceraldehyde 3-phosphate dehydrogenase (G3P), translation elongation factor 1-alfa (EFA) and transcription factor (TF). The species relationships were demonstrated by haplotype network and Bayesian phylogram construction for the combined data set. The distribution of each species was demonstrated. *Heterobasidion annosum* s.s., which had the greatest host range, was found on thirteen genera (*Pinus, Picea, Fraxinus, Betula, Corylus, Alnus, Abies, Acer, Salix, Ligustrum, Quercus, Larix and Prunus*). *Heterobasidion parviporum* was found on spruce (*Picea abies*), fir (*Abies alba*), apple tree (*Malus sylvestris*), *Vitis vinifera* and *Fagus sylvatica,* and *Heterobasidion abietinum* was observed on fir (*Abies*), spruce (*Picea*), pine (*Pinus*) and maize (*Zea mays*). Sequences from different genes yielded conflicting results for six specimens, which were interpreted as belonging to interspecific hybrids. These were collected from *A. alba, P. abies* and *Chamaecyparis lawsoniana*. The occurrence of *Heterobasidion annosum* s.l. is strongly associated with the natural distribution of its hosts and the occurrence of *H. abietinum* appears to be related to the historical occurrence of *A. alba* in stands now reforested with *P. abies*. No statistically significant differences in the vertical distributions within the species complex were detected.

1 Introduction

Root and butt rot caused by *Heterobasidon annosum* (Fr.) Bref. sensu lato (Basidiomycota, *Bondarzewiaceae*) is one of the most destructive diseases of conifers in the temperate regions of the Northern Hemisphere, particularly in Europe (Woodward et al. 1998). The fungi belonging to this group occur in most regions in which coniferous forests are present and cause damage in both managed and unmanaged forests after thinning and harvesting. In Europe, three species have been identified, based on mating differentiation: *H. parviporum* Niemelä and Korhonen, which primarily infects *Picea abies* (L.) Karst; *Heterobasidion abietinum* Niemelä & Korhonen, which mainly infects *Abies* spp. and *H. annosum* sensu stricto, which is prevalent on *Pinus* spp. (Niemelä and Korhonen 1998). To a lesser extent, *Heterobasidion* also causes root rot on some deciduous trees, for example, species of *Betula* and *Quercus* (Korhonen and Stenlid 1998).

The species in the *H. annosum* species complex emerged during the Palearctic era, approximately 60 Ma after the radiation of their host genera. The emergence of the most recent common ancestor for *H. annosum* s.l. has been estimated to be 75–85 Ma, while the separation of the more closely related *H. parviporum* and *H. abietinum* is estimated to have occurred 25–28 Myr ago (Dalman et al. 2010).

Heterobasidion annosum spreads mainly by airborne basidiospores and conidia that germinate on fresh stump surfaces. The mycelium then grows through the stump into the root system, where it can invade healthy roots of adjacent trees by means of root grafting (Blanchard and Tattar 1997). Significant economic losses result from the consequent root rot, butt rot, suppressed growth and tree mortality. *Heterobasidion* root rot disease is common in spruce and pine stands established on former agricultural lands (Oliva et al. 2008). It also occurs frequently in plantations and monocultures of conifers in Scandinavia, the Baltic countries and Poland (e.g. Łakomy and Werner 2003; Lygis et al. 2004; Oliva et al. 2008; Vasaitis et al. 2008). The problem is caused by high fertility (high nitrogen concentration) or by sandy loam containing little organic matter and a fluctuating water supply in such soils throughout the year (Woodward et al. 1998).

The disease caused by *Heterobasidion* rot has been studied in many parts of Europe, particularly in Fennoscandia, Italy, Britain, Lithuania, Poland, Switzerland and Spain (Korhonen and Stenlid 1998; Gonthier et al. 2001, 2003; Bendel et al. 2006; Mesanza and Iturritxa 2012). Research on the disease has also been conducted in other countries, such as Bulgaria, Belarus, Slovenia and Estonia (Korhonen et al. 1992; Munda 1994; La Porta et al. 1998).

In the Czech Republic (and former Czechoslovakia), *Heterobasidion* is found mainly on *Picea* spp., particularly *P. abies* (Černý 1989), but the pathogen has also been reported on other conifer species, as well as some hardwood species, such as *Acer pseudoplatanus*, *Betula pendula, Cerasus avium, Fraxinus* spp., *Populus tremula, Sambucus nigra* and *Salix caprea* (Kotlaba 1984). At lower elevations (<600 m), *Heterobasidion* root rot disease occurs mainly in secondary stands of Norway spruce, whereas at higher elevations, first-rotation stands planted on steep agricultural sites are most affected (Černý 1989). However, more precise information on the distribution of the pathogen and the occurrence of the three species in the country is unavailable. The aim of our study was therefore to obtain data on the distribution, genetic diversity and ecology of each species of *H. annosum* s.l. in the Czech Republic.

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2 Material and methods

2.1 Fungal material

Basidiocarps of *Heterobasidion* spp. were collected by arbitrary sampling in natural forest stands and artificial forests in the Czech Republic. Wind throws, symptomatic trees and decayed stumps were checked for the presence of fruit bodies at each site visited. Care was taken to collect samples at locations at least 30 m apart to avoid repeated sampling of the same genotype and to obtain a representative collection of the local population (La Porta et al. 1998). Each sample location was georeferenced using a GPS (Oregon 300, Garmin). After collection, each sample was dried for approximately 24 h at 54°C.

Cultures were isolated from some field specimens on malt extract agar (HiMedia, India). We also examined *Heterobasidion* isolates held in the Czech culture collections CCF (Charles University, Prague) and CPPF (Crop Research Institute, Prague), some of which were obtained from unusual hosts: grape wine (*Vitis vinifera*), maize (*Zea mays*) and apple tree (*Malus sylvestris*). The fungal isolates, their origins and their substrates are summarized in Table 1. In additional, four basidiocarp collections were borrowed from the herbaria BRNM (the Moravian Museum, Brno), PRM (the National Museum, Prague) and MJ (The Museum of the Bohemian-Moravian Highlands, Jihlava). A total of 110 specimens were studied.

2.2 Distribution patterns

The mapping of sampling sites was conducted using the ARCGIS 10 software Environmental Systems Resource Institute (ESRI 2012). All of the statistical analyses were performed using the STATISTICA 10 software (StatSoft, Inc. 2011). The influence of altitude on the distribution of the three species was tested using a one-way analysis of variance (ANOVA). The null hypothesis of no relation between altitude and distribution of the species was not rejected.

2.3 DNA extraction PCR and DNA sequencing

DNA was isolated from pure cultures and dried basidiocarps using the PowerSoil™ DNA Isolation Kit (Mo-Bio, Carlsbad, CA, USA) and the QIAGEN DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA, USA), according to the manufacturers' instructions.

To identify the samples at the species level and assess their genetic diversity, we sequenced three nuclear gene regions: glyceraldehyde 3-phosphate dehydrogenase (G3P), translation elongation factor 1-alfa (EFA) and transcription factor (TF) (Linzer et al. 2008; Dalman et al. 2010). In some cases in which we were not able to amplify the G3P locus, we first amplified the GPD region flanking an extended part of the same gene (Linzer et al. 2008) and used its product as a template for G3P amplification. PCR was performed in a 25 μl reaction mixture containing approximately 50 ng of template DNA, 0.5 μmol of each primer, 5× MyTaq Reaction Buffer and 1U MyTaq DNA Polymerase (Bioline, London, UK). The PCR was amplified using a Mastercycler® ep thermocycler (Eppendorf, Hamburg, Germany). The PCR programme was as follows: 3 min at 95°C, followed by 37 cycles of 40 s at 95°C, 55 s at various temperatures, depending on the amplified gene (59°C for G3P, 60°C for GPD, 60°C for TF and 66°C for EFA) and 55 s at 72°C with a final extension of 7 min at 72°C. The PCR products were electrophoresed in 1% agarose gel in TBE buffer at 5 V/cm for approximately 1 h. The amplified fragments were sequenced by the DNA Sequence Service of Macrogen Inc. (Seoul, Korea).

The sequences were aligned manually using the freeware BioEdit (Hall 1997). Sequences published by Dalman et al. (2010) from GenBank Numbers FJ627391, FJ627418, FJ627421, FJ627425, FJ627397, FJ627416, FJ627419, FJ627393, FJ627411, FJ627430, FJ627387 and FJ627404 in the case of EFA; sequence numbers FJ627465, FJ627499, FJ627505, FJ627510, FJ627477, FJ627497, FJ627503, FJ627471, FJ627491, FJ627515, FJ627461 and FJ627484 in the case of the G3P gene; and sequence numbers FJ627732, FJ627695, FJ627690, FJ627693, FJ627699, FJ627702, FJ627704, FJ627709, FJ627710, FJ627719, FJ627724 and FJ627737 in the case of the TF gene were added to the alignment to ensure identification of the *Heterobasidion* species.

The minimum-evolution phylogenetic trees were constructed using MEGA software version 5 (Tamura et al. 2011) for EFA, G3P and TF separately (see Figure S1a–c). Bootstrap tests of 1000 replicates were used.

2.4 Sequence data analysis

The sequence data obtained were analysed using two different approaches, Bayesian analysis and haplotype network construction. To determine whether the three genetic markers were in significant conflict, the partition homogeneity test in PAUP 4.0b10 (Swofford 2003) was used between the markers in all possible pairwise combinations, using 100 replicates and the heuristic general search option. The null hypothesis of congruence was rejected if $p < 0.001$. The alignments of all three genes were connected to one alignment using the online utility FABOX Fasta alignment joiner (Villesen 2007). The phylogeny was generated using MRBAYES 3.2.1 (Ronquist and Huelsenbeck 2003). MrModelTest (Nylander 2004) was used to determine the best-fitting model and the parameters used in the analyses. The haplotype networks were constructed using NETWORK 4.6.1.1 (Fluxus Technology Ltd., Kiel, Germany), including only sequences without heterozygous positions.

2.5 Nucleotide and haplotype diversity and gene flow rates

The DNA divergence among populations and the average level of gene flow were measured using DNA SP v. 5.10.01 (Librado and Rozas 2009). The number of haplotypes (h), the number of polymorphic/indel/missing sites (S), the haplotype

Table 1. List of specimens included in the study.[1]

Specimen	Herbarium/ Culture collection	Location	Substrate	Altitude	Identification		
					EFA	TF	G3P
CZ134	–	Rotava	*Picea abies*	613	*a*	*a*	*a*
CZ135	–	Nejdek	*Picea abies*	835	*p*	*p*	*p*
CZ133	–	Nejdek	*Picea abies*	772	*p*	*p*	*p*
CZ136	–	PLA Slavkovský forest, Nová Ves	*Picea abies*	840	*p*	*p*	*p*
CZ139	–	Stříbro	*Pinus sylvestris*	498	*a*	*a*	*a*
CZ137	–	Pluhův bor NNR, Louka	*Picea abies*	784	*a*	*a*	*a*
CZ70	–	Louny	*Picea abies*	420	*a*	*a*	*a*
CZ79	–	NP Bohemian forest, Stožec	*Picea abies*	800	*p*	*p*	*p*
CZ44	–	NP Bohemian forest, Trojmezná	*Picea abies*	1220	*p*	*p*	*p*
CZ119	–	NP Bohemian forest, Černý Kríž	*Picea abies*	740	*p*	*p*	*p*
CZ110	–	Řepešín	*Picea abies*	750	*a*	*a*	*a*
CZ145	–	Peruc	*Picea abies*	320	*a*	*a*	*a*
CZ130	–	Peruc	*Larix decidua*	320	*a*	*a*	*a*
CZ129	–	Peruc	*Pinus sylvestris*	325	*a*	*a*	*a*
CZ126	–	Kačice	*Picea abies*	425	*a*	*a*	*a*
CZ107	–	Husinec	*Pinus sylvestris*	540	*a*	*a*	*a*
CZ3	–	Český Krumlov	*Picea abies*	680	*a*	*a*	*a*
CZ143A	CPPF 143	Prague, Ruzyně	*Zea mays*	345	*b*	*b*	*b*
CZ3368	CCF 3368	Prague	indoor air	–	*p*	*p*	*p*
CZ345	CPPF 345	Karlštejn	*Vitis vinifera*	–	*p*	*p*	*p*
CZ111	–	Hluboká nad Vltavou	*Picea abies*	385	*p*	*p*	*p*
CZ112	–	Hluboká nad Vltavou	*Pinus sylvestris*	390	*a*	*a*	*a*
CZ148	–	NP Bohemian Switzerland	*Pinus sylvestris*	425	*a*	*a*	*a*
CZ81	–	NR Baba, Dobřejovice	*Picea abies*	300	*p*	*p*	*p*
CZ115	–	Libníč	*Picea abies*	505	*p*	*p*	*p*
CZ95	–	PLA Třeboňsko, Chlum u Třeboně	*Picea abies*	475	*a*	*a*	*a*
CZ57	–	Žofínský prales NNR	*Picea abies*	799	*p*	*p*	*p*
CZ59	–	Žofínský prales NNR	*Abies alba*	852	*b*	*b*	*b*
CZ58	–	Žofínský prales NNR	*Picea abies*	826	*p*	*p*	*p*
CZ31	**MJ 877450**	**Žofínský prales NNR**	***Abies alba***	**750**	***p***	***a/p***	***a***
CZ92	–	Žofínský prales NNR	*Picea abies*	750	*p*	*p*	*p*
CZ93	–	Žofínský prales NNR	*Abies alba*	750	*p*	*p*	*p*
CZ94	**-**	**Žofínský prales NNR**	***Picea abies***	**750**	***p***	***a/p***	***a***
CZ96	–	Žofínský prales NNR	*Picea abies*	750	*p*	*p*	*p*
CZ98	**-**	**Žofínský prales NNR**	***Picea abies***	**750**	***p***	***a/p***	***a***
CZ108	–	Klec	*Pinus sylvestris*	445	*a*	*a*	*a*
CZ125	–	Vestec	*Pinus sylvestris*	415	*a*	*a*	*a*
CZ123	–	Vestec	*Picea abies*	460	*a*	*a*	*a*
CZ25	PRM 915910	Rataje nad Sázavou	*Abies alba*	400	*b*	*b*	*b*
CZ40	–	Černousy	*Picea abies*	350	*a*	*a*	*a*
CZ38	–	Oldřichov v Hájích	*Picea abies*	550	*p*	*p*	*p*
CZ51	–	Slavonice	*Picea abies*	610	*a*	*a*	*a*
CZ50	–	Slavonice	*Picea abies*	605	*p*	*p*	*p*
CZ257	CPPF 257	Bítouchov u Semil	*Malus sylvestris*	430	*p*	*p*	*p*
CZ64	–	Cejle	*Picea abies*	808	*a*	*a*	*a*
CZ63	–	Stonařov	*Picea abies*	705	*p*	*p*	*p*
CZ69	–	Želetava	*Picea abies*	717	*p*	*p*	*p*
CZ5	–	Třebelovice	*Picea abies*	545	*a*	*a*	*a*
CZ48	–	Jackov	*Picea abies*	490	*a*	*a*	*a*
CZ72	–	Moravské Budějovice	*Picea abies*	560	*a*	*a*	*a*
CZ07H	–	NNR Ransko	*Picea abies*	560	*p*	*p*	*p*
CZ117	–	NP Podyjí, Čížov	*Pinus sylvestris*	405	*a*	*a*	*a*
CZ19	–	Slavice	*Picea abies*	590	*a*	*a*	*a*
CZ118	–	NP Podyjí, Hnanice	*Pinus sylvestris*	340	*a*	*a*	*a*
CZ03	–	Křižánky	*Picea abies*	640	*p*	*p*	*p*
CZ02	–	Křižánky	*Picea abies*	630	*p*	*p*	*p*
CZ7	–	NNR Adršpach rocks, Zdoňov	*Betula pendula*	600	*a*	*a*	*a*
CZ122	–	Jinošov	*Picea abies*	520	*a*	*a*	*a*
CZ149	–	PLA Orlicke Mts., Lukavice	*Picea abies*	360	*p*	*p*	*p*
CZ16	–	Vysoké Popovice	*Pinus sylvestris*	513	*a*	*a*	*a*
CZ1078	–	Lískovec, Štěpánov nad Svratkou	*Salix caprea*	470	*a*	*a*	*a*
CZ1194	–	PLA Orlické Mts., Uhřínov	*Picea abies*	450	*b*	*b*	*b*
CZ75	–	Březová nad Svitavou	*Picea abies*	470	*p*	*p*	*p*
CZ55	–	Brno, Kamenný vrch	*Picea abies*	300	*b*	*b*	*b*
CZ6	–	Kuřim, Podlesí	*Picea abies*	470	*b*	*b*	*b*
CZ78	–	Kuřim, Podlesí	*Picea abies*	330	*b*	*b*	*b*

Table 1. Continued

Specimen	Herbarium/ Culture collection	Location	Substrate	Altitude	Identification EFA	TF	G3P
CZ76	-	Letovice	*Picea abies*	370	*p*	*p*	*p*
CZ1810	-	Brno, Mahenova stráň	*Picea abies*	300	*p*	*p*	*p*
CZ2409	-	Brno, Wilsons forest	*Pinus sylvestris*	250	*a*	*a*	*a*
CZ2309	-	Brno, Wilsons forest	*Picea abies*	252	*p*	*p*	*p*
CZ91	-	Brmo, Ořešín	*Picea abies*	345	*b*	*b*	*b*
CZ1709	-	Brno, Zaječí hora	*Picea abies*	290	*p*	*p*	*p*
CZ1910	-	Brno, Čertova rokle	*Picea abies*	280	*p*	*p*	*p*
CZ35	-	Brno, NR Coufavá	*Pinus sylvestris*	514	*b*	*b*	*b*
CZ80	-	Brno, NR Coufavá	*Fagus sylvatica*	510	*p*	*p*	*p*
CZ36	-	Brno, NR Coufavá	*Abies alba*	485	*b*	*b*	*b*
CZ77	-	Řícmanice	*Larix decidua*	380	*a*	*a*	*a*
CZ1	-	Kanice	*Picea abies*	315	*a*	*a*	*a*
CZ54	-	Řícmanice	*Larix decidua*	390	*a*	*a*	*a*
CZ1909	-	Brno, Říčka valley	*Picea abies*	300	*a*	*a*	*a*
CZ74	-	Lanškroun	*Picea abies*	335	*p*	*p*	*p*
CZ73	-	Babice	*Picea abies*	430	*a*	*a*	*a*
CZ1210	-	Brno, Říčka valley	*Picea abies*	350	*a*	*a*	*a*
CZ86	-	Lednice	*Pinus sylvestris*	165	*a*	*a*	*a*
CZ84	-	Lednice	*Pinus sylvestris*	160	*a*	*a*	*a*
CZ85	-	Lednice	*Pinus sylvestris*	185	*a*	*a*	*a*
CZ82	-	Lednice	*Pinus sylvestris*	180	*a*	*a*	*a*
CZ83	-	Lednice	*Pinus sylvestris*	180	*a*	*a*	*a*
CZ2710	-	Lednice	*Corylus avellana*	169	*a*	*a*	*a*
CZ100	-	Drahany	*Picea abies*	669	*b*	*b*	*b*
CZ87	-	Lednice	*Betula pendula*	160	*a*	*a*	*a*
CZ88	**-**	**Lednice**	***Chameacyparis law.***	**160**	***p***	***a/p***	***a***
CZ103	-	Ruprechtov	*Picea abies*	520	*b*	*b*	*b*
CZ104	-	Husinec	*Pinus sylvestris*	620	*a*	*a*	*a*
CZ46	-	Bučovice	*Picea abies*	370	*a*	*a*	*a*
CZ11	-	Bzenec	*Pinus sylvestris*	307	*a*	*a*	*a*
CZ14	-	Uherské Hradiště	*Picea abies*	295	*a*	*a*	*a*
CZ141	-	PLA White Carpathians, Suchov	*Quercus robur*	430	*a*	*a*	*a*
CZ04	-	Budišov nad Budišovkou	*Picea abies*	565	*a*	*a*	*a*
CZ144	-	PLA White Carpathians, Vápenky	*Picea abies*	440	*p*	*p*	*p*
CZ142	-	PLA White Carpathians, Vápenky	*Picea abies*	450	*a*	*a*	*a*
CZ143	-	PLA White Carpathians, Vápenky	*Larix decidua*	440	*a*	*a*	*a*
CZ89	-	NR Tesák, Vičanov	*Picea abies*	655	*a*	*a*	*a*
CZ90	**-**	**NR Tesák, Vičanov**	***Picea abies***	**630**	***p***	***a/p***	***a***
CZ01	-	Hradec nad Moravicí	*Fraxinus excelsior*	380	*a*	*a*	*a*
CZ21	-	Oznice	*Picea abies*	570	*a*	*a*	*a*
CZ131	-	Rožnov pod Radhoštěm	*Picea abies*	510	*p*	*p*	*p*
CZ42	-	Ostrava, Bělský forest	*Picea abies*	350	*a*	*a*	*a*
CZ43	-	Ostrava, Bělský forest	*Picea abies*	355	*p*	*p*	*p*
CZ30	**BRNM 732935**	**PLA Razula, Javorníky**	***Abies alba***	**760**	***b***	***a/p***	***b***

[1]The abbreviations *a*, *b* and *p* refer to *Heterobasidion annosum* s.s., *H. abietinum* and *H. parviporum*; NP, NNR, NR and PLA refer to National Park, National Nature Reserve, Nature Reserve and Protected Landscape area. Interspecific hybrids highlighted with bold font.

diversity (Hd) and the average number of nucleotide differences (K) determined the genetic diversity for each species. The genetic differentiation between the species for each locus was estimated using a database containing haploid genomic information for each organism. From nucleotide data information, Wright's F-statistic (F_{ST}) was derived from equation 3 of Hudson et al. (1992).

3 Results

In total, 110 samples, comprising 330 sequences, were used in this study. All loci were successfully amplified, with the exception of the TF and G3P regions from 12 herbarium specimens, due to the lower quality of their DNA. The partition homogeneity tests showed no significant conflict between the three genetic markers used ($p = 0.01$). This allowed a combined analysis of the TF-G3P-EFA sequence data set generated. The three-marker alignment was 1042 bp long, 944 bp were conserved, 98 bp were variable and 91 bp were parsimon informative.

Based on the Akaike information criterion results calculated using MrModeltest, the HKY+I+G model was selected for the Bayesian analysis. Markov chains were initiated from a random tree and were run for 5 million generations. Samples were taken from every 100th generation. The first 500 000 generations were excluded as a burn-in.

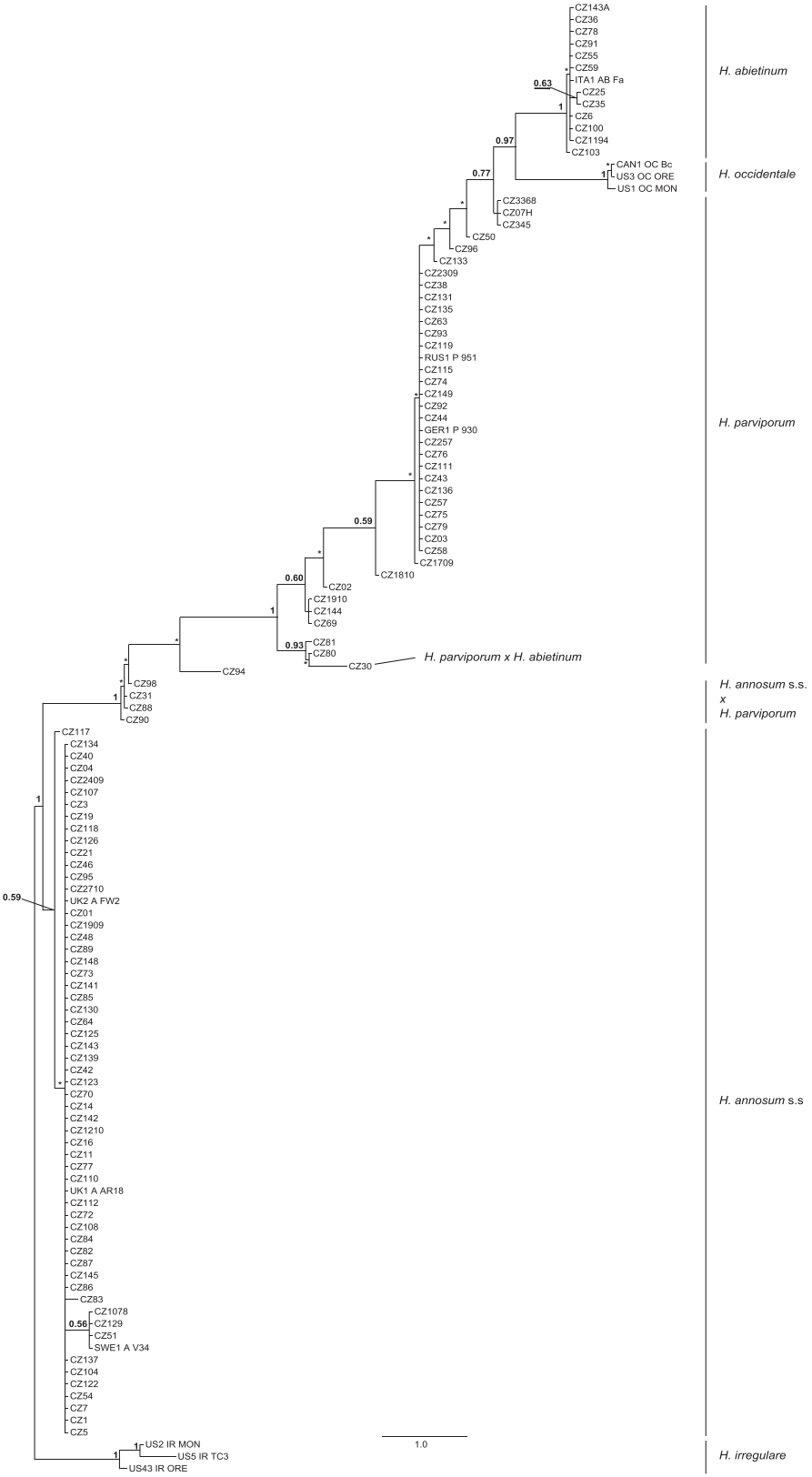

Fig. 1. The phylogram inferred from the Baesyan analysis of aligned sequences of EFA, G3P and TF. Numbers at branches indicate Bayesian PPs values higher than 50%, asterisk indicates a value lower than 50%. The bar indicates the number of expected substitutions per position. ITA1 in *H. abietinum* clade includes Italian strain; CAN1, US3, US1 in *H. occidentale* clade include North American strains, RUS1 and GER1 in *H. parviporum* clade include European strains; UK1, UK2, SWE1 in *H. annosum* s.s. clade include European strains; US2, US43, US5 in *H. irregulare* clade include North American strains.

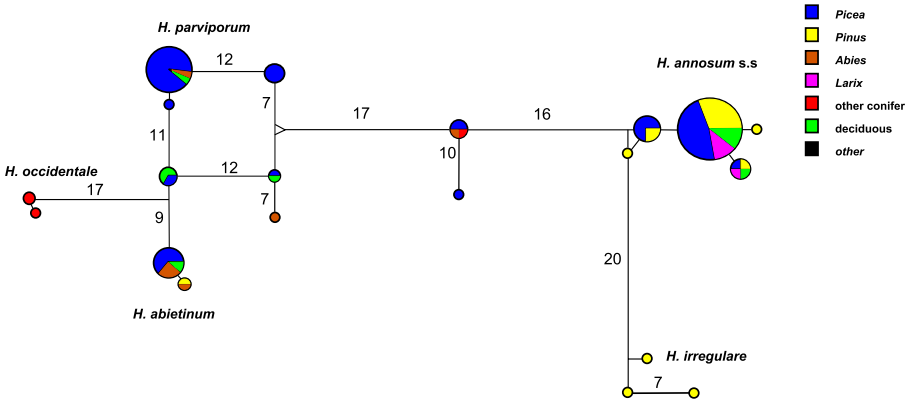

Fig. 2. Unrooted median-joining (MJ) haplotype networks for connected alignment of TF, G3P and EFA were constructed using Network 4.6.1.1. (Fluxus Technology Ltd.). The sizes of the nodes are proportional to the number of isolates in each haplotype. Numbers indicate the numbers of mutations between haplotypes and values above one are shown.

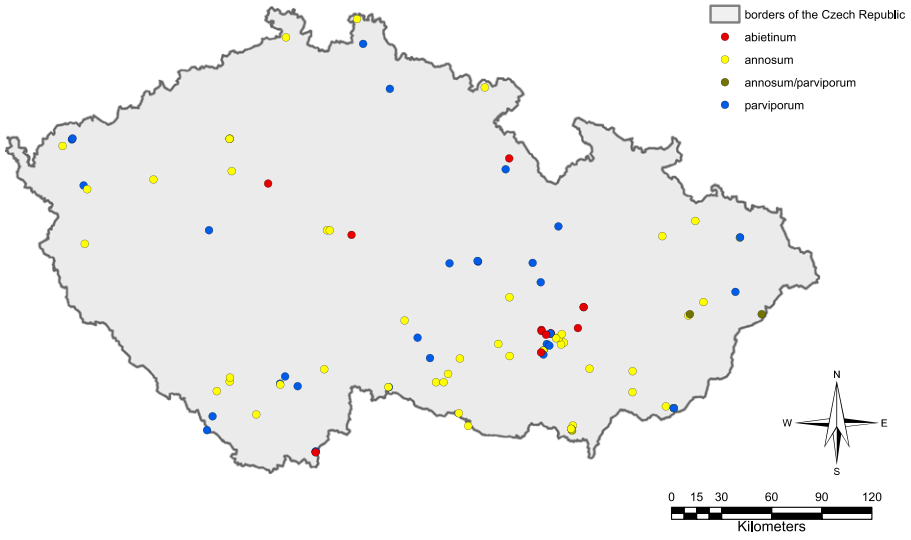

Fig. 3. Spatial distribution map of samples included in the study.

Our data recognized the occurrence of all three European *Heterobasidon* species in the Czech Republic. Some specimens included either sequences with heterozygous nucleotides at species-specific positions or genotypes of different genes corresponding to different species. Such specimens were treated as interspecific hybrids. The hybrid specimens were well separated in both the phylogenetic tree and the median-network diagrams (Figs 1 and 2). The geographical distribution of the samples used in this study is shown in Fig. 3. Most of the specimens analysed (56 specimens, 51% of the total of 110) were identified as *H. annosum* s.s., while 36 specimens (33%) fell into the *H. parviporum* clade, 12 specimens (11%) fell into *H. abietinum* and six specimens (5%) were interspecific hybrids.

Heterobasidion annosum s.s. was collected from the widest range of hosts: *P. abies, Pinus sylvestris, Larix decidua*, *Betula pendula*, *Alnus glutinosa*, *Corylus avelana*, *Fraxinus excelsior* and *Quercus robur*. Some herbarium specimens, which we were able to amplify only with EFA primers and whose sequences were identified as *H. annosum* s.s., were collected from the following hosts: *Abies alba*, *Ligustrum vulgare, Acer* spp., *Prunus domestica*, *Pinus nigra* and *Prunus avium*. These sequences were not included in our Bayesian and haplotype network analyses. We recorded *H. parviporum* on *P. abies*, *A. alba, Fagus sylvatica, Vitis vinifera* and *M. sylvestris* (Fig. 4). *Heterobasidion abietinum* was collected on *P. abies*, *A. alba* and *P. sylvestris* and was documented on *Z. mays*. The interspecific hybrids were found on the following hosts: *A. alba, P. abies* and *Chamaecyparis lawsoniana*.

In the Czech Republic, *H. annosum* s.l. has been found at a wide range of altitudes (160–1220 m) and in various types of soil. The median altitudes at which *H. annosum*, *H. parviporum* and *H. abietinum* has been found are 463.5 msl, 560 msl and 477.5 msl, respectively. Furthermore, there is a statistically significant difference in the vertical distribution only within *H. annosum* and *H. parviporum* (one-way ANOVA; $F = 8.7061$, $p = 0.0037$).

A comparison of the haplotype diversity of the three species showed that *H. annosum* is the most diverse, due to it being the most commonly occurring of the three. The use of three different genes for the analyses revealed interesting differences

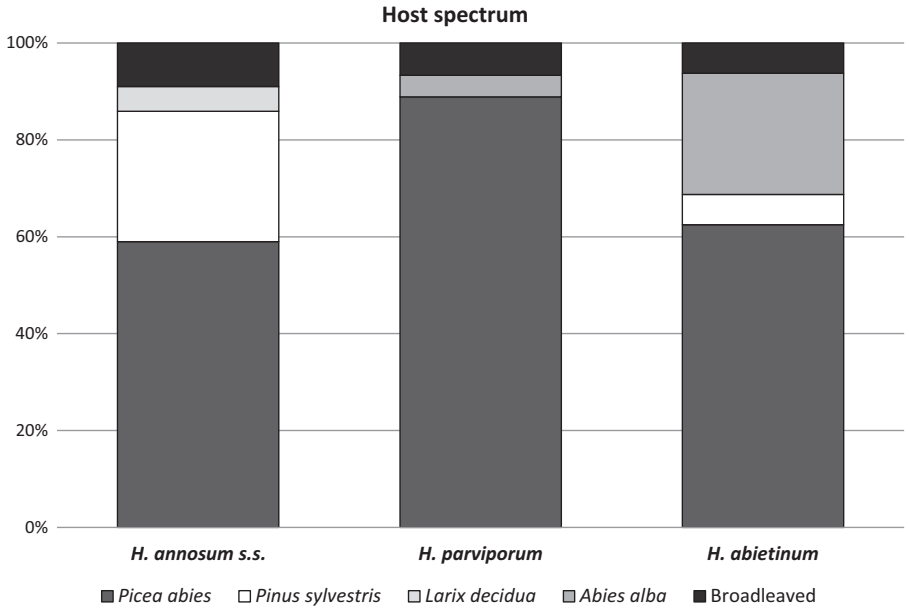

Fig. 4. Host spectrum of the three European species in the Czech Republic.

Table 2. Genetic diversity within species.

		EFA	G3P	TF
	Total number of sequences	110	110	110
	Total number of sites	331	394	323
N	*H. annosum* s.s.	56	56	56
	H. parviporum	36	36	36
	H. abietinum	12	12	12
h	*H. annosum* s.s.	5	3	2
	H. parviporum	1	3	6
	H. abietinum	1	3	1
S	*H. annosum* s.s.	4	26	1
	H. parviporum	0	9	12
	H. abietinum	0	22	0
Hd	*H. annosum* s.s.	0.31	0.13	0.036
	H. parviporum	0	0.18	0.48
	H. abietinum	0	0.62	0
K	*H. annosum* s.s.	0.63	1.4	0.036
	H. parviporum	0	1.51	4.31
	H. abietinum	0	4.12	0

N, sequences studied per population; h, number of haplotypes; S, number of polymorphic/indel/missing sites; Hd, haplotype diversity; K, average number of nucleotides differences.

Table 3. F_{ST} values estimated between *Heterobasidion* species.

	F_{ST}		
	EFA	G3P	TF
H. annosum vs. H. parviporum	0.952	0.933	0.769
H. annosum vs. H. abietinum	0.958	0.862	0.998
H. abietinum vs. H. parviporum	1	0.711	0.194

in the Hd values of the three species (Table 2). *Heterobasidion abietinum* seems to be more variable in G3P, and *H. parviporum* seems to be more viable in the TF locus.

The total haplotype diversity was relatively high in the EFA and TF genes (Table 2). G3P was the most polymorphic ($S = 33$ and $K = 12.1$), followed by TF ($S = 19$ and $K = 5.68$) and EFA ($S = 22$ and $K = 4.27$) (Table 2). It seems that the polymorphism is distributed primarily among the three groups in the case of G3P. In each case, the occurrence of linkage

disequilibrium between mutations within each gene was significant, according to the results of a chi-square test (p-value = 0.0000). The genetic differentiation between the species is high, according to F_{ST} in the three genes (Table 3).

4 Discussion

The results of our study confirm the presence of all three European species of *H. annosum* s.l. in the Czech Republic. The samples were collected over a range of altitudes (160–1220 m) from conifer and broadleaved hosts. In the Czech Republic, the most widespread species with the widest host range is *H. annosum* s.s. Despite the fact that the prevalent host of *H. annosum* in Europe is usually *Pinus* spp. (La Porta et al. 1998; Niemelä and Korhonen 1998), the dominant host of *H. annosum* in the Czech Republic was found to be *P. abies* (59%). This is likely due to the abundance of spruce stands in this country. Although the potential natural distribution of *P. abies* is only 6.3% (Žárník and Křístek 2007), there has been extensive planting of this species in the Czech Republic. Artificial afforestation with spruce began in the present area of this country at the beginning of the 18th century and increased further at the end of 19th century to limit damage caused by serious floods. The conifers *P. abies, P. sylvestris, Larix decidua, Pinus nigra, Pinus banksiana* and *Pseudotsuga menziesii* were primarily planted in newly afforested areas, starting at the beginning of the 19th century (Špulák and Kacálek 2011). Norway spruce is now a dominant tree species in the Czech Republic, covering 47.7% of forested areas (NIF 2007). The samples of *H. annosum* s.s. were primarily obtained from forests on formerly agriculture lands and from lower altitudes (<500 m). The distribution of *H. annosum* corresponds to the indigenous distribution of its main host, *P. sylvestris*, which covers 17.2% of the forested land in the country, although the original natural occurrence of *P. sylvestris* in the Czech Republic has been estimated to be approximately 4% (Musil and Hamerník 2007). *Heterobasidon annosum* was not found on either *Pinus mugo* or *Pinus uncinata* var. *uliginosa*, other native *Pinus* species in the Czech Republic, which occur here near the timber line in high mountains and at the margins of peat bogs. More strict host specificity was found for *H. parviporum*. This species was found on *P. abies* (89%), only two records on *A. alba* and one record from *F. sylvatica*. On the other hand, *H. parviporum* was found on unusual hosts: one sample comes from *M. sylvestris*. This record from apple tree originated from a living branch, which was formerly investigated for the presence of endophytic fungi, and the tree was situated near the conifer forest with prevalence of Norway spruce and European larch. One specimen belonging to *H. parviporum* was collected from vine trunk (*Vitis vinifera*). The vineyard was situated on limestone and loess surface close to mixed forest with current presence of Scots pine and sessile oak but historical plantations of Norway spruce are reported from the area. The records of *H. parviporum* on hardwoods in the literature are less frequent than are those of *H. annosum*, but Luchi et al. (2011) reported collecting *H. parviporum* from *Fraxinus* in Belarus. *H. parviporum* was observed over a wide range of elevations (160–1220 m) with a median value of 560 msl. The indigenous altitudinal distribution of *P. abies* in the Czech Republic is from 165 msl to the timber line (1350 msl), with an ecological optimum at 900 msl. Nevertheless, its natural occurrence at lower elevations is restricted to sites with stagnant water and colder temperatures (Musil and Hamerník 2007). In fact, the vertical distribution of *H. parviporum* corresponds to the distribution of *P. abies*. Similarly, *H. abietinum* was found primarily on *P. abies* (63%), while records from *A. alba* comprised only 25%.

The occurrence and host affinity of *H. abietinum* to spruce is worth a detailed discussion. The specimens of *H. abietinum* found on spruce were collected from secondary spruce stands, in some of which fir or beech and fir were formerly (1950–1960) the dominant tree species. Málek (1983) mentions *A. alba* as the main tree species present in the 15th and 16th centuries, covering between 40 and 60% of the forested area of the current Czech Republic. Furthermore, fir started to decline in occurrence during the 18th century (Málek 1983). The historical forest management data from the area of Plumlov (49.430291N, 16.916519E) indicate a steady decrease in silver fir composition from the 1940s (85%) to the 1980s (5%) and the 1990s (0%), along with continuous occurrence of *Heterobasidion* rot. Currently, the silver fir does not occur in the locality, but *H. abietinum* is markedly common there. According to forest management data from 1948, a decline in fir had been observed at the beginning of the 20th century. Dry seasons and an inability to regenerate naturally are mentioned as the main reasons for the decline. Therefore, our results confirm that *H. abietinum* can persist in stands with a previous history of *A. alba* after reforestation has occurred. Our only record of *H. abietinum* was observed at the site where *A. alba* occurred. Lakomy (1996) also documented *H. abietinum* on both *A. alba* and *P. abies* in the Carpathian Mountains of southern Poland. This finding supports the notion that *H. abietinum* can survive in localities with a changed tree composition. Noteworthy, *H. abietinum* was isolated as endophyte from maize ear (*Z. mays*). The maize field was located apart from any coniferous stand, but an ornamental park where mostly conifer tree species occur is situated up to 2 km from the locality.

Our results confirm the occurrence of hybrids among the three *Heterobasidion* species, although the F_{ST} values of nucleotide data indicate only rare gene flow. In Europe, 4–10% interfertility between *H. parviporum* and *H. annosum* has been reported, and even higher value of 37% has been recorder when analysing *H. parviporum* and *H. abietinum* (Korhonen and Stenlid 1998). The possibility of hybridization between *H. abietinum* and *H. parviporum* due to partial compatibility of interspecific mating *in vitro* was repeatedly confirmed (Dai et al. 2003; Oliva et al. 2011). We also revealed sympatrical co-occurrence of both species in some localities, which enables interspecific mating to occur. Due to high frequency of contact between *Heterobasidion* species, an occasional interspecific mating is probable and it was confirmed by our results. Modern forest management introduced during the last six decades has enlarged the infection possibilities for *Heterobasidon* through the formation of increasing number of stump surfaces and stem and root wounds as result of more intensive management. The increased area susceptible to infection increases the possibility co-infection of the same substrate by two species, which is the crutial prerequisite for hybridization (Olson and Stenlid 2002). Therefore, we confirmed the occurrence of hybrids in the *H. annosum* group in natural habitats, although they are of limited fitness in comparison with their parent genotypes (Garbelotto et al. 2007).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The minimum evolution phylograms for three separate genes: EFA, G3P and TF.

Table S1. List of specimens included in the study and NCBI GenBank accession numbers.

Paper II

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PAPER II

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Underground spaces as neglected niche for occurrence of *Heterobasidion annosum* complex

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Summary

The serious forest pathogen *Heterobasidion annosum sensu lato* was found during mycological exploration in caves and underground tunnels. Seventeen isolates of *Heterobasidion annosum* s.l. were obtained in these habitats in the Czech Republic, Slovakia, Romania and Spain during 2008–2012. Three species, *H. abietinum, H. annosum sensu stricto,* and *H. parviporum*, were identified by analyses of DNA sequences of three gene regions: glyceraldehyde 3-phosphate dehydrogenase, translation elongation factor 1-alfa and transcription factor. Isolates of *H. parviporum* were obtained from moonmilk, air and underground gallery wall. *H. parviporum* recorded from Spain, Castañar de Ibor Cave, was obtained far from the natural distribution of its main host, *Picea abies*. *Heterobasidion annosum* was obtained from cave air and sediment, isopod faeces and underground gallery wall. *Heterobasidion abietinum* was isolated from bat guano and outdoor air. The origin and ways of fungal dispersal are discussed. The forest stands surrounding caves and tunnels are hypothesized as the potential source of inoculum to inhabit these unusual spaces, although the real paths of the pathogen to the habitats remain unclear.

1 Introduction

Heterobasidion annosum (Fr.) Bref. sensu lato (Basidiomycota, *Bondarzewiaceae*) is among the most devastating forest pathogen widely distributed throughout the boreal and temperate zones of the Northern Hemisphere (Korhonen and Stenlid 1998). The fungus occurs in most regions where coniferous forests are present, and it causes serious damage in managed forests after thinning and harvesting and has become an important risk factor on spruce or pine stands placed on former agricultural lands (Oliva et al. 2008). *Heterobasidion annosum* spreads mainly by airborne basidiospores that germinate on fresh stump surfaces and grow through the stumps into the root system, where it can invade healthy roots from adjacent trees via root grafts (Blanchard and Tattar 1997; Korhonen and Stenlid 1998; Garbelotto and Gonthier 2013). The primary damage is tree mortality and deterioration of timber by decay. The secondary damage can be attributed to higher risks of windthrow and stem breakage and growth reduction of infected trees (Bendz-Hellgren and Stenlid 1995). The fungus has been classified into five species; two of them occur in North America: *H. irregulare* Otrosina & Garbelotto and *H. occidentale* Otrosina & Garbelotto (2010), and three in Europe (Niemelä and Korhonen 1998). The European species are *H. annosum* (Fr.) Bref. sensu stricto (s.s.), *Heterobasidion parviporum* Niemelä & Korhonen and *H. abietinum* Niemelä & Korhonen.

Since the first description of *H. annosum* as a destructive root and butt rot of European conifers (Hartig 1874), the recorded host range has broadened considerably to include many other gymnosperms as numerous hardwood, woody shrubs and herbaceous hosts. It has been reported from more than 200 species of woody plant including c. 45 species of pine 25 species of fir and 10 species of spruce (Pilát 1936; Reid 1978; Kotlaba 1984; Webb and Alexander 1985). Many of these records come from introduced tree species in areas outside of original distribution. Among native tree species, it attacks especially *Picea abies* (mainly *H. parviporum*) and *Pinus* spp. *(H. annosum* s.s.*)*, *Abies* spp. (*H. abietinum*), *Larix* spp. and *Juniperus communis* (both, *H. annosum* s.l.). Deciduous trees are much less susceptible to *Heterobasidion* infections, but *Betula pendula*, the introduced *Quercus rubra*, less frequent *Populus tremula* and other species can be attacked when growing in mixtures with conifers or on unsuitable sites or when suffering from heavy industrial pollution (Korhonen and Stenlid 1998). During an extensive research of mycobiota in caves and mining galleries in the last years (Nováková 2012a; Nováková unpublished), the isolates of *Spiniger meineckellus* (A.J. Olson) Stalpers (the anamorph of *H. annosum* complex) were occasionally recorded (Fig. 2a). The aim of this study was to identify the isolates at species level and to discuss the ecological preferences of *Heterobasidion* species.

2 Material and methods

2.1 Sampling and isolation of cultures

Seventeen *Heterobasidion* isolates were obtained from 14 different caves and underground tunnels in the Czech Republic, Slovakia, Romania and Spain during 2008–2012, see Table 1. Samples from caves and tunnels (cave sediment, bat droppings and guano, moonmilk, isopods faeces, wall swabs) were collected aseptically into sterile plastic bags, vessels or microtubes, and they were kept cold during the transport to the laboratory. Microscopic fungi were isolated in situ or

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immediately after the return to the laboratory (i.e. at most 5 days after sampling) either directly by transferring a small amount of collected material into Petri dishes filled with Dichloran rose bengal chloramphenicol (DRBC) agar or Sabouraud glucose agar (SGA) or using the dilution plate method (Garrett 1981; Kreisel and Schauer 1987). Number of taken samples depended on the character and size of caves and the number of viable sources of inoculum but at least three replicates of Petri dishes were carried out for each isolate and substrate. Airborne microfungi were isolated using the gravity settling culture plate method (Buttner and Stetzenbach 1991) and SGA or DRBC agar (Atlas 2010) as the isolation media. Petri dishes were incubated in the dark at 10 and 25°C for 7–28 days. Additionally, soil samples from aboveground environment in selected localities were analysed, and microfungal isolations from the outdoor air were carried out too by the gravity settling technique. Sampling was performed out of the range of tourists influence.

Isolated strains were deposited at the Culture Collection of Fungi (CCF), Department of Botany, Charles University in Prague and at the Collection of Microscopic Fungi ISB (CMF), České Budějovice, Czech Republic (Nováková 2012a).

The forest conditions within 200 m of the sampled cave entrances were as follows: The caves in the Moravian Karst (the Výpustek Cave, the New Amateur Cave, the Kateřina′s Cave), the Bozkov Dolomite Caves, the Demänovská Piece Cave and the Bobačka Cave are surrounded by Norway spruce even-aged stands with minor presence of deciduous trees. The Balcarka Cave is situated in forest–steppe with mix of different tree species (birch, Norway spruce, Scots pine). Broadleaved forest, dominated by beech, surrounded the Javoříčko Caves. The Chýnov Cave was situated in mixed forest. An olive orchard and cork oaks were situated nearby the Castañar de Ibor Cave, but no conifers were observed in the close

Table 1. List of examined specimens of *Heterobasidion* spp.

Isolate	Identification	Locality	Substrate	Year of isolation	GenBank accession number (EFA)	GenBank accession number (G3P)	GenBank accession number (TF)
CMF 1808	*H. parviporum*	Spain, The Castañar de Ibor Cave, Laberinto Norte	Moonmilk	2009	KJ816768	KJ832111	KJ832128
CMF 1829	*H. annosum*	Czech Rep., Moravian Karst, The New Amateur Cave, Písečný dome	Cave air	2009	KJ816769	KJ832112	KJ832129
CMF 1952	*H. parviporum*	Czech Rep., The Javoříčko Caves, passage to emergency exit	Cave air	2010	KJ816770	KJ832113	KJ832130
CMF 1956	*H. parviporum*	Czech Rep., Moravian Karst, The Výpustek Cave, German Boiler Room	Cave air	2010	KJ816771	KJ832114	KJ832131
CMF 1957	*H. parviporum*	Czech Rep., Moravian Karst, The Balcarka Cave, Gallery	Cave air	2010	KJ816772	KJ832115	KJ832132
CMF 2084	*H. abietinum*	Slovakia, The Demänovská Piece Cave	Outdoor air	2011	KJ816773	KJ832116	KJ832133
CMF 2143	*H. abietinum*	Slovakia, The Bobačka Cave, near the site "Whale"	Bat guano	2011	KJ816774	KJ832117	KJ832134
CMF 2369	*H. annosum*	Romania, The Movile Cave, Lake Room	Isopod faeces	2011	KJ816775	KJ832118	KJ832135
CMF 2377	*H. annosum*	Romania, The Movile Cave, Lake Room	Isopod faeces	2011	KJ816776	KJ832119	KJ832136
CMF 2519	*H. parviporum*	Czech Rep., Moravian Karst, The Výpustek Cave, Low Passage	Cave air	2012	KJ816777	KJ832120	KJ832137
CMF 2520	*H. parviporum*	Czech Rep., The Bozkov Dolomite Caves	Outdoor air	2012	KJ816778	KJ832121	KJ832138
CMF 1953	*H. annosum*	Czech Rep., Moravian Karst, The Chýnov Cave, passage to Dragon′s Head.	Cave air	2010	KJ816779	KJ832122	KJ832139
CMF 2522	*H. parviporum*	Czech Rep., Moravian Karst, The Kateřina′s Cave, entrance corridor	Cave air	2012	KJ816780	KJ832123	KJ832140
CMF 2521	*H. parviporum*	Czech Rep., Moravian Karst, The Výpustek Cave, Henry′s Hall	Cave air	2012	KJ816781	KJ832124	KJ832141
CMF 1740	*H. annosum*	Spain, The Altamira Cave, Walls Hall	Cave sediment	2008	KJ816782	KJ832125	KJ832142
CCF 4741	*H. parviporum*	Czech Rep., Solenice, abandoned tunnel	Wall	2010	KJ816783	KJ832126	KJ832143
CCF 4742	*H. annosum*	Czech Rep., Nový Knín, abandoned tunnel	Wall	2010	KJ816784	KJ832127	KJ832144

surroundings. Broadleaved trees, grassland, shrubs but no conifers were presented in the surroundings of the Altamira Cave. The Moville Cave was situated in a locality with grassland and shrubs with Maritime pine.

2.2 DNA extraction and amplification

Mycelium was scrapped off from overgrown Petri dishes and homogenized by grinding the tissue in liquid nitrogen using sterile mortar and pestle. DNA was extracted using QIAGEN DNeasy Plant Mini Kit, according to the manufacturers' instructions. To identify the samples at the species level, we sequenced three nuclear gene regions: glyceraldehyde 3-phosphate dehydrogenase (G3P), translation elongation factor 1-alfa (EFA) and transcription factor (TF) (Linzer et al. 2008; Dalman et al. 2010). PCR was performed in a 25-μl reaction mixture containing approximately 50 ng of template DNA, 0.5 μmol of each primer, 5× MyTaq Reaction Buffer and 1 U MyTaq DNA Polymerase (Bioline, London, UK). The PCR was amplified using a Mastercycler® ep thermocycler (Eppendorf, Hamburg, Germany). The PCR programme was as follows: 3 min at 95°C, followed by 37 cycles of 40 s at 95°C, 55 s at various temperatures, depending on the amplified gene (59°C for G3P, 60°C for GPD, 60°C for TF and 66°C for EFA) and 55 s at 72°C with a final extension of 7 min at 72°C. The PCR products were electrophoresed in 1% agarose gel in TBE buffer at 5 V/cm for approximately 1 h (Linzer et al. 2008; Dalman et al. 2010). The amplified fragments were sequenced by the DNA Sequence Service of Macrogen Inc. (Seoul, Korea).

2.3 Sequence data analysis

To determine whether the three genetic markers were in statistically significant conflict, the partition homogeneity test in PAUP 4.0b10 (Swofford 2003) was used between the markers in all possible pairwise combinations, using 100 replicates and the heuristic general search option. The null hypothesis of congruence was rejected if $p < 0.001$. The alignments of all three genes were connected to one alignment using the online utility FABOX Fasta alignment joiner (Villesen 2007). Maximum likelihood (ML) analyses of the combined data set were performed using the online version PhyML 3.0 at Phylogeny.fr (Dereeper et al. 2008) using the 'A la Carte' module. The GTR substitution model was selected for the data set, and bootstrap branch support values (BP) were estimated in PHYML under the maximum likelihood criterion using default 100 replicates. Sequences published by Dalman et al. (2010) with GenBank numbers: FJ627430, FJ627391, FJ627387 and FJ627404 in the case of EFA; sequence numbers FJ627465, FJ627515, FJ627461 and FJ627484 in the case of G3P; and sequence numbers FJ627732, FJ627719, FJ627724 and FJ627737 in the case of TF gene were added to the alignment to ensure the identification of the *Heterobasidion* species. Sequences published by Gonthier et al. (2007) of *Heterobasidion insulare* (with GenBank numbers: DQ916091, DQ916105) were used as an out-group.

3 Results

In total, 17 isolates originated from caves or underground galleries were examined in this study (see Table 1). The partition homogeneity tests showed no significant conflict between the three genetic markers used ($p = 0.01$). This allowed a combined analysis of the TF-G3P-EFA sequence data set generated. The three-marker alignment was 1042 bp long, of which 953 bp were conserved, 89 bp were variable, and 67 bp were parsimony informative. Most from the isolates analysed, most (9) were identified as *H. parviporum* (Fig. 1), with isolates obtained from moonmilk, cave and outside air, and wall from underground gallery. Six isolates were identified as *H. annosum*. These isolates were obtained from cave air and sediment, isopod faeces and underground gallery wall. *Heterobasidion abietinum* was obtained from Slovakian caves and was isolated from bat guano and outdoor air.

4 Discussion

Records of *Heterobasidion* from underground spaces as caves and tunnels are rather rare. Detailed studies of microscopic fungi in remote sediments of West Virginia caves, USA (Rutherford and Huang 1994), in various cave substrates including cave air in The Domica Cave, Slovakia (Nováková 2009), and in three other caves of the NP Slovak Karst, the Ardovská Cave, the Stará brzotínska Cave and the Drienovská Cave (Nováková 2012b), and the Doña de Trinidad Cave, Spain (Hermosín et al. 2010), as well as in cave air and bat guano in Gruta Lapa Nova, Brazil (Taylor et al. 2013), or in the Nazi military complex Osówka in Poland (Pusz et al. 2014), did not report this fungus. Vanderwolf et al. (2013) in their extensive worldwide review cited eight records of *H. annosum* from underground spaces, but only from decayed wood (Great Britain, Poland, Austria, Slovenia, Croatia, Bosnia, Czech Republic and Romania). The record of *H. annosum* basidiocarps on spruce wood in mines of the Kladno coal district (Czech Republic, formerly Czechoslovakia) was mentioned by Příhoda (1965). The report of *Spiniger meineckellus* from the air of an underground tunnel under Bedřichov, Czech Republic was published by Kubátová et al. (2005).

The origin and the main ways that *Heterobasidion* isolates spread in underground spaces are not clear. The fungal basidiospores or conidia may persist in underground environment in air on sediment or on wood substrates as mycelium with conidiophores. Most of the inspected caves are surrounded by woods where conifers are present, so short-distance dispersal of fungal spores from the wood to underground spaces is plausible. But the identified isolates from the Výpustek Cave (Czech Rep.) do not confirm this theory. While isolates from the caves were identified as *H. parviporum*, isolates collected from roots and stumps of *Picea abies* in the neighbourhood of the Výpustek Cave were identified as *H. annosum* (Sedlák and Tomšovský 2014). However, the Výpustek Cave has an exceptional history due to its utilization for military

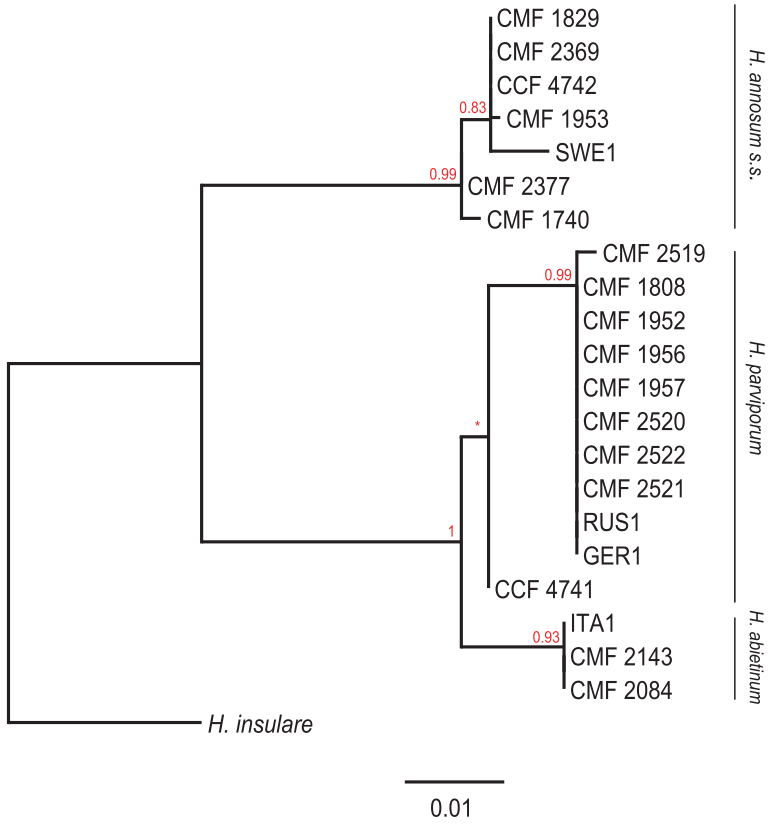

Fig. 1. The phylogram inferred from maximum likelihood analysis of aligned sequences of EFA, G3P and TF genes. Numbers at branches indicate Bayesian PPs values higher than 50%, asterisk indicates a value lower than 50%. The bar indicates the number of expected substitutions per position. Asian *H. insulare* (Gonthier et al. 2007) was added as outgroup. ITA1 in *H. abietinum* clade includes Italian strain; RUS1 and GER1 in H. parviporum clade include European strains; SWE1 in *H. annosum* s.s. clade include European strains; ITA1, RUS1, GER1, SWE1 are sequences by Dalman et al. (2010).

purposes in 1936–2001 (Cave Administration of the Czech Republic 2014). During this time, spruce wood for human activities infected by *H. parviporum* may have been transferred in the cave.

The record of *H. parviporum* from Castañar de Ibor Cave also contradicts a local spore source. Oliva et al. (2010) reported known distribution of *H. parviporum* in Spain as co-occurrence with its preferential host – *Picea abies*. This locality is several hundred kilometres away from natural localities of *Picea abies* in Spain (Skrøppa 2003). Likely, *H. parviporum* had been introduced to this cave by man, because *Picea abies* as the preferential host has never been presented in Central Spain at least since the beginning of the Quaternary period (Taberlet et al. 1998). Most likely, *H. parviporum* was transported to the site with infected wood.

The Movile Cave in Romania is the most unique among the mentioned caves (Chen et al. 2009) characterized by another food web based on chemolithotrophic and chemoautotrophic bacteria that is different from aboveground ecosystems. The cave became isolated from the surface (thick layer of loess had isolated the cave fauna from the surface forms) at the end of the Miocene (5.5–5.2 Mya) resulting in a unique ecosystem. The cave had been discovered accidentally in 1986, when an artificial shaft intercepted a low cave passage and had been closed again immediately. Despite the fact that the cave has no natural entrance and consequently there is no input of food from the surface, both the aquatic and the terrestrial communities consist of many species occurring in large populations (Sarbu 1991). Isolates of *S. meineckellus* come from *Trachelipus troglobius* faeces (Fig. 2c) from more than 25 m underground level of The Movile Cave. *Trachelipus troglobius* (Fig. 2b) *(Isopoda, Oniscidea)* is an endemic troglobiont (cave-dwelling animal) inhabiting permanently the subterranean environment of the Movile Cave where the base of the trophic chain appears to be chemoautotrophic microbiota that uses H_2S from the thermomineral stream in the cave as a source of energy (Sarbu 1991; Brustur et al. 1999). Its probable sources of nutrients are dead bodies of other animals present in the cave or a microbial mat (Fig. 2d) covering the cave walls and lakes in thick layers. Organic compounds such as cellulose, lignin and other organic compounds typical for plant tissues are absent in this cave system which may affect enzyme production in *Heterobasidion* isolates. The occurrence of the anamorphic stage of *Heterobasidion* in such ecosystem is rather surprising. This may be a unique, relict population of Tertiary origin or a result of the contamination by a recent inoculum (although conditions of access to the cave are strict, human-mediated transfer is less probable).

Heterobasidion annosum s.l. is usually being reported from the forest environment (rotting roots, butts, trunks of forest trees and dead wood). The range of varied environment and diverse substrate and hosts that this fungus can colonize and

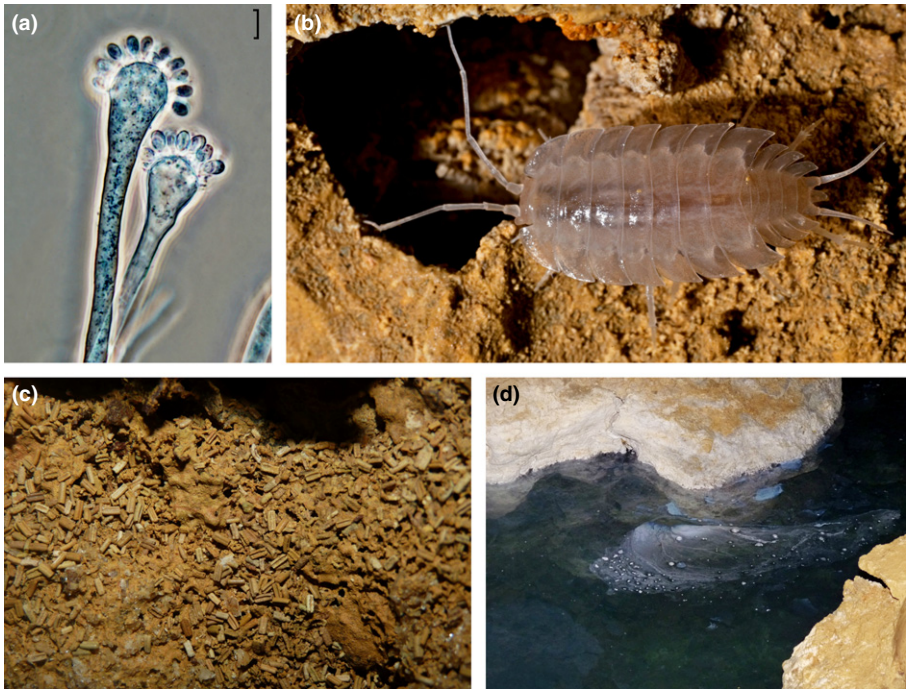

Fig. 2. (a) Conidiophores with conidia of the anamorph of *H. annosum*, CCF 4742, 1000× magnification, scale bar-10 um (Photo by A. Kubátová), (b) *Trachelipus troglobius* (photo by Petr Zajíček), (c) Faecal pellets (faeces) of *Trachelipus troglobius*, (d) Microbial mat floating on the surface of underground lake (photos c, d by A. Nováková).

survive on seems to be astonishingly wide. Männiste and Hanso (2006) reported *Heterobasidion* spp. in Italy and Estonia on green symptomless needles of young *Abies alba* trees and *Picea abies* trees from natural regeneration, respectively. In the previous study, Sedlák and Tomšovský (2014) documented *H. annosum* s.l. on various unusual hosts. Incidentally, during the investigation on agriculture land for the presence of endophytic fungi *Heterobasidon* spp., it had been found on the trunk of vine (*Vitis vinifera*) and on the ear of maize (*Zea mays*). The present study adds to the wealth of unusual habitats for this forest pathogen.

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ACHIEVEMENTS AND STUDY ACTIVITIES

09/2011 – Species distribution and host specificity of *Heterobasidion annosum* s.l. in the Czech Republic. Lecture at XIII IUFRO conference "Root and Butt Rot of Forest Trees"in Firenze and S. Martino di Castrozza, Italy.

10/2011 – *Heterobasidion annosum* s.l. and its rapid identification using molecular methods. Lecture at 12th International scientific conference of PhD. students, young scientists and pedagogues, Nitra, Slovakia.

01/2012 - 04/2012 – Research stay at SILAVA, *Phlebiopsis gigantea* – biological control agent of root rot and butt rot disease – participating on different experiments. Forestry Institute in Salaspils, Latvia.

03/2012 – *Heterobasidon annosum* s.l. and *Armillaria* as dangerous species for short rotation plantations; Lecture on International Student Workshop at Latvia University of Agriculture, Forest Faculty, Jelgava, Latvia.

04/2012 – Root rot and butt rot consequences in modern forestry; lecture for students and staff at Latvia University of Agriculture, Forest Faculty, Jelgava, Latvia.

2011 – 2013 – Member of organization committee of student conference SilvaNET – WoodNET, held on Mendel University in Brno.

05/2013 – Species variation and disposition of *Heterobasidion annosum* sensu lato in the Czech Republic, Lecture at IUFRO 2013 WORKING PARTY 7.02.02 Foliage, shoot and stem diseases of Forest trees. Biosecurity in natural forests and plantations, genomics and biotechnology for biosecurity in forestry. Brno May 20. Czech Republic.

08/2013 - 10/2013 – Research stay at SLU, Participating on: Different treatments as possible methods of controlling root and butt rot in forests of Scandinavia, SLU Uppsala.

05/2014 – Research visit of BFU, studying herbarium specimens of East Asian *Heterobasidion* species, Beijing Forest University, The People's Republic of China.

06/2014 – Short research stay at SLU. Studying of the possibilities in identification of wood-inhabiting fungi in various environmental material, using the ION Torrent, 454 and Illumina sequencing. Uppsala.

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