MENDEL UNIVERSITY IN BRNO

FACULTY OF FORESTRY AND WOOD TECHNOLOGY

DEPARTMENT OF FOREST PROTECTION AND WILDLIFE MANAGEMENT

Distribution of *Heterobasidion* and *Armillaria* root rots in Vallombrosa fir forest, Italy

DIPLOMA THESIS

2015/16 Ing. László Benedek Dálya

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DIPLOMA THESIS TOPIC

Author of thesis:	Ing. László Benedek Dálya
Study programme:	European Forestry
Field of study:	European Forestry
Thesis supervisor:	prof. Dr. Ing. Libor Jankovský
Consultant:	Prof. Paolo Capretti, Ing. Tomáš Májek, Ing. Petr Sedlák, PhD.
Title of the thesis:	**Distribution of Heterobasidion and Armillaria root rots in Vallombrosa fir forest, Italy**

Guides to writing a thesis:

1. 25 years ago - in 1990 - was done a survey in Vallombrosa forest, Central Italy, which is a reference forest for research in Italy, to register the occurrence of Heterobasidion on Abies and other species. The goals of these Thesis are to refresh data from 1990 and to compare situation in plots after 25 years in cooperation with Italian partner. The aim of theses is to describe health of forest and to give some suggestions for management. External tutor is Prof. Paolo Capretti, University of Florence.
2. To process literature review on research in Vallombrosa forest, especially focused to fungal pathogens and theirs impact to forest ecosystem.
3. To do sampling for further laboratory analyses compatible to sampling in 1990. Collect fruiting bodies, soil samples, dead wood, rhizomorphs etc.
4. On the bases of DNA analyses to identify Armillaria and Heterobasidion species, present in soil, rotten wood, taken from plots.
5. On the bases of obtained results to evaluate role of root rots in Vallombrosa forest and to suggest some practical measures for the forest.
6. To do general overview of health of Vallombrosa forest on the bases of markers as defoliation, etc.
7. Theses write in the structure of scientific paper:1. Introduction, 2. Material and Methods, 3. Results, 4. Discussion, 5. Conclusions, 6. Literature, 7. Appendixes

Length of thesis: min. 50 pages

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Date of entry: December 2014

Date of submission: April 2016

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Statutory declaration

I hereby declare that I compiled the diploma thesis on the topic of *"Distribution of* Heterobasidion *and* Armillaria *root rots in Vallombrosa fir forest, Italy"* by myself and have stated all sources used. I agree to my thesis being published in accordance with §47(b) of the Act No. 111/1998 Coll. on Higher Education Institutions including amendments to some other acts and in compliance with Mendel University Chancellor's decree on publishing final theses.

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Abstract

Ing. László Benedek Dálya

Distribution of Heterobasidion *and* Armillaria
root rots in Vallombrosa fir forest, Italy

This work intends to describe the present condition of Vallombrosa forest (Tuscany, Italy) from the phytopathological point of view. The chronic disease caused by *Heterobasidion* and *Armillaria* root rots is a key factor affecting the vitality of silver fir plantations of the region. Detailed knowledge about their distribution could help to control the pathogens. Systematic sampling and survey of damages on trees were undertaken at 52 points. Identification of different species from soil and fungal samples was accomplished by DNA-based methods (TSCP, nested PCR, RFLPs analysis). The high presence of both parasitic fungi was detected under a wide range of ecological conditions. Data analysis indicates the strong spreading potential of the pathogens even into new habitats, especially in connection with water stress of their hosts.

Keywords: *Heterobasidion annosum* (Fr.) Bref.*, Armillaria,* silver fir (*Abies alba* Mill.), root rot, butt rot, polymerase chain reaction (PCR)

Abstrakt

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Rozšíření kořenových hnilob Heterobasidion *a* Armillaria *v jedlovém lese Vallombrosy, Itálie*

Diplomová práce je zaměřena na studium aktuální fytopatologické charakteristiku porostů ve Vallombrosa Forest (Toskánsko, Itálie). Klíčovým faktorem, který ovlivňuje vitalitu jedle bělokoré v regionu jsou chronické infekce kořenovými hnilobami způsobené kořenovníkem *Heterobasidion* spp. a václavkami *Armillaria* spp. Podrobné znalosti o jejich rozšíření by mohly napomoci k implementaci ochranných opatření proti těmto patogenům. Zkoumané vzorky byly odebrány ze stromů z 52 míst. Byla provedena identifikace hub z půdy metodami založenými na DNA (TSCP, nested PCR, analýza RFLP). V širokém rozsahu ekologických podmínek lokality byla zjištěna vysoká frekvence obou původců kořenových hnilob. Analýza dat ukazuje na silný potenciál obou patogenů dále se na lokalitě šířit, a to především v důsledku stresové zátěže hostitelů nedostatkem vody.

Klíčová slova: *Heterobasidion annosum* (Fr.) Bref., *Armillaria*, jedle bělokorá (*Abies alba* Mill.), kořenové hniloby, hniloby kmene, polymerázová řetězová reakce (PCR)

List of abbreviations

a.s.l.	above sea level
bp	base pair
DBH	diameter at breast height
DNA	deoxyribonucleic acid
GPS	Global Positioning System
ISG	intersterility group
ITS	Internal Transcribed Spacer
mya	million years ago
PCR	polymerase chain reaction
rDNA	ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
s.l.	sensu lato
s.s.	sensu stricto
TBE	Tris-borate buffer
TSCP	taxon-specific competitive-priming

Table of contents

1. Introduction

Silver fir (*Abies alba* Mill.) is one of the dominant forest species in the mountainous regions of Central and Southern Europe. Despite its excellent silvicultural qualities and multi-purpose wood, the species has been on the decline throughout Europe for the last two centuries, due to direct (conversion of stand structure, clearcutting, grazing) and indirect (climate change, air pollution, overpopulated game species) human influences.

Another destructive factor comes into play in Italy, where the main problem for the conservation of silver fir stands has long been conceived pathogenic agents, in particular *Heterobasidion annosum* s.l. and to a lesser extent, the *Armillaria* species complex. These parasitic fungi cause root rot and decay of the stem, which typically leads to uprooting under intense mechanical stress, by reason of the decreased stability of the tree.

On 5th March 2015, the Nature Reserve of Vallombrosa was hit by a severe windstorm (Fig. 1), which reached a peak velocity of 160 km/h, causing considerable damages to the forest stands. According to preliminary calculations, 50 ha of forest, equivalent to 20000 m^3 of timber, had been destroyed by windthrow. This event gave actuality to a complex monitoring in order to shed light on the key factors determining the susceptibility of the forest to such catastrophic damages (CIBECCHINI ET AL. 2015; CHIRICI ET AL. 2015).

FARINA ET AL. (1990) reported the massive presence of an intersterility group (ISG) of *H. annosum* in Vallombrosa, specialized to silver fir. During the data collection of present work, the same sampling grid had been used, so the spatial distribution of the fungus can be compared over the past 25 years. In addition, the distribution of *Armillaria* spp. has been investigated. The several DNA-based diagnostic techniques, which have been being developed since the above study, allowed of a more straightforward identification of wood rotting fungi.

Figure 1: Silver fir stand in Vallombrosa forest, after the windthrow of 2015. (Photo by Paolo Capretti)

1.1. Aims of the diploma thesis

1. Creating distribution maps of *Heterobasidion* and *Armillaria* species in Vallombrosa forest.
2. Comparing the situation in sampling plots after 25 years.
3. Evaluating the role of root rots in Vallombrosa forest.
4. Describing the general forest health in Vallombrosa.
5. Making suggestions for using the results in forestry management practice.

1.2. Description of the study area

The Vallombrosa forest is a favoured target area for forestry research compared to other regions. Moreover, it serves as a reference forest for studies conducted in various disciplines. Several publications have been issued about the history of local forests in recent years (ELDER 2008; CIANCIO 2009; CIANCIO AND NOCENTINI 2009; BOTTALICO ET AL. 2012).

1.2.1. Location

The study area is the Vallombrosa forest (43° 44' N, 11° 34' E, Fig. 2); a biogenetic reserve located about 50 km east-southeast of Florence, in Tuscany, Italy. The forest extends on the western flank of the Pratomagno massif, a north-south oriented spur of the Apennines Mountains. The forest covers an area of 1273 ha.

Figure 2: Satellite view of Vallombrosa.
(Source: http://www.tageo.com/index-e-it-v-00-d-m200547.htm)

1.2.2. Climate

According to the daily temperature and precipitation data of the Thermopluviometric Station of Vallombrosa (980 m a.s.l.), the climate at Vallombrosa is humid temperate, characterized by a mean annual air temperature of 9.8°C and a mean annual precipitation of 1275 mm. January is the coldest month (1.9°C), August is the warmest one (19.0°C). Snowfall occurs during the winter and early spring (CERTINI ET AL.

2007). The vegetative season begins in late April and lasts till late September (CERTINI ET AL. 2003a).

1.2.3. Topography

As regards the main features of relief, western and northwestern slopes dominate. The average inclination is 18.8%. Slopes steeper than 30% represent 8% of the total area. The altitude ranges between 470 and 1440 m a.s.l., with an average of 990 m.

1.2.4. Pedology

The single parent material of the soils of the area is Oligocene sandstone, chiefly composed of quartz, feldspars and phyllosilicates, intercalated with thin layers of siltstone (calcite, quartz, plagioclases and phyllosilicates) that represents 7-10% of the rock (CERTINI ET AL. 2003a). The sandstone layers are up to several metres thick (CERTINI ET AL. 2003b). The addition of wind-blown material has probably not played a major role in the soil formation at Vallombrosa, as shown by the continuous mineralogy of the profiles opened by CORTI ET AL. (2001).

At higher elevations of the Vallombrosa forest, evidence of paleosols has been found by CORTI ET AL. (2001). The data for these soil profiles show reversed depth trends, suggesting a history of periglacial phenomena such as soil truncation and deposition of soliflucted material in which the modern soil horizons have since developed. These events are considered to date from the Würm glaciation.

Many soils at Vallombrosa have a dense horizon close to the surface, as reported by CERTINI ET AL. (2000a). Such a layer is fragipan, a diagnostic subsurface horizon formed by the mechanical compaction of mineral particles, probably under periglacial conditions. It is characterized by the lack of cementation among the grains, a thickness of at least 15 cm, evidence of pedogenesis, and no reaction to dilute HCl. Measuring samples from Vallombrosa, CERTINI ET AL. (2007) determined an oven-dried bulk density as high as 1.95 g/cm^3, and 1.7-1.8 g/cm^3 at the original moisture content.

Fragipans show an irregular distribution at Vallombrosa, being present in approximately 30% of the forest, at altitudes between 800 and 1100 m. Typically they form large lobes that elongate on the flanks of the Pratomagno Ridge, parallel to the maximum slope gradient, and end in flatter surfaces (BOLLA 2001). This "digitate" distribution supports a hypothesis that downslope earthflows emplaced the material that, once deposited, acquired high density through dewatering and rearrangement of particles (CERTINI ET AL. 2007).

Based on the soil map of Tuscany Region, 5 distinct soil units can be found in the Vallombrosa forest. The following list contains their names according to the Italian soil classification system and the SOIL SURVEY STAFF (1999), their extent at Vallombrosa and basic characteristics.

Pontepetri (PON1): Typic Dystrudepts, loamy-skeletal, mixed, mesic. Cover 38.9% of the area. Moderately deep, A-Bw-C-R profile, from gravelly and stony to very gravelly and stony, sandy loam and loamy texture, non-calcareous, highly to moderately acid, low base saturation, from well drained to sometimes excessively drained. Located on slopes with medium to long subparallel valleys, often with strongly to moderately steep erosion channels of considerable size. Subject to strong water erosion mainly of channel type. High amount of large stones and low to moderate rockiness. Very common soils primarily occupied by beech, chestnut and conifer reforestations, secondarily by pastures and former pastures colonized by broom species.

Giunchete (GIU1): Ultic Hapludalfs, fine-loamy, mixed, mesic. Occupy 36.3% of the area. Deep, A-AB(E)-Bt-C profile, from less gravelly to gravelly and stony, silty loam and loamy texture, non-calcareous, slightly acid, low base saturation, well drained. Generally located in stable positions on linear, moderate to steep slopes or valleys prevalently exposed to the north. Little to high amount of stones and low rockiness. Subject to moderate water erosion mainly of channel type. Mostly covered by chestnut and mesophilic broadleaves and are frequent.

Maresca (MRS1): Humic Dystrudepts, coarse-loamy, mixed, mesic. Extend on 20.6% of the area. Moderately deep, A-Bw-C-R profile, rich in organic matter in the A horizon, from less gravelly to gravelly and stony, sandy loam and loamy texture, non-calcareous, highly to moderately acid, low or medium base saturation, well-drained. Present on mostly linear, strongly to moderately steep slopes, sometimes in instability, at altitudes above 800 m. Subject to moderate erosion prevalently of diffuse type. Little to high amount of large stones and low rockiness. Infrequent soils primarily utilized as pasture or covered by montane grasslands.

Monte Bastione (MBS1): Typic Hapludalfs, coarse-loamy, mixed, mesic. Found on 2.6% of the area. Deep, A-AB-Bt-C profile, from less gravelly to gravelly, sandy loam and loamy texture, non-calcareous, from slightly acid to neutral, high base saturation, well-drained.

Poggio di Petto (PGG1): Lithic Dystrudepts, coarse-loamy, mixed, mesic. Present on 1.7% of the area. Shallow, A-Bw-BC-R profile, gravelly and stony, sandy loam and loamy texture, non-calcareous, highly to moderately acid, very low base saturation, from well drained to sometimes excessively drained. Normally situated on very steep slopes with valleys, in all aspects. Subject to strong water erosion of diffuse and channel type. High amount of large stones, moderate to high rockiness and rocky outcrops. Uncommon soils often populated by sparse and degraded forest formations.

1.2.5. Vegetation

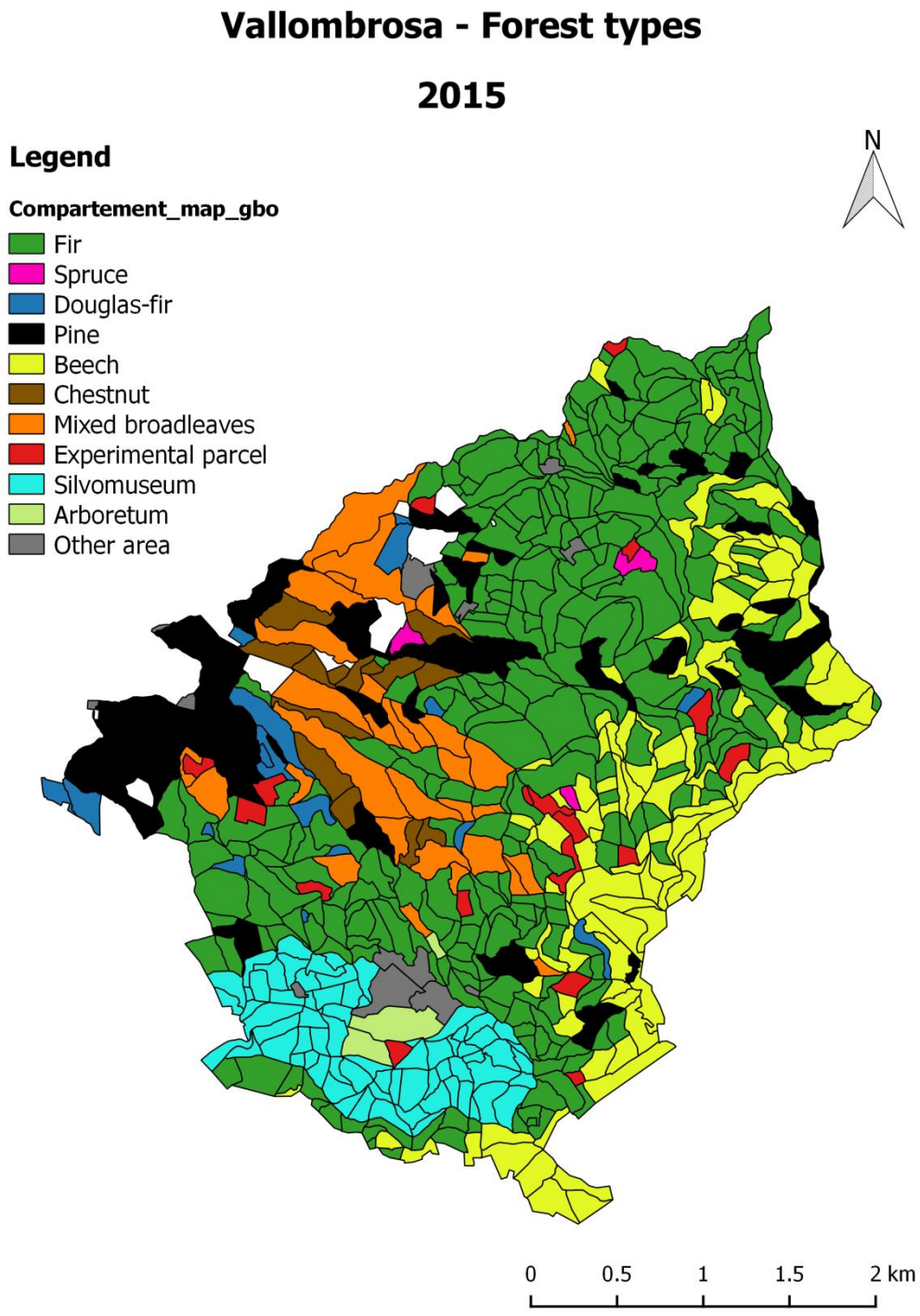


Figure 3: Forest types in Vallombrosa, indicating the prevailing species of each stand. (Source: Database of the forest management plan 2006-2025 of Vallombrosa)

The most common tree species in the forest is silver fir (Fig. 3), which occupies 681 ha (53.5% of the total area), typically with an admixture of different broadleaved species or – less commonly – conifers, though 39.1% of the fir stands are monocultures. European beech (*Fagus sylvatica*) dominates 15.8% of the Reserve, occurring in its natural range at higher elevations. Various species of pine are prevalent on 12.2% of the area, usually forming mixed stands. Corsican pine (*Pinus nigra* ssp. *laricio*) and Austrian pine (*Pinus nigra* ssp. *nigra*) are widespread, while Scots pine (*Pinus sylvestris*) has only two occurrences. Other introduced conifers include coast Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*, 2.4%) and Norway spruce (*Picea abies*, 0.4%). At lower altitudes, broadleaves represent the native vegetation. Chestnut (*Castanea sativa*) is growing on 3.1% of the Reserve. Other mixed deciduous stands (9.6%) are mostly composed of oaks (*Quercus* spp.), maples (*Acer* spp.), European hornbeam (*Carpinus betulus*) and hop hornbeam (*Ostrya carpinifolia*).

1.2.6. Forest management

The management history of the Vallombrosa forest goes back to the eleventh century when the Benedictine Monks settled there. Taking care of the forests was one of the functions of the abbots, for whom the timber served as a necessary resource to furnish the Vallombrosian Abbey. The fir trunks were an important commodity, used for the construction and restoration of buildings in Florence, so the Monks bent every effort to propagate and enhance the development of fir trees (ELDER 2008).

The early emphasis on coppicing and chestnut production for food gave place to the cultivation of silver fir, which has been the preferred tree species since 1350. This attitude change caused that pure stands of fir pushed in the higher-elevation beech forest, while at the same time gradually appeared in place of the chestnuts of the lower elevations (ELDER 2008). Commercial cultivation of silver fir plantations started in 1600, when the Monks introduced the clear-cut system in lieu of selection cuttings, replacing the natural regeneration to artificial and broadleaves to conifers (CIANCIO 2009).

The forest management was executed by the Monks until 1866, the unification of Italy and the subsequent expropriation of church lands to state property (ELDER 2008). The State Administration continued the propagation of fir. The first three regulation plans (1876-1886-1896) solely dealt with the 200-300 ha of fir stands surrounding the Abbey, for which the prescribed treatment was clear-cut and artificial regeneration with 80-90 year rotation period. The fourth plan, issued by DI TELLA in 1923, also included in the management the coppice and high forests of beech, chestnut coppice and pine forests, with a change of property limits that became similar to the actual one, covering an area of approximately 1200 ha. 100 year rotation and a gradual conversion of beech stands into fir-beech mixed forest were proposed. The plan of PATRONE in 1960 incorporated natural regeneration even for fir stands and tried to attain their transformation into fir-beech mixed forest. Such a choice was based on the beech's higher resistance and resilience to biotic and abiotic damages over fir (CIANCIO 2009). In 1977, the entire area became Biogenetic Nature Reserve, authorized by Law No. 394/91. After the Conference of Rio de Janeiro in 1992 and the adoption of Agenda 21, the woodland of Vallombrosa and Saint Anthony has been recognized as a Site of Community Importance.

Analysing the period between 1923 and 2006, BOTTALICO ET AL. (2012) pointed out the expansion of fir (from 504.6 to 684.4 ha) and pine stands (from 9.4 to 142.6 ha) and the decrease of chestnut woods (from 245.3 to 37.9 ha) and beech forests (from 400.7 to 201.3 ha). The fir grown in purity, lying at the limit of its distribution has often been affected by root rot. Starting in 1960, plantations and under-plantations of conifers and deciduous trees were carried out to encourage the natural regeneration of silver fir, thus seeking to establish mixed, stable and self-perpetuating populations. Today, in older fir stands, the spontaneous appearance of other species such as beech and sycamore is common. An important change brought by past choices is the introduction of Austrian pine and Corsican pine at lower elevations. In some of these stands the spontaneous spread of broadleaves is observed today, increasing the degree of biodiversity of these artificially created stands (BOTTALICO ET AL. 2012).

In 2006, the forest management plan 2006-2025 of the State Biogenetic Nature Reserve of Vallombrosa was compiled by the University of Florence. This plan attains a significant change of all instructions prescribed in previous plans. According to the traditional regulation, the forest had to reach the normal state, providing a maximum and almost constant annual increment, and the regeneration was predetermined; in the new plan, the forest is considered a complex biological system that ensures and fulfills multiple functions (ecosystem services), the management is based on the principles of minimal intervention (CIANCIO 2009). In this perspective, three main directions are defined: the preservation of historical fir stands around the Abbey of Vallombrosa, the enhancement of the natural growth in one part of the Reserve and the re-naturalization of a large part of the Vallombrosa forest (BOTTALICO ET AL. 2012).

The purpose of the Silvomuseum is to maintain the landscape through the preservation of silvicultural methods applied in the past. Its regulation plan, described by CIANCIO AND NOCENTINI (2009) aims to conserve a dynamic mosaic of even-aged silver fir monocultures, where all age classes are represented. 70 fir stands are included, for a total of 105 ha. Choosing the rotation period has been considered from the social point of view instead of maximizing wood production. Silver fir has a lifespan beyond 400 years, and the abundance of old fir stands throughout the forest testifies the adequacy to manage this species with a much longer rotation than before. In fact, the lack of cuts since the 1970s has led to a situation where more than 45% of fir stands in the Silvomuseum are older than 100 years. Proposing a 100 year rotation would infer the obligation to regenerate all these stands in a short time with a significant and adverse impact on the landscape and the environment. For all these reasons, a 150 year rotation was chosen. The plan prescribes clear-cut with follow-up artificial regeneration. The area of the cut must not exceed 3000 m^2. Preferably the cuts will be executed in those parts of the forest where openings already exist or fir saplings are established.

1.3. *Heterobasidion annosum* s.l.

Heterobasidion annosum s.l. (Fr.) Bref. is one of the most important specialized, root-inhabiting pathogens in the temperate and boreal regions of the Northern Hemisphere. This fungus causes a root and butt rot, primarily infecting coniferous trees. It represents a major threat to intensively managed forest stands, while playing a more subordinate role in natural ecosystems. Financial losses attributable to the species complex in the European Union are approximately 790 million € annually (WOODWARD ET AL. 1998). This estimate includes losses due to decay as well as reduction in diameter growth of infected trees but excludes windthrow and reduction of resistance of stands to storm damages caused by the fungus, which may be locally significant (GONTHIER AND THOR 2013). As a facultative necrotroph, *H. annosum* s.l. is able to survive saprotrophically on dead wood, and the same individual can switch from one mode of lifestyle to the other (GARBELOTTO AND GONTHIER 2013).

1.3.1. Taxonomy and distribution

H. annosum s.l. had long been regarded as a single species until mating experiments among different individuals revealed the occurrence of ISGs (KORHONEN 1978b). All of them – three in Eurasia and two in North America – have obtained formal description as species. The Eurasian groups were named *H. annosum* s.s., *H. abietinum* Niemelä & Korhonen and *H. parviporum* Niemelä & Korhonen (NIEMELÄ AND KORHONEN 1998), while the North American groups were described as *H. irregulare* (Underw.) Garbel. & Otrosina and *H. occidentale* Otrosina & Garbel. (OTROSINA AND GARBELOTTO 2010). Although defined on the basis of partial reproductive isolation and morphology, further supported by phylogenetic analyses based on a range of markers, these species are also characterized by a distinct host preference. *H. annosum* s.s. is mostly associated with pines, especially Scots pine, but attacks several other conifers and even some broadleaved tree species. *H. parviporum* shows a relatively strict specialization for Norway spruce, while *H. abietinum* is commonly associated with silver fir and other species of the genus *Abies* (GONTHIER AND THOR 2013).

The distribution of *H. annosum* s.l. species reflects that of their main host species. *H. annosum* s.s. is found throughout Europe, except on sites north of 66°N, and its distribution area extends east to the Altai region in southern Siberia. *H. parviporum* occurs from the northernmost parts of Europe to the Southern Alps and from Western Europe to China, Japan, and southern Siberia. *H. abietinum* is distributed in Central and Southern Europe and in the Mediterranean Basin. Nevertheless, *H. irregulare* was introduced into central Italy during World War II (GONTHIER ET AL. 2004), and subsequently became invasive by spreading in Italian stone pine (*Pinus pinea*) stands (GONTHIER ET AL. 2007). The current distribution area of *H. irregulare* includes an area of approximately 100 km along the Tyrrhenian coast around Rome (D'AMICO ET AL. 2007; GONTHIER ET AL. 2007).

The evolutionary history and biogeography within the species complex were recently clarified (LINZER ET AL. 2008; DALMAN ET AL. 2010). Speciation within *H. annosum* s.l. started approximately 60 mya, well after the radiation of host genera. The last emerging species was *H. abietinum*, which arose 14–31 mya. Although *H. annosum* s.l. species are characterized by partial interfertility in laboratory experiments and by overlapping ranges, interspecific hybrids have rarely been reported and only between the sympatric North American *H. irregulare* and *H. occidentale* (GARBELOTTO ET AL. 1996; LOCKMAN ET AL. 2011).

1.3.2. Diagnosis

H. annosum s.l. causes a white rot through selective delignification. During this process, lignin is decomposed more rapidly than cellulose and hemicelluloses. Chemicals secreted by both host and pathogen in the early stages of infection result in a darker stain in the wood. The pockets that develop in the advanced stages of decay often contain black specks, while the rot becomes soft, fibrous and stringy in texture, light brown or straw coloured and dry in nature. Eventually a hollow may be formed in the centre of the tree. Decay can be detected in primary roots, at the root crown, and in the stem, sometimes several meters away from the ground (WOODWARD ET AL. 1998).

Symptoms caused by the fungus vary depending on the ISGs involved, the tree species infected, the age and previous use of the forest stand, the soil type, the local climate, and possibly atmospheric pollution. In species characterized by a resinous heartwood (e.g. *Pinus* spp.), both young and mature trees are susceptible to a root rot and may be killed by the pathogen. Mortality occurs very soon (i.e. one season) in young trees, and proceeds more slowly in older trees. Symptoms before death may include the decline of annual shoot growth and the shading of old needles, resulting in the so-called lion-tailing phenomenon in a thinner crown. Up to two-thirds of a root system may be killed by *H. annosum* s.l. before symptoms appear in the crown. In species characterized by a non-resinous heartwood (e.g. *Picea* spp., *Abies* spp.), the fungus is generally responsible for extensive heart rots in the roots, the butt and the stem. When heart rot develops, external symptoms are rarely visible and mortality does not occur. Occasionally, exudation of resin or butt swelling may indicate the presence of decay within the stem (GONTHIER AND THOR 2013).

However, these symptoms are not characteristic and can be related to other root diseases – a definitive field diagnosis of *Heterobasidion* infection requires the identification of a fruiting body. 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 irregularly shaped, 3.5(-7) cm thick and up to 40 cm in diameter. The anamorphic stage is described as *Spiniger meineckellus* (A.J. Olson) Stalpers.

1.3.3. Biology and epidemiology

H. annosum s.l. is able to infect a tree by airborne propagules which colonize freshly exposed wood surfaces: stumps or wounds in the stem or roots. The period of susceptibility of stumps to infection rarely exceeds a month after cutting. Once primary mycelia have established and eventually formed secondary mycelia, colonization proceeds downward to the root system at a rate of up to 20 cm per month.

Subsequently, the fungus can spread to living trees over the root system. Direct root contacts are crucial for secondary infection since the pathogen is incapable of freely growing in the soil. Although the fungus produces both sexual spores and conidia, only the former seem to cause infections in nature (WOODWARD ET AL. 1998). Normally, trees die in clusters and mortality progresses over time, spreading outward in concentric rings.

Infection risk highly depends on the weather. Under Mediterranean climate, GARBELOTTO ET AL. (2010) detected high levels of spore deposition of *H. annosum* s.s. in the winter and significantly lower levels in summer. The root rot intensifies after the second and subsequent thinnings in the stands because of local production of spores. Damage caused by *Heterobasidion* is generally greater on fertile soils and on sandy soils with low organic matter content. Besides, high calcium content and pH favour the occurrence of the disease by reducing the activity of antagonistic fungi in the soil. Former agricultural lands or pasture lands pose a higher infection risk than forest soils (WOODWARD ET AL. 1998).

1.3.4. Control

Several methods have been developed aiming to prevent the spore dispersal to control the disease. 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 applied techniques to minimize the disease spread (ASIEGBU ET AL. 2005). Integrated management systems combining different approaches to fight *Heterobasidion* root and butt rots are generally more effective and even cheaper than single control methods (GONTHIER AND THOR 2013). These often include silvicultural means such as: (a) wide spacing between planted trees to decrease the probability of root contacts, (b) delay of thinnings and selecting the suitable season for thinning, when spore production halts, (c) reduction of rotation period, (d) admixing broadleaved species into the stand. This latter strategy may not completely eliminate the parasite after a single rotation (LYGIS ET AL. 2004) but should

significantly and progressively reduce disease incidence (GARBELOTTO AND GONTHIER 2013).

1.3.5. Impact in Vallombrosa

Heterobasidion annosum s.l. is one of the most important basidiomycetes causing damage to conifers in Italy (CAPRETTI 1998). It spreads under all ecological conditions and all the European ISGs are present, affecting the main native host species: *Abies*, *Picea* and *Pinus*. Along the Apennines, the disease incidence is particularly high in silver fir plantations that are older than 50-60 years (CAPRETTI 1998). Sanitary cuts in this area often amount for more than 50% of the annual cut (MORIONDO AND TIBERI 2000). According to CANTIANI (1960), the parasite caused 10.2% loss of the growing stock in the first rotations. However, the main economic loss lies in the higher logging expenses associated with windthrows (LA MARCA 1979).

FARINA ET AL. (1990) investigated the occurrence of *H. annosum* s.l. at Vallombrosa, performing a systematic sampling of the whole area. The presence of the pathogen has been ascertained in 81.0% of thinned coniferous stands, but it was absent in areas with broadleaved growth. The highest frequency of colonization (56.1%) was recorded on silver fir stumps. The most widespread species was *H. abietinum*, which besides its main host, fir, was occasionally found on other species: Douglas-fir, Norway spruce, Austrian pine, chestnut and Japanese red cedar (*Cryptomeria japonica*). *H. annosum* s.s. was very rare, only detected on Austrian pine and Scots pine, while *H. parviporum* was absent. It was established that the colonization of stumps does not necessarily mean a close host-pathogen relationship. In fact, the Douglas-fir despite showing the presence of the parasite on the stumps is among the undamaged species at Vallombrosa.

In the Vallombrosa forest, the most common and alarming phenomenon is the dieback of silver fir, observed during the 1920s, 40s, then the 70s and 80s. It manifests itself in a thinner crown, defoliation, drying branches, epicormic shoots and the so-called

"stork's nest" due to the extreme reduction of vertical growth (MORIONDO AND TIBERI 2000). INTINI AND MORIONDO (1989) found an annual tree mortality of 2% in the area. The root rot caused by *Heterobasidion abietinum* is a chronic disease in all fir stands, and undoubtedly the most important factor of the species' decline. Typically, the epidemic begins right after the first thinning, because the remaining live stumps are a favourable substrate for the vegetative spread of the parasite (CERTINI ET AL. 2000a). Locally, a number of other pathogenic agents contribute to the fir dieback, such as *Armillaria ostoyae* (INTINI 1988), xylophagous insects and defoliators (MORIONDO AND TIBERI 2000). Moreover, the major declines had always been preceded by a drought period which could increase the susceptibility of the trees to the parasite attack. BONGIANNI AND SULLI (1992) verified that water stress during the vegetative season increases the damage on silver firs by *H. abietinum*.

The previously mentioned symptoms are normally visible only in the final stage of decline. In fact, the most frequent type of damage is the uprooting of apparently healthy trees mainly during the winter after abundant snowfalls or squalls (CERTINI ET AL. 2000a; MORIONDO AND TIBERI 2000). However, the incidence of windthrow does not necessarily indicate the extent of root rot in any given stand. The locality of Croce Vecchia was completely destroyed due to its exposition to strong winds. On other parts of the forest, 120-150 year old trees do not show any symptoms of the disease (MORIONDO AND TIBERI 2000). In addition, the physical properties of the soil are crucial for stand stability. Compacted soil horizons are impervious for the roots. If such a layer occurs closer than 30-40 cm to the surface, the root system of firs develop poorly and cannot provide a sufficient anchorage to the ground (CERTINI ET AL. 2000a; CERTINI ET AL. 2000b).

1.4. *Armillaria* spp.

The genus *Armillaria* (Basidiomycota, Agaricales, *Physalacriaceae*), commonly known as honey fungus, is one of the most studied fungi in the world. The main ecological role of *Armillaria* spp. is the decomposition of dead wood, but some species turn to necrotrophic parasitism. The resulting root rot disease affects a broad host range of woody plants and causes extensive economic losses, especially in managed forests and orchards (GUILLAUMIN AND LEGRAND 2013).

1.4.1. Taxonomy and distribution

Armillaria is distributed on all continents, with at least 40 species worldwide. In Europe, seven biological species have been distinguished by KORHONEN (1978a), based on sexual incompatibility. Five of them have annulate carpophores: *A. mellea* (Vahl:Fr.) Kummer, *A. gallica* Marxm. & Romagn., *A. cepistipes* Velen., *A. borealis* Marxm. & Korhonen and *A. ostoyae* (Romagn.) Herink, and two are exannulate: *A. socialis* (DC.: Fr.) Fayod and *A. ectypa* (Fr.) Lamoure. *A. socialis* is frequently mentioned as *A. tabescens* in the international literature. Nevertheless, ANTONÍN ET AL. (2006) suggest using the name *A. socialis*, arguing that the first author's description (DE CANDOLLE 1815) agrees well with the fungus. *A. mellea* is distributed in the Atlantic and Mediterranean parts of Europe, in various deciduous forests (SHAW AND KILE 1991). *A. ostoyae* is less thermophilic and fundamentally linked to conifers (SHAW AND KILE 1991; GUILLAUMIN AND LEGRAND 2013), following the distribution area of *Abies* spp. in Southern Europe and that of Norway spruce to the north (PERSONAL COMMUNICATION: JANKOVSKÝ 2016). *A. borealis* mainly populates coniferous forests of Northern Europe (GUILLAUMIN AND LEGRAND 2013), but it is common in highlands of Central Europe, too (JANKOVSKÝ 2003). *A. gallica* is regarded as a low-elevation species typical in floodplain forests, while *A. cepistipes* occurs most frequently in the zone of beech (SHAW AND KILE 1991; ANTONÍN ET AL. 2009; JANKOVSKÝ 2003). *A. socialis* is a thermophilic species of Southern Europe (ANTONÍN ET AL. 2006). *A. ectypa* is confined to wetlands and extremely rare (IUCN Red List).

1.4.2. Diagnosis

Colonization of the roots can cause an overall growth loss and a discoloration of the foliage, evolving year by year. Stress-induced reproduction is common, especially on conifers. Resin exudation is particularly obvious at the level of the collar, where sometimes cankers or cracks can also be seen. However, these more generic symptoms only imply that the root system has lost a great part of its functionality. In order to confirm *Armillaria* root disease, the root collar and lower bole must be examined for specific signs to the fungus. Infected roots are frequently blackened and their diameter is increased. In the advanced stage of infection, white mycelial fans form under the bark. *Armillaria* spp. cause a white rot of woody tissues which can intrude more than 2 meters up the stem (MORIONDO AND TIBERI 2000). Subterranean rhizomorphs can often be observed at the surface of the roots, but the most aggressive species (*A. mellea* and *A. ostoyae*) are not the most rhizomorphogenic and often the *Armillaria* species that has initiated rhizomorphs around the roots is not the one responsible for the main infection (SHAW AND KILE 1991; GUILLAUMIN AND LEGRAND 2013).

Identification of the various *Armillaria* spp. present on a site can be of great practical importance because the virulence and host range markedly differ among species. The morphology of the fruiting bodies and rhizomorphs, when present, can give some information on the field. Carpophores grow in clusters from mycelial fans in the host or in small numbers from rhizomorphs. Fruiting occurs from mid-summer to mid-winter. The pileus is fleshy; its colour is highly variable from yellow to brown, tawny or olivaceous, according to the species and environment. It generally carries squamules darker than the ground colour, and these remain at the surface of the cap at maturity. The stipe is central, fibrous to fleshy, often becoming hollow in old samples. The lamellae are adnexed, sub-decurrent or decurrent (SHAW AND KILE 1991; GUILLAUMIN AND LEGRAND 2013).

1.4.3. Biology and epidemiology

All *Armillaria* spp. are able to directly invade dead woody substrates, as well as to continue to exploit wood as saprophytes after they have killed a host as parasites. The colonization of stumps and dead wood in the soil maintains an inoculum potential that threatens the roots of living trees. The fungal mycelium can be preserved in stumps over several decades. Basidiospores are unable to directly attack trees. There are two modes of infection: either the rhizomorph grows actively in the soil and adheres to roots, or the growing root reaches a source of inoculum (a stump or an infected root). Wounds do not seem to play an important role in infection. Defence mechanisms of the host can result in the formation of latent lesions where the mycelium remains alive and represents a threat for the next stand after felling (GUILLAUMIN AND LEGRAND 2013).

Disease caused by the same *Armillaria* species may be expressed differently on various hosts of the ecosystem. Some species considered pathogenic on broadleaves or conifers, respectively, may opportunistically infect both tree types while others can routinely infect plants of both groups. Stress may extend the host range of some species (SHAW AND KILE 1991). The development and severity of the disease largely depend on quantity, nature and distribution of primary inoculum, which consists of the contaminated stumps and woody remnants present in the soil (GUILLAUMIN AND LEGRAND 2013).

Occurrence of *Armillaria* root rot is generally the consequence of droughts as a starting stressor, so these fungi are just an element of complex declines in which other abiotic and biotic factors also play a major role (GUILLAUMIN AND LEGRAND 2013). Through destabilization of spruce stands, the pathogen can considerably increase their susceptibility to bark beetle invasions (JANKOVSKÝ ET AL. 2003). According to the scheme of MANION (1981), *Armillaria* spp. appear sometimes as a predisposing factor, through numerous early infections of the root systems, and sometimes as a contributing factor giving the deathblow to weakened trees.

The most pathogenic European species appear to be *A. ostoyae* and *A. mellea*. The former mainly damages artificial stands of *Pinaceae*, while the latter is a strong parasite of a wider host range, especially fruit trees (GUILLAUMIN AND LEGRAND 2013). *A. gallica* and *A. cepistipes* are weakly pathogenic species (ANTONÍN ET AL. 2009).

1.4.4. Control

Controlling a root disease is technically difficult and expensive, and the control strategy will be different according to the degree of damage caused or expected in the forest stand. In establishing new plantations, the choice of the site and the planting material are both important. *Armillaria* is generally absent from former agricultural lands, but can reach a high inoculum potential in clear-felled forest sites. Seedlings in containers, from locally adapted seed sources are recommended to use. Creating mixed stands, particularly broadleaves with conifers, reduces root contacts and the propagation of the fungus. Direct reduction of inoculum by the physical removal of stumps and roots using an excavator during site preparation has proved to be efficient (GUILLAUMIN AND LEGRAND 2013).

2. Material and Methods

2.1. Data acquisition

Figure 4: Field work in a destroyed fir stand. (Photo by Erico Kutchartt)

During the data collection the methodology of FARINA ET AL. (1990) was followed. Their work serves as a benchmark for present study to compare the distribution of *Heterobasidion annosum* s.l. in the Nature Reserve of Vallombrosa over 25 years. The systematic sampling was done through surveys carried out at points taken at regular intervals. For this purpose, the topographic map of the study area was covered by a 500×500 m grid identical to the one used by FARINA ET AL. (1990), which resulted in 53 points in total (Fig. 5, Annex 1). Point No. 45 lies in built-up area next to Vallombrosa Abbey, and only served as a reference for computing coordinates.

The 52 sampling points were visited between 7th and 30th July 2015 (Fig. 4). Points were identified in the field with a handheld GPS navigator (Garmin GPSMAP 62s, containing the forest management map of the Reserve) and a topographic map.

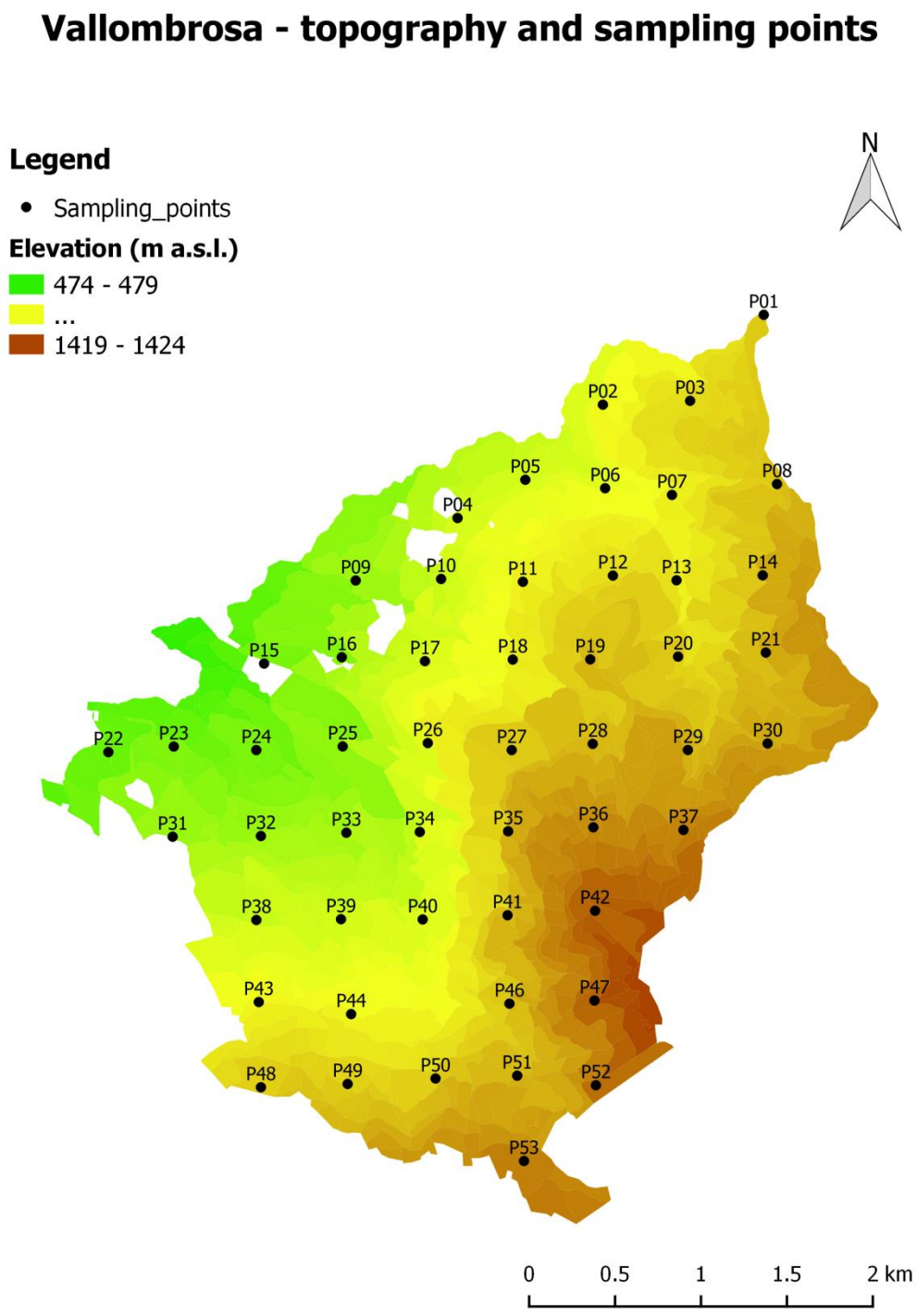


Figure 5: Topographic map of Vallombrosa forest with the location of sampling points. (Source: Database of the forest management plan 2006-2025 of Vallombrosa)

Comprehensive assessment of 4 individual trees' health condition was carried out at each point as described by CAPRETTI ET AL. (2009). The examined trees were located at about 13 metres from the centre of point towards the main cardinal directions (N, S, E, W). The majority of them represented the prevailing tree species of the stand, having dominant or intermediate social position. The central tree and the 4 trees subjected to the survey were signed by tape or paint. The measured/assessed dendro-morphological parameters of each tree were the following: DBH (with caliper or tape measure; 5 mm precision), height (with Suunto; 0.5 m precision), social position (on a scale of 1 to 3), crown insertion (1-3), crown compressed (0-4 sides), transparency (1-3) and decay level (1-9, Fig. 6) according to HUNTER (1990). On a scale of 1 to 4, the damages suffered by the trees were estimated separately on each of their aboveground organs: new foliage, old foliage, top shoot, twigs (<5 cm in diameter), branches (between 5-10 cm in diameter), branches (>10 cm in diameter), stem, collar roots. The type of damage was specified (discolouration, epicormic shoots, exudation/resin flow, desiccation/dry branches, partial/total loss of organ, necrosis of organ, canker, wounds/injuries, rot, signs of insects, signs of fungi) as well as the damage agent when it was definable. The species composition of the stand was noted as additional information.

It was impossible to approach points No. 27 and 43 because of the windthrow, and point No. 15 because it lies in a chasm (only shrubs are present). In the vicinity of these points, soil sampling was done and main tree species recorded without any further data.

The presence of deadwood or partially degraded woody material in a site provides useful information for us, especially from the pathological point of view. Therefore in each sampling point the first 5 (in some cases only 4) stumps or fallen trees were taken into consideration that were met by performing a spiral movement starting from the centre and proceeding outwards. The registered data were: species, DBH for fallen trees, diameter at base for stumps, decay level. At points No. 33, 37 and 42 neither stumps nor fallen trees were present.

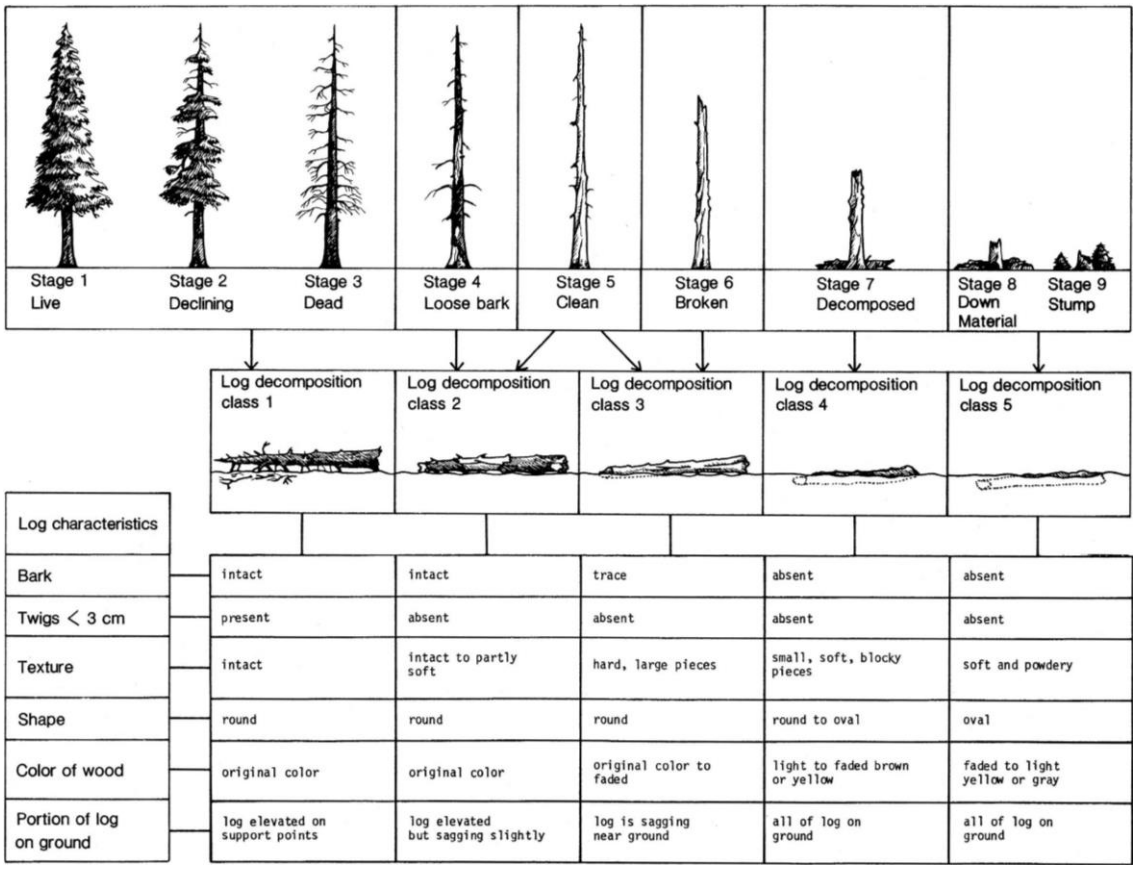


Figure 6: Decay classes following HUNTER (1990).

A major goal of this research is to create a map which shows the occurrence of parasitical root destroying fungi in Vallombrosa. In order to achieve this, various types of samples were collected (Table 1). Soil sampling was carried out close to the central tree, through hammering a standard metal cylinder of approximately 200 cm^3 in volume to the topmost layer of soil, excluding the organic horizon. Decayed stumps, windthrown and symptomatic trees were checked for the presence of *Heterobasidion* and *Armillaria* at each site visited. Pieces of *Heterobasidion* fruiting bodies were cut by knife. In case of *Armillaria*, rhizomorphs were gathered because carpophores usually only appear in the autumn. Samples were put into plastic bags or microtubes and placed in a fridge, at 4°C on the day of collection, for further laboratory analysis.

Table 1: Number of samples collected from Vallombrosa.

Soil	*Heterobasidion*	*Armillaria*	∑
52	18	19	89

2.2. DNA isolation from samples

Conventional identification methods of wood decay fungi mostly rely on visual analysis of carpophores. Dichotomous keys to species are based on macromorphology of the carpophore and hymenophores, and on micromorphology of hyphal system, hymenial organs, and spores. This diagnostic method often requires a deep mycological background and rarely allows for early identification of wood rotting fungi, since fruiting bodies usually emerge at advanced stages of the infection. Analysis of pure fungal cultures isolated from mycelium and/or decayed wood may be used when no fruiting bodies are available, based on growth rate, microscopic features, and enzymatic capabilities of mycelia. Sexual compatibility tests, by means of pairing the unknown isolate with known haploid testers, have been extensively used for the discrimination of species within *Armillaria* and *Heterobasidion annosum* species complexes (ANDERSON 1986; KORHONEN 1978b). Although these diagnostic tools may be efficient, they are time-consuming and impractical due to the presence of fast growing fungal contaminants (NICOLOTTI ET AL. 2010).

The great potential of DNA-based techniques relies on the chance to select diagnostic markers in coding as well as noncoding DNA regions of the nuclear and mitochondrial genome. Techniques based on polymerase chain reaction (PCR) are valuable tools for specific, sensitive, and rapid routine diagnoses of wood decay fungi. The identification becomes possible directly from environmental samples, without the need of any pure fungal culture isolation step (NICOLOTTI ET AL. 2010).

The principle of PCR is quite simple. The DNA solution is heated until it denatures and becomes single strained (ssDNA). A pair of primers (short oligonucleotides complementary to either side of the sequence in interest) is allowed to anneal to ssDNA under primer-specific temperature. A thermostable DNA polymerase uses single nucleotides to build a complementary strand. The procedure is repeated and the newly synthesized strand serves, together with the original DNA, as a template for the following cycle. This procedure is repeated 25-40 times, thereby sufficiently increasing

the concentration of DNA to be visualized in agarose gel stained with DNA-intercalating reagents such as ethidium bromide (MÁJEK 2006).

All types of samples (*Heterobasidion* carpophores, *Armillaria* rhizomorphs and soil) were chosen for DNA isolation. Soil samples were mixed; fungal samples were homogenized by grinding the tissue in liquid nitrogen using sterile mortar and pestle. Approximately 0.25 g of samples was used. DNA was isolated using the PowerSoil™ DNA Isolation Kit (Mo-Bio, Carlsbad, USA), according to the manufacturer's instructions. The extracted DNA solution was conserved at -25°C.

2.3. Identification of *Heterobasidion annosum* s.l.

The identification of species within *H. annosum* s.l. was traditionally based on mating tests, i.e. sexual compatibility tests, through which an unknown isolate is paired on agar medium with homokaryotic tester strains of the different species. The partial in vitro interfertility among species may occasionally lead to ambiguous diagnosis (GONTHIER AND THOR 2013). A number of molecular diagnostics assays based on both nuclear and mitochondrial DNA have been developed for the identification of species within *H. annosum* s.l. (WOODWARD ET AL. 1998). The complete genome sequence of the fungus is now available, making it the first sequenced plant pathogenic homobasidiomycete (OLSON ET AL. 2012).

For rapid identification of *Heterobasidion annosum* s.l., taxon-specific competitive-priming (TSCP-PCR) method was used according to GONTHIER ET AL. (2003). For amplifying DNA, a mix of four primers (MLS, MLF, Mito 5 and Mito 7) was applied. PCR was performed in a 25-µl reaction mixture containing approximately 50 ng of template DNA, 0.5 µmol of each primer, 5x 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 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.

2.4. Identification of *Armillaria* spp.

Conventionally, the distinguishing of *Armillaria* spp. was achieved by sexual compatibility tests. This method requires some experience and also the maintenance of the tester species in culture (GUILLAUMIN AND LEGRAND 2013). The new approaches are mostly based on the analysis of DNA sequences. To determine *Armillaria* species, the most commonly used DNA regions for amplification are Internal Transcribed Spacer (ITS) and Intergenetic Spacer (IGS). Both are highly polymorphic and provide a useful tool for taxonomic and phylogenetic studies (CHILLALI ET AL. 1998). LOCHMAN ET AL. (2004a) have developed new specific AR1 and AR2 primers based on conserved sequence of ITS1 and ITS2 region of European *Armillaria* (Fig. 7). These primers gave selective PCR products and enabled identification on the species level by a subsequent RFLP and sequence analysis of the amplicons. This technique enables the identification of *Armillaria* spp. within one day directly from soil samples without the need for previous isolation and cultivation of mycelium (LOCHMAN ET AL. 2004a). The method was used efficiently by TOMŠOVSKÝ ET AL. (2006); ANTONÍN ET AL. (2009).

Figure 7: Location of PCR primers AR1 and AR2 on nuclear rDNA.
The arrowheads represent the 3' end of each primer (LOCHMAN ET AL. 2004a).

We have to take into account the differences in the infection biology of *Armillaria* spp. Some species like *A. cepistipes* propagate directly in soil, whereas others such as *A. ostoyae* are restricted to spreading through roots (PROSPERO ET AL. 2006). Therefore, the presence of DNA of distinct *Armillaria* spp. in soil does not necessarily correlate with their prevalence in the ecosystem. Another limitation of DNA isolation technique is its lack of ability to make a distinction between monocaryotic and dicaryotic mycelium. As a result, what we treat as a positive sample might be only spores. This problem can be resolved by choosing the right time of year for sampling, preferably

summer months when the spore load is minimal in the environment, due to *Armillaria* life cycle (SHAW AND KILE 1991).

Internal transcribed region (ITS) was selectively amplified from soil and rhizomorph samples by nested PCR. The first reaction was carried out with external primers ITS1 and ITS4 used for amplification of ITS region of fungi (WHITE ET AL. 1990). In the second reaction, the internal primers AR1 and AR2 for *Armillaria* ITS region were used (LOCHMAN ET AL. 2004a). In both reactions, 1 µl of isolated DNA was used in 25 µl PCR mixture containing 0.5 µmol of each primer, 5x MyTaq Reaction Buffer and 1U MyTaq DNA Polymerase (Bioline, London, UK). Amplifications were carried out in a Mastercycler® ep Thermocycler (Eppendorf, Hamburg, Germany) with thermal cycling parameters: initial denaturation at 94°C for 2.5 min, followed by 35 cycles of heat denaturation at 94°C for 30 s, annealing at 55°C for 40 s, extension at 72°C for 30 s and final extension at 72°C for 5 min for ITS-PCR; initial denaturation at 94°C for 2.5 min, followed by 35 cycles of heat denaturation at 94°C for 30 s, annealing at 60°C for 40 s, extension at 72°C for 30 s and final extension at 72°C for 7 min for AR-PCR. In some cases, due to scarce visibility on agarose gel, it was necessary to repeat the PCR with higher volume (2 µl) of extracted DNA. Negative samples were discarded after each reaction, positive results were further processed.

During Restriction Fragment Length Polymorphism (RFLP) analysis, DNA is cut into shorter strands by restriction enzymes that can be visualized after gel electrophoresis. Digestion of unpurified PCR products was carried out using restriction endonuclease *Hin*fI (Fermentas, Lithuania). As shown by LOCHMAN ET AL. (2004b), this enzyme is able to discriminate the six main European *Armillaria* species (Table 2, Fig. 8). The restriction mixtures containing 19 µl of PCR product with 1 µl of buffer R and 1 µl of the enzyme *Hin*fI were incubated for 12 h at 37°C (LOCHMAN ET AL. 2004a).

Table 2: Length of restriction fragments typical for Armillaria *spp. (LOCHMAN ET AL. 2004a)*

Isolate - species	Length of amplicons (bp) ITS/AR	Restriction fragments *Hin*fI (bp)
A. borealis	868/711	293, 172, 56, 31, 75, 68
A. cepistipes	868/711	293, 227, 43, 132
A. gallica	868/711	294, 227, 43, 63, 69
A. mellea	882/724	148, 159, 401
A. ostoyae	870/713	294, 228, 31, 75, 69
A. socialis	847/690	295, 125, 93, 32, 129

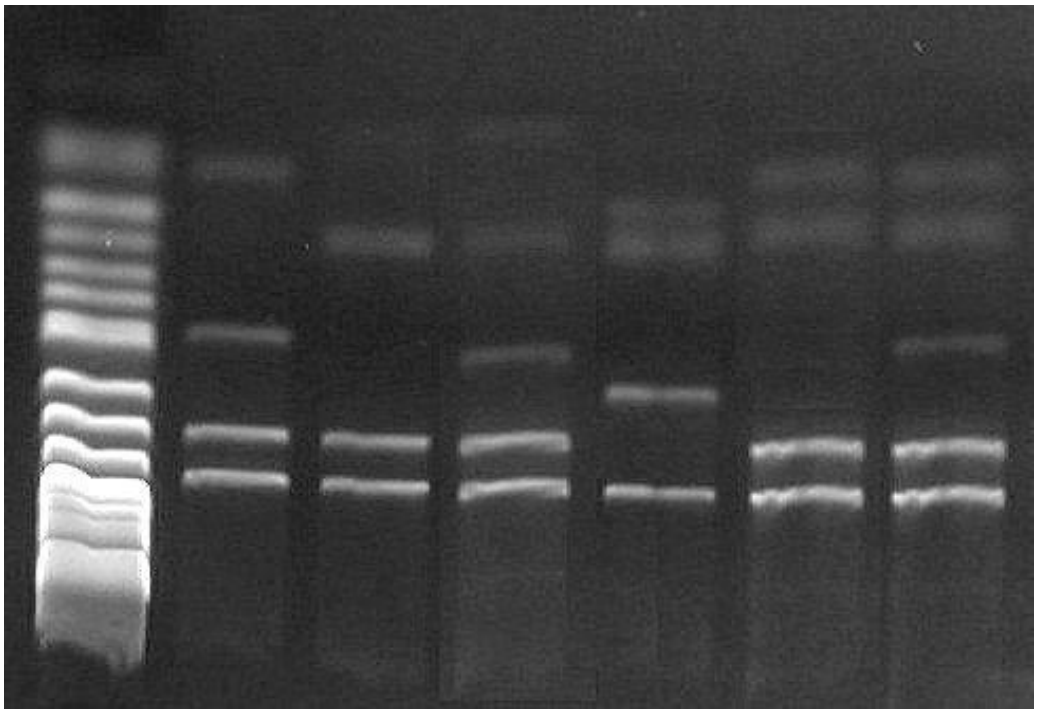


Figure 8: RFLP-PCR products visualized on agarose gel after electrophoresis. From left to right: DNA ladder (ΦX174, Fermentas, Lithuania), A. cepistipes*,* A. ostoyae*,* A. ostoyae *(second type),* A. borealis*,* A. gallica*,* A. cepistipes x gallica*. (Source: ANTONÍN ET AL. 2007)*

2.5. Visualization of PCR products

After each reaction, PCR products were electrophoresed in agarose (Serva, Heidelberg, Germany) gel in TBE buffer at 5 V/cm for approximately 35 min (ITS, AR) or 80 min (TSCP, RFLP). 1% gel was used for simple identification (ITS, AR) and 2% for species gel identification (TSCP, RFLP). 1 µl of ethidium bromide or Serva DNA Stain

G was added to the gel. 6 µl of each sample and control, as well as 3 µl of 100 bp DNA Ladder (BioLabs, New England), to determine the fragment size, were mixed with loading dye (BioLabs, New England) and loaded into the wells. In case of RFLP-PCR products, DNA fragment sizes were estimated using ΦX174 DNA/*Hin*fI Marker (Fermentas, Lithuania) as DNA molecular size marker. Amplified DNA of *H. annosum* s.s. (for TSCP) and *A. borealis* (for ITS, AR and RFLP) served as positive controls. To visualize DNA fragments, the gel was placed on an ultraviolet transilluminator and documented by digital camera (Olympus C8080WZ, Japan, Fig. 9-12, Annex 2).

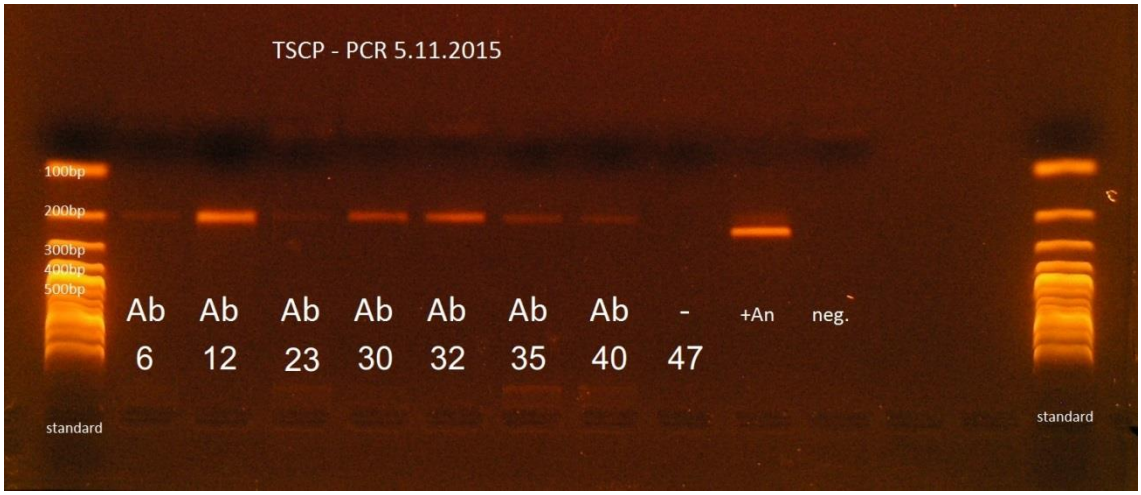


Figure 9: Result of gel electrophoresis under UV light.[1]

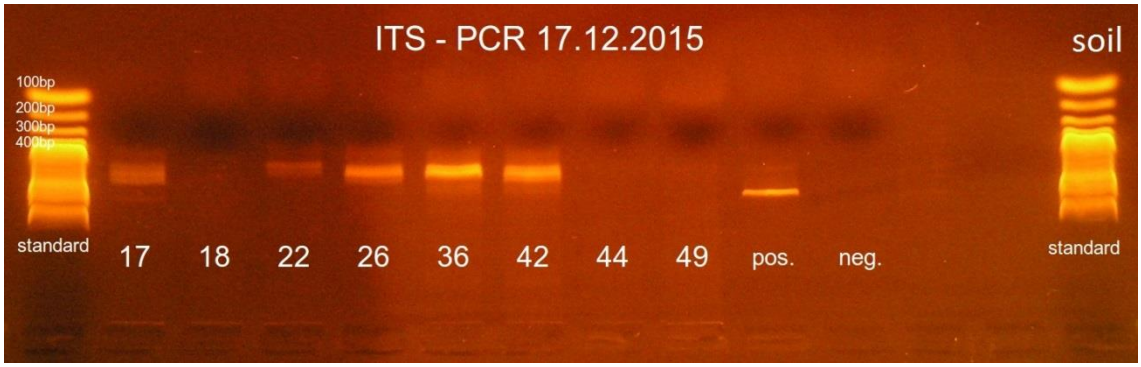


Figure 10: Result of gel electrophoresis under UV light.

[1] Legend to all photos of gel electrophoresis: numbers refer to No. of point in the field where the sample is originated from (see Fig. 5); standard – DNA molecular size marker; pos., +An, +B – positive controls; neg. – negative control; Ab – *Heterobasidion abietinum*; Ar – *Armillaria* spp.; C – *A. cepistipes*; G – *A. gallica*; M – *A. mellea*; O – *A. ostoyae*; - – negative result; ? – we were not able to identify the species, so repeating the PCR was necessary.

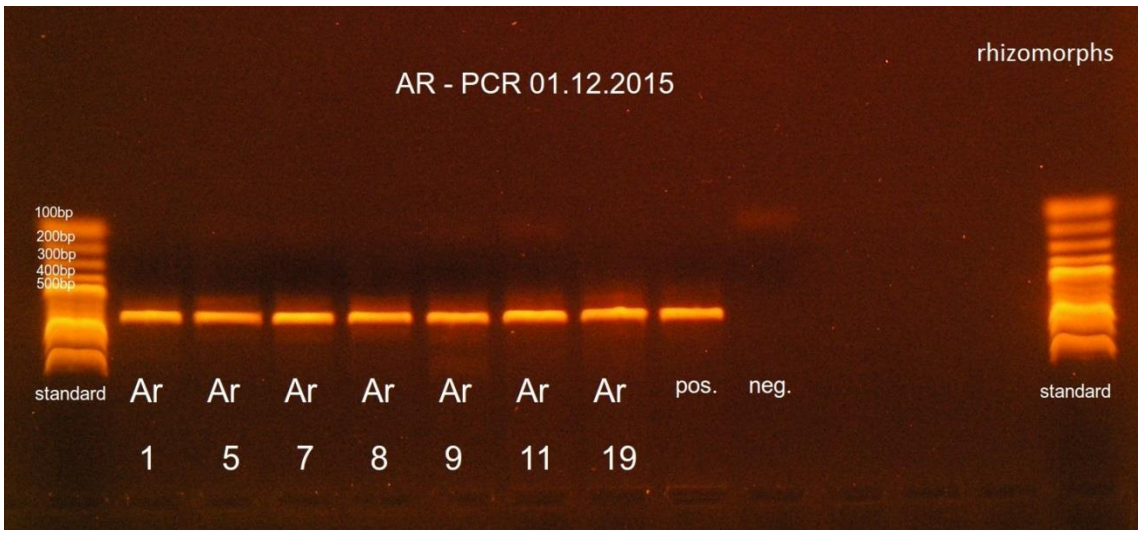


Figure 11: Result of gel electrophoresis under UV light.

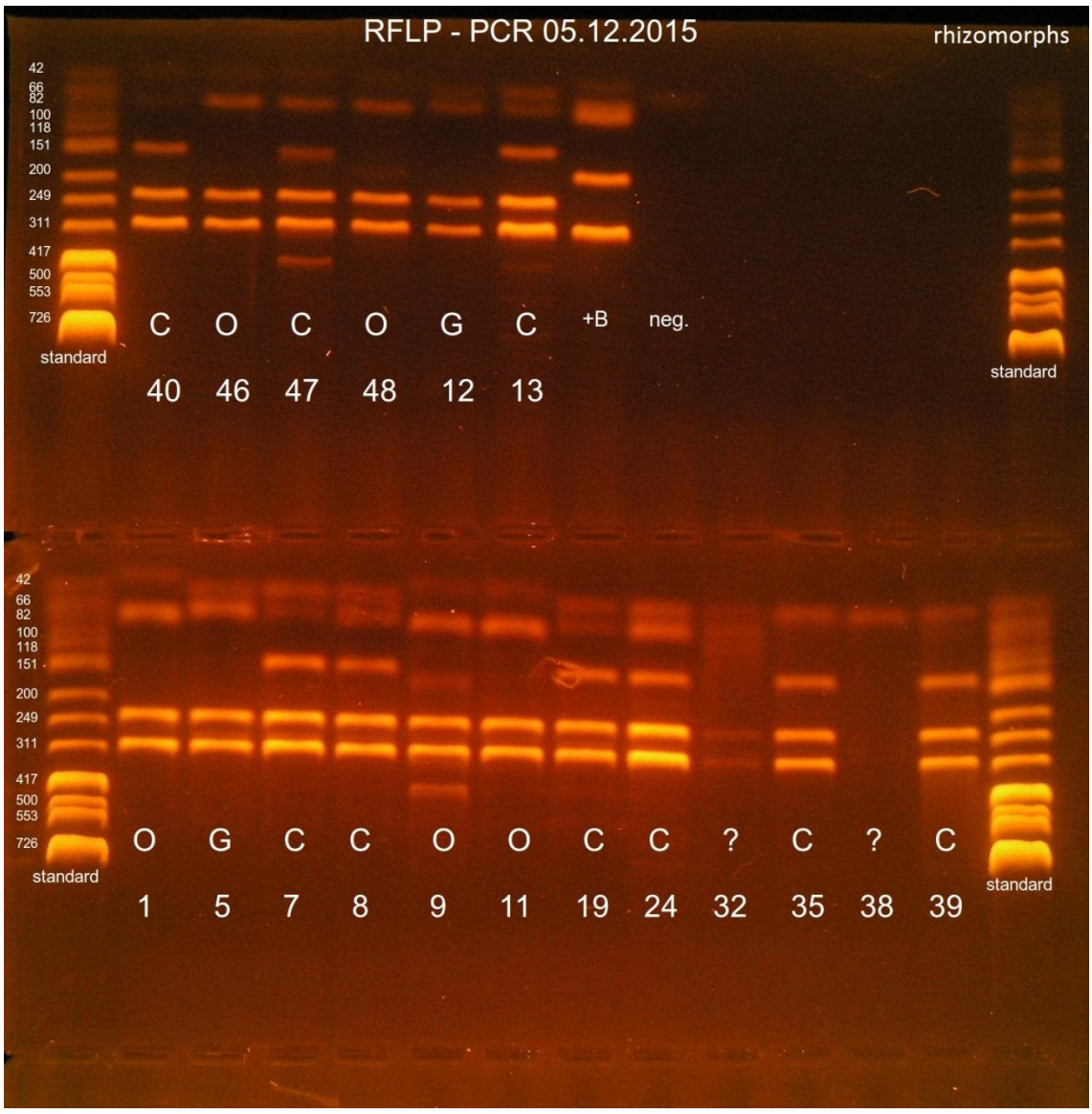


Figure 12: Result of gel electrophoresis under UV light.

2.6. Data processing

The mapping of sampling sites was conducted using the QGIS 2.12.3 ‘Lyon’ software. Spatial data transferred from GPS was linked with the collected attributive data in the program. After georeferencing each sampling location, thematic map layers were created. Data was processed by means of the statistical functions and graphical capabilities of Microsoft Excel 2010 to produce results. The influence of altitude, site fertility, and age of forest stand on the distribution of *Armillaria* spp. was tested using a one-way analysis of variance (ANOVA). The null hypothesis of no relation between the above variables and distribution of the species was only rejected in the case of stand age.

3. Results

3.1. Distribution of *Heterobasidion annosum* s.l.

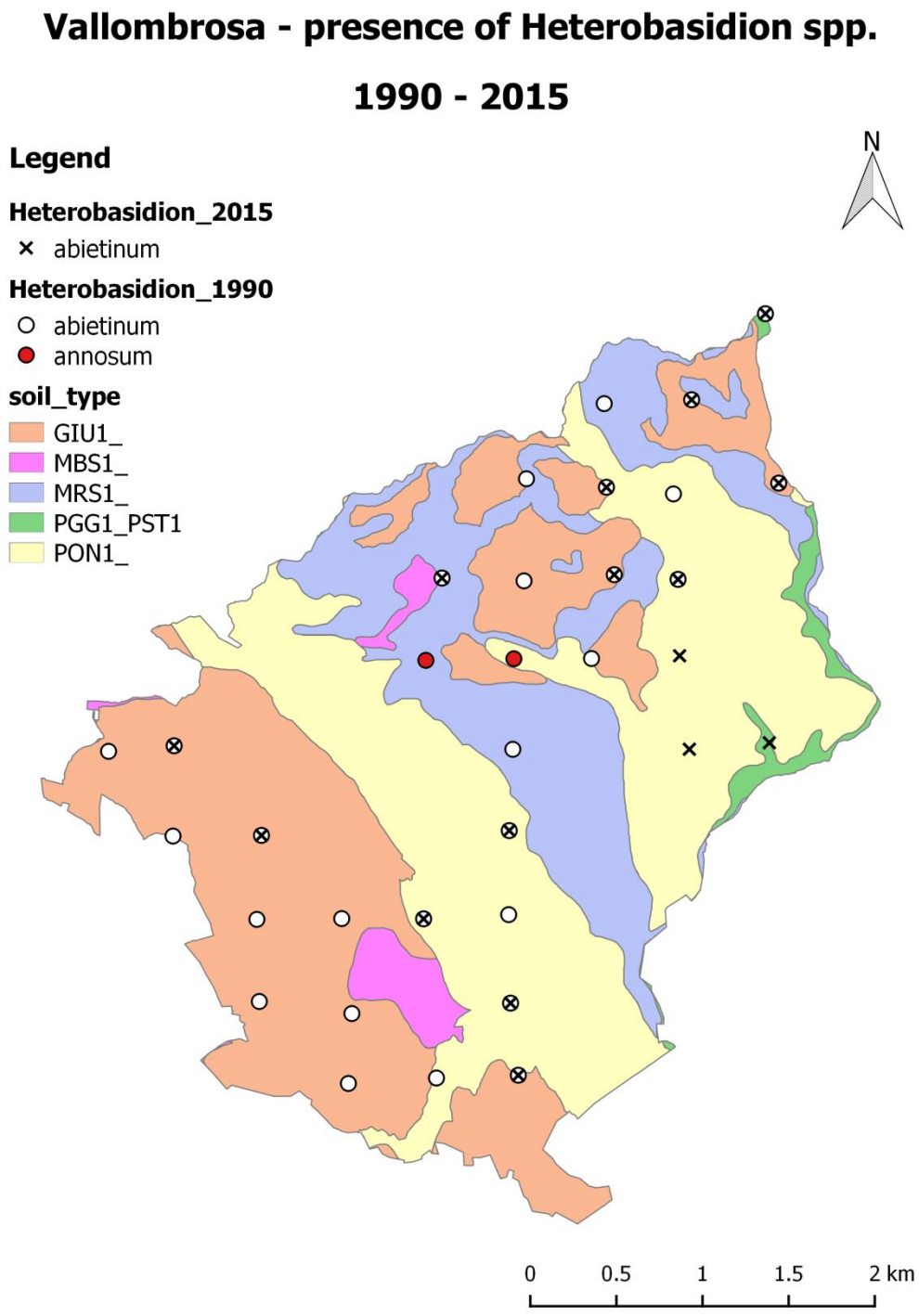


Figure 13: Spatial distribution map of Heterobasidion *samples identified by* FARINA ET AL. *(1990) and by present study (2015). (Source: http://159.213.57.101/pmapper/map.phtml)*

Out of 18 analysed *Heterobasidion* fruiting bodies, 16 samples turned out positive, and all of them belong to *H. abietinum*. Identification of *H. abietinum* was confirmed by sequencing of ITS region of some obtained amplicons by the DNA Sequence Service of Macrogen Inc. (Seoul, Korea). The geographical distribution of the samples used in this study, plus the ones distinguished by FARINA ET AL. (1990) is shown in Fig. 13.

Fig. 14 shows that 75% of *Heterobasidion abietinum* carpophores were found in fir stands. Relative to elevation and soil type, no clear tendency can be interpreted, but the shallow Lithic Dystrudepts seem to ensure favourable conditions for the spread of the fungus.

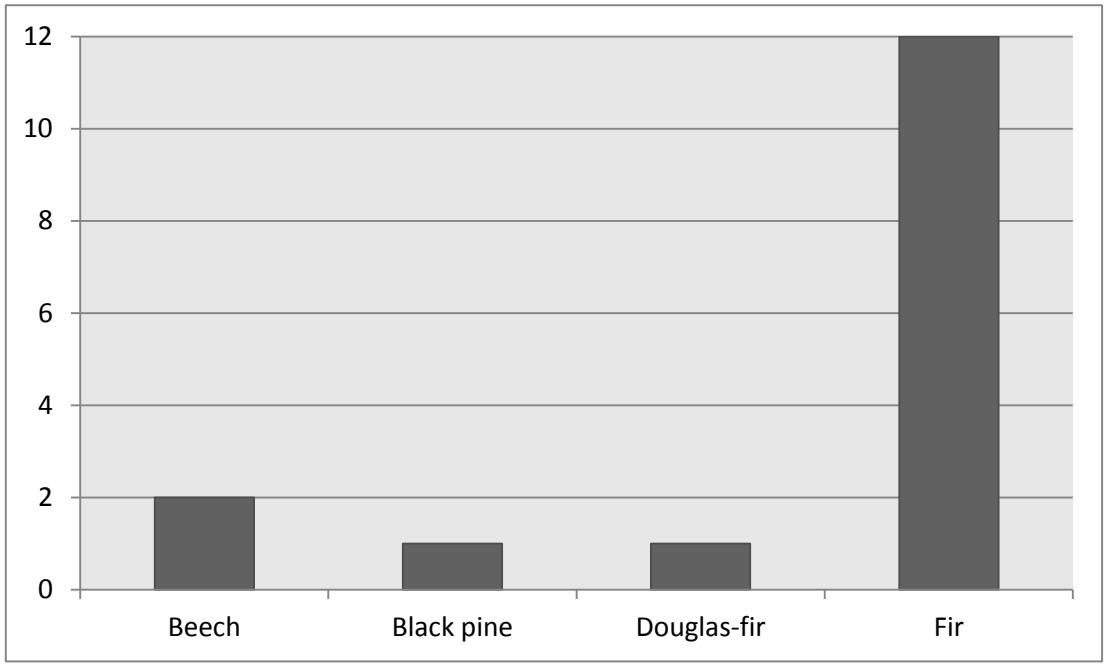


Figure 14: Distribution of Heterobasidion abietinum *in different forest types.*

3.2. Distribution of *Armillaria* spp.

Table 3: Identified samples of Armillaria *spp.*

Species	Rhizomorphs	Soil samples	∑
A. cepistipes	9	13	22
A. gallica	3	14	17
A. mellea	0	2	2
A. ostoyae	5	17	22
∑	17	46	63

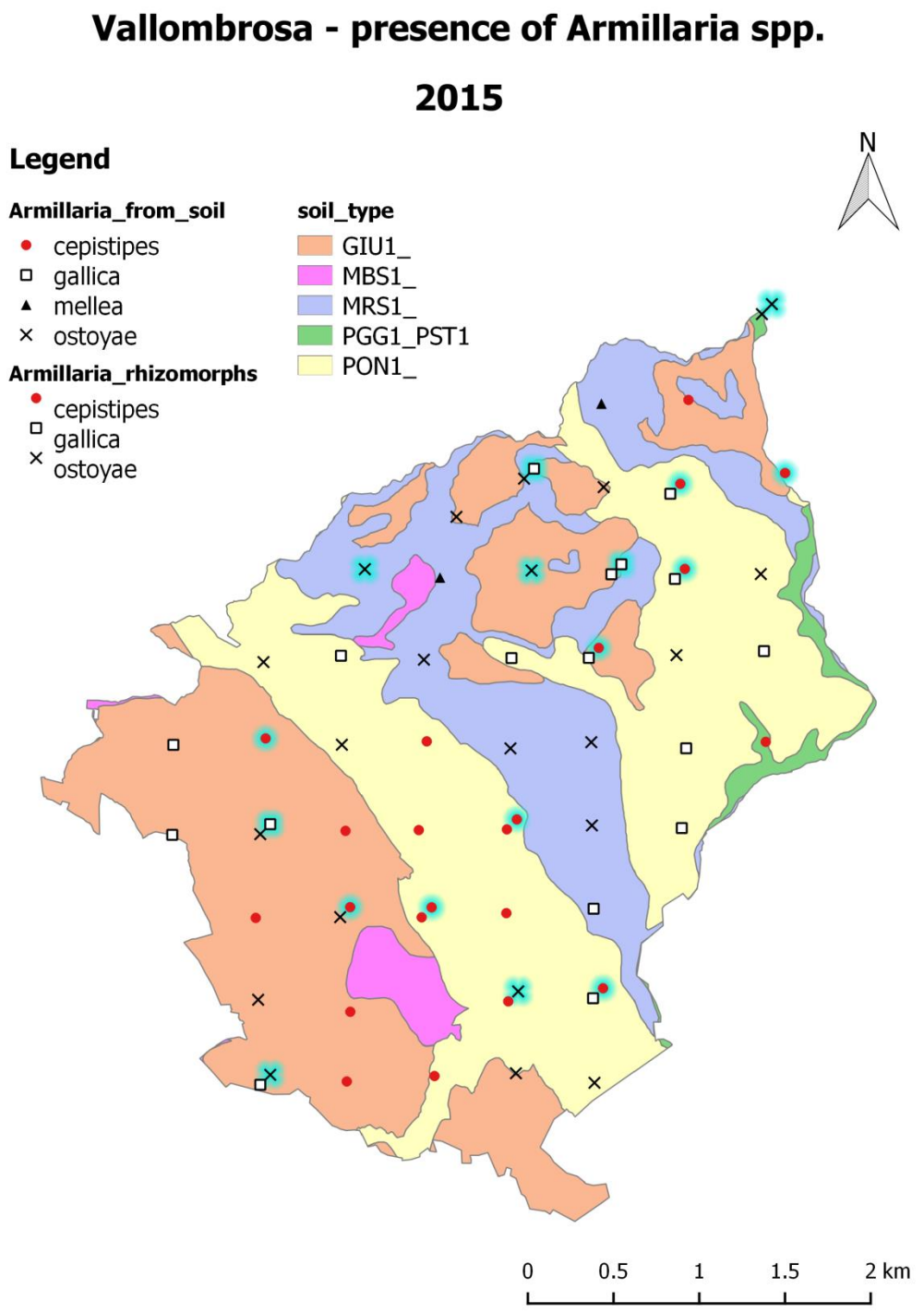
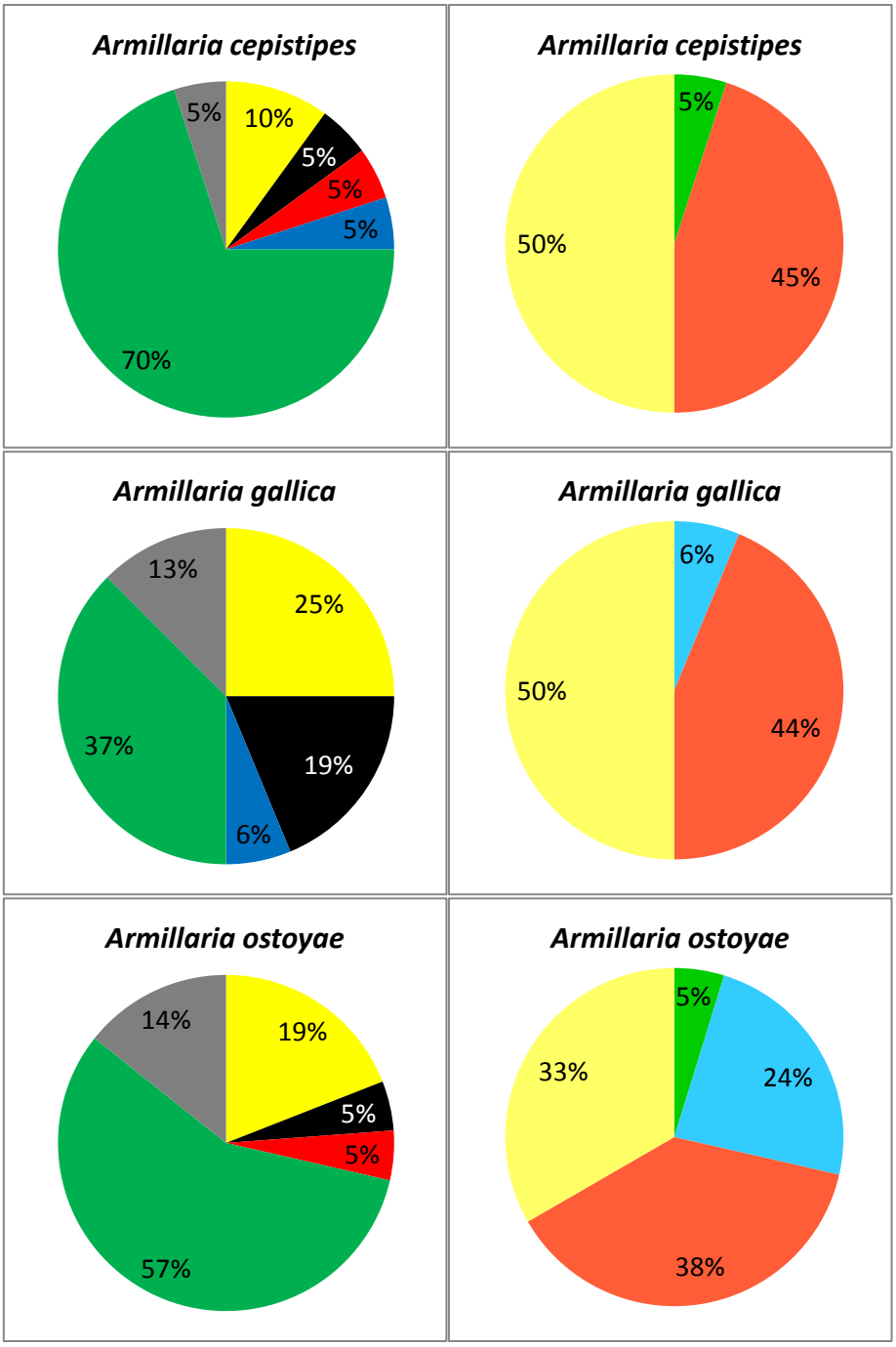


Figure 15: Spatial distribution map of Armillaria *samples included in the study. Points with an outer glow indicate rhizomorph samples. (Source: http://159.213.57.101/pmapper/map.phtml)*

89.5% of rhizomorphs and 88.5% of soil samples have been successfully identified. Table 3 shows the number of different *Armillaria* species distinguished by restriction. *A. ostoyae* and *A. cepistipes* have the most occurrences. *A. gallica* is also common, while *A. mellea* was present in only two samples. *A. cepistipes* was relatively twice more frequent among rhizomorph samples than in soil. The spatial distribution of both types of samples is shown in Fig. 15.



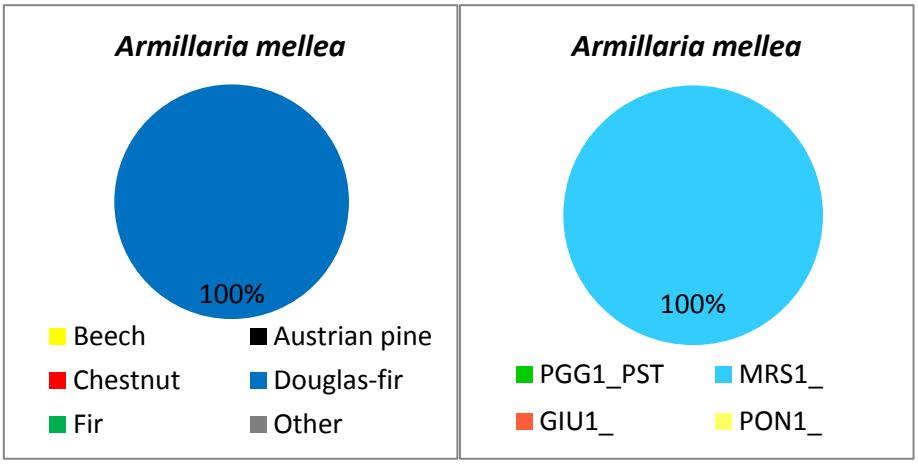


Figure 16: Distribution of Armillaria *spp. in different forest types (left column) and soil units (right column).*

Fig. 16 demonstrates how the individual *Armillaria* species are distributed in the main forest types and soil units of Vallombrosa. These diagrams represent all the identified samples (rhizomorphs and soil) together, because analysing them separately gave almost the same results. *A. mellea* was found only in Douglas-fir stands, in Humic Dystrudepts. The other species were recorded in almost every type of forest and soil. There is no significant difference in their distribution, except that *A. cepistipes* shows a high preference for fir stands (70%), while *A. ostoyae* and especially *A. gallica* more abundantly occur in forests dominated by other tree species, particularly beech.

Armillaria spp. are present at a wide range of elevations and various forest sites. There has been no statistically significant difference found neither in the altitudinal distribution of *Armillaria* species (one-way ANOVA; F=0.308, p=0.820), nor in their presence in site fertility classes (one-way ANOVA; F=1.976, p=0.130). The single parameter which has a statistically significant effect on the distribution of the pathogen is the age of the stand (one-way ANOVA; F=3.402, p=0.024). *A. mellea* is only present in pole stands. The majority of *A. cepistipes* are found in the 61-80 years age class, while the occurrence of *A. gallica* and *A. ostoyae* are shifted towards the oldest stands (Fig. 17).

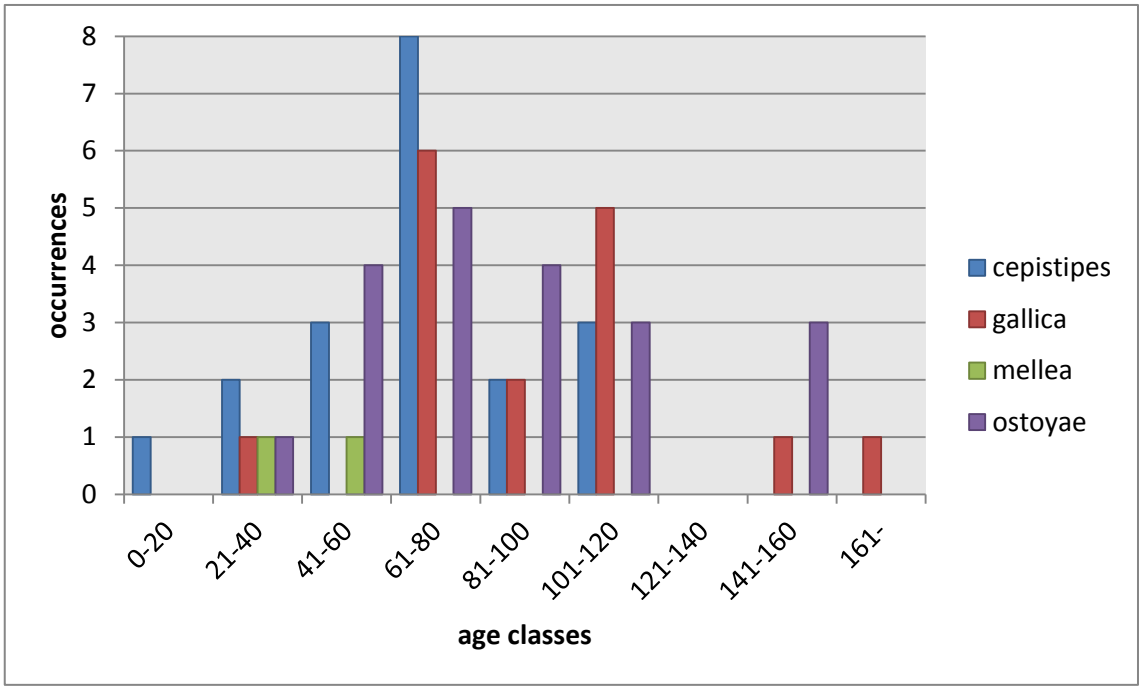


Figure 17: Distribution of Armillaria *spp. according to age classes.*

3.3. Forest health in Vallombrosa

We have data about the health condition of 196 trees; almost half of them are *Abies alba* (48.5%). *Fagus sylvatica* (20.4%), *Pinus nigra* (12.2%) and *Pseudotsuga menziesii* (5.1%) are other important species. Among the 218 stumps/fallen trees *Abies alba* (57.8%) was predominant as well, followed by *Pinus nigra* (16.1%), *Castanea sativa* (10.1%) and *Fagus sylvatica* (7.8%). *Abies alba* dominated nearly half of the stands, ahead of *Fagus sylvatica* (19.2%) and *Pinus nigra* (11.5%) forests.

Among the species for which we have sufficient data, *Pinus nigra* stumps and fallen trees were the most degraded with an average value of 6.4. *Castanea sativa* and *Abies alba* had a moderate decay level (5.0 and 4.9 respectively), while *Fagus sylvatica* (4.0) and *Pseudotsuga menziesii* (3.7) were in a more initial stage of decomposition.

Summarizing the data of damages, stem was far the most frequently damaged organ: 46.9% of the trees showed some degree of stem damage. The second organ in this respect was new foliage, damaged on only 13.3% of trees. While being quite uncommon (present on 5.6% of all trees), the damages of top shoot were twice as

severe as those of the stem. Specifying the types of damages, 32 different forms can be distinguished. Among these, there is also one prominently common, namely the epicormic shoots, detected on 24.0% of the trees. Wounds were present on 14.3% of individual stems, followed by canker on the stem (8.2%) and desiccation of the twigs (7.1%). Rot of either the stem or the collar roots was found on 6.6% of all trees, being most frequent and severe on *Pinus nigra*. Interesting fact, that discolouration of the foliage was detected only on *Abies alba*, affecting 23.2% of the trees in some extent. In two cases, the loss of the stem could be observed as the most serious damage. 40.8% of trees proved to be totally healthy. As a rough estimation of overall damage, the severity values of different kinds of damages suffered by each individual tree were summarized. In this comparison, the average value is 2.48. The most damaged tree species are *Pinus sylvestris* (10.0), *Castanea sativa* (8.3) and *Fraxinus excelsior* (6.3), while the least affected ones are *Acer platanoides*, *Acer pseudoplatanus* without damage, *Pseudotsuga menziesii* (0.6) and *Picea abies* (1.0).

Some of the damage agents were recognized in the field. On a few *Abies alba* trees, *Heterobasidion*, *Armillaria*, *Lirula nervisequia*, bacterial and mechanical damage were recorded. *Apiognomonia errabunda*, *Mikiola fagi*, defoliators and frost damage were detected on *Fagus sylvatica.* Half of the examined *Castanea sativa* trees suffered from *Cryphonectria parasitica*. Some *Pinus nigra* individuals were affected by *Armillaria*, bark beetles and ants. *Armillaria* infection developed in form of mycelial fans under the bark of dead trees. This symptom was found on *Pinus sylvestris*, too. Galls were observed on the twigs of a *Quercus cerris* tree. Additionally, the carpophores of another decay fungus, *Fomitopsis pinicola* were found at 10 points, mainly on *Abies alba* stems.

Correlations were not found between the trees' dendro-morphological parameters, suffered damages and characteristics of the stands.

4. Discussion

FARINA ET AL. (1990) recorded the presence of *Heterobasidion annosum* s.l. in 57.7% of all investigated points in Vallombrosa forest. The results of present study confirmed the incidence of the pathogen in substantially less areas (30.8% of total). It must be pointed out, that this value is very likely an underestimation, because the survey was based solely on the apparent fruiting bodies. The particularly hot and dry summer of 2015 could restrict the formation of carpophores to protected niches (WOODWARD ET AL. 1998). Indeed, most of the samples were collected from roots of windthrown trees. In contrast, the research of FARINA ET AL. (1990) involved isolating the fungus from wood samples gathered at each point. In their study, carpophores only accounted for 37% of all detections of *H. annosum* s.l. Taking this into consideration, we cannot conclude that the occurrence of the fungus has decreased since 1990.

As opposed to the work of FARINA ET AL. (1990), *H. annosum* was not found in the area in 2015; all analysed samples were identified as *H. abietinum*. As visible in Fig. 13, the spatial distribution of *H. abietinum* correlates very well with that outlined by FARINA ET AL. (1990). However, the fungus was recorded at 3 new locations near Stefanieri and Croce Vecchia. These detections clearly indicate the expansion of the parasite up to the beech zone, which may be associated with the climate change. The marked long-term increase of the mean annual air temperature at Vallombrosa (Fig. 18) may enable the pathogen to invade new habitats. No relationship has been found between soil type and *Heterobasidion* occurrence, which corresponds to SEDLÁK (2015).

Regarding the host range of *H. abietinum*, direct conclusions cannot be drawn since the substrate of the collected fruiting bodies was not registered during the survey. We can only rely on the species composition of the stands. However, there are some interesting findings. Although the majority of *H. abietinum* occurrences fell to fir stands, carpophores of the fungus occasionally appeared in forests dominated by Douglas-fir,

Austrian pine and beech. In most cases, there was admixture of fir in these stands, except point No. 23 (Austrian pine with chestnut) and 29 (pure beech stand). The latter finding contradicts the previous results of GONTHIER AND THOR (2013), who consider broadleaved species immune to *H. abietinum* infection, either FARINA ET AL. (1990) have not found this parasite on beech. This can be explained by a really high inoculum potential on the site.

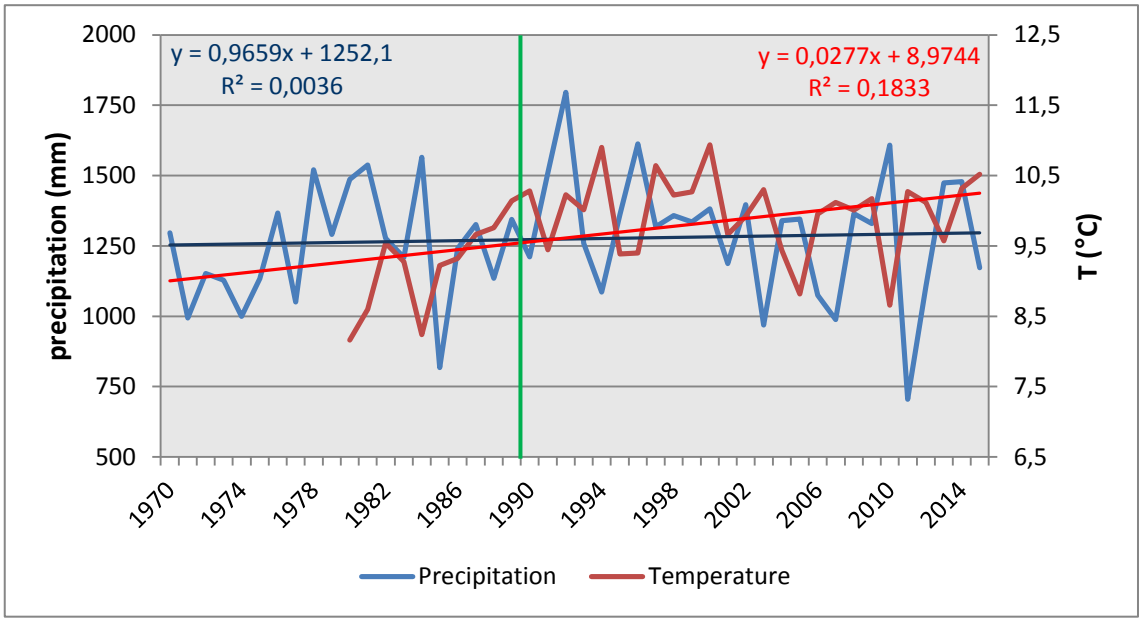


Figure 18: Annual precipitation (1970-2015) and mean air temperature (1980-2015) at Vallombrosa.
(Source: Thermopluviometric Station of Vallombrosa, 980 m a.s.l.)

Armillaria spp. are present in the entire area of the Nature Reserve of Vallombrosa. In many cases, the DNA of the fungus was amplified from both the rhizomorph and the soil sample collected at the same location, and these isolates often belong to different species. This type of coexistence within the same forest stand is well documented in the literature (PROSPERO ET AL. 2006; MÁJEK 2006; ANTONÍN ET AL. 2009), and has been explained by the different ecological strategy of the species, i.e. their specialization to saprophytic or parasitic behaviour.

In many respects, *Armillaria cepistipes* seems to be the most influential honey fungus in the Vallombrosa forest. The fact that more than half of the identified rhizomorph samples belong to this species is consistent with many authors who describe it as

highly rhizomorphogenic (SHAW AND KILE 1991). Its thick, strong rhizomorphs formed a large net under the bark of Austrian pines at point No. 7, and a Scots pine at point No. 19. In both locations, the root disease killed the infected trees. Most probably, *A. cepistipes* finds its ecological optimum in the area and consequently shows pathogenicity in weakened hosts. Given its dominance in fir stands, this species is considered potentially hazardous in Vallombrosa. It deserves especially high attention due to its prevalence in middle-aged stands where tree mortality is a bigger problem from both the ecological and economical point of view.

Armillaria ostoyae is equally common as *A. cepistipes*, but its DNA was more often isolated from soil. Despite this species is usually associated with conifers, 38% of its occurrences are broadleaved stands. More research is needed to assess its pathogenicity in Vallombrosa.

The third important species is *A. gallica*. This facultative parasite did not show any preference to forest type, is evenly distributed in the area. In the points where both species are present, *A. cepistipes* was found as rhizomorphs while *A. gallica* was determined from soil. This points to the fact that *A. cepistipes* acts as a parasite whereas *A. gallica* plays a major ecological role as a decomposer of woody debris in soil, which is in line with the abovementioned theory of PROSPERO ET AL. (2006).

A. mellea has only two records, both from Douglas-fir stands. European plantations of this species are considered resistant to *Armillaria* root rot (GUILLAUMIN AND LEGRAND 2013). It is reasonable to assume that *A. mellea* is a residual from the previous rotation of broadleaves in these quite young stands rather than a colonizer of conifers (PERSONAL COMMUNICATION: JANKOVSKÝ 2016). Further investigations on forest history are required to verify this theory. The climate of Vallombrosa is probably not warm enough to favour the spread of *A. mellea*, however this can change in the future.

The Ministerial Conference on the Protection of Forests in Europe (MCPFE) uses defoliation as an indicator for forest health and vitality. Transparency of the crown was

19.4% on average throughout the Reserve. Severe loss of foliage was observed only on conifers. 5.3% of silver fir individuals and 11.1% of pine trees were affected by moderate to total defoliation. Other general markers such as the decay level of trees and stumps or discolouration of the foliage also highlight the vulnerability of these species. Epicormic shoots were also ubiquitous, though these may also signal the unfavourable structure of the forest stand. Broadleaved species have much better health condition and appear to be less susceptible to diseases than conifers. The reason should be that the potential vegetation of the area, consisting of mixed beech and hornbeam-oak forests, is naturally adapted to the site. On the other hand, chestnut stands are also highly damaged, being infected by *Cryphonectria parasitica* and showing severe symptoms of desiccation.

Heterobasidion abietinum and *Armillaria* spp. constitute an integral part of the biotopes in Vallombrosa. As decomposers, they contribute to nutrient recycling, while the root rot caused by these fungi plays a significant role in the succession of forest habitats, altering their structure and species composition. Nevertheless, since these forests are utilized by human for a long time, their ecological balance is disturbed, and one-time natural and harmless elements of the ecosystem have become a destructive factor. Environmental effects may further exacerbate the problems. In the last decades, dozens of Italian scientists made efforts to explore the reasons of silver fir decline. Besides and in connection with root rot disease, two factors are conceived to be crucial.

Soil properties decisively influence silver fir growth. This tree requires deep, well-structured and moist soils. At Vallombrosa, the high bulk density and low penetrability of the BC horizon has two negative effects. Firstly, it hinders deep rooting, thus limiting the stability of trees. On the other hand, the almost impermeable layer prevents the soil from accumulating adequate amounts of water. The trees suffering from water stress are unable to produce inhibitor metabolites for *Heterobasidion abietinum*, so their susceptibility increases to the infection (CERTINI ET AL. 2000a).

This is the reason why the propagation speed of *Heterobasidion* is inversely proportional to the water-holding capacity and organic matter content of the soil (CERTINI ET AL. 2000b). According to CERTINI ET AL. (2000b), fir can also grow in shallow soils where the bedrock is porous and fractured. The roots penetrate the cracks of the rock, taking up the water retained in pores. Of course this only works if precipitation is sufficient, which leads us to the third key factor of fir dieback.

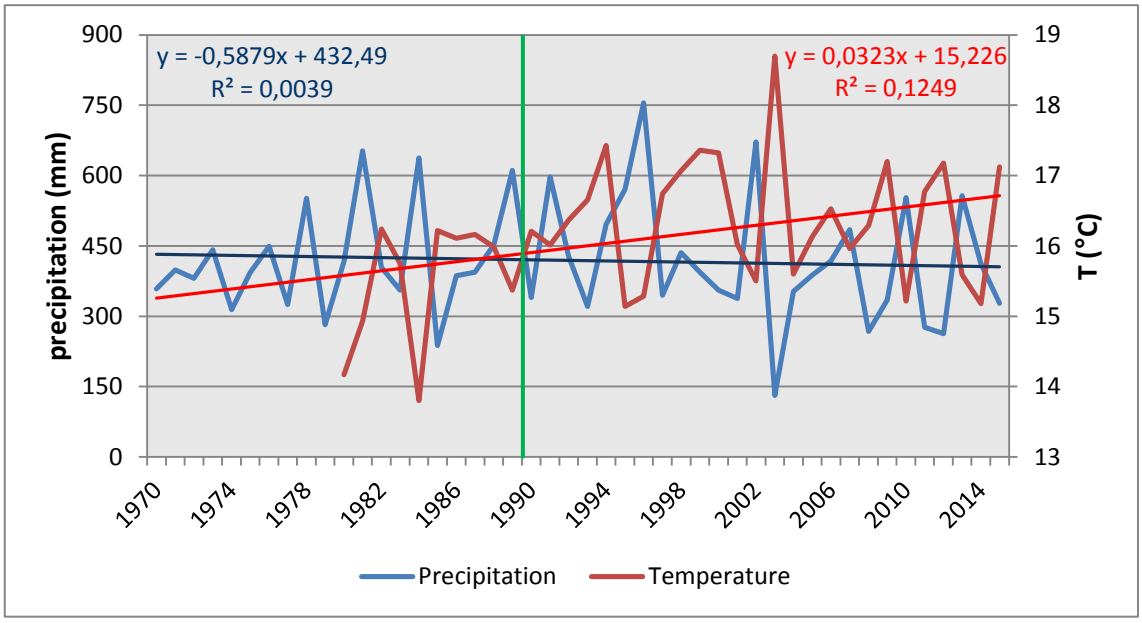


Figure 19: Precipitation (1970-2015) and mean air temperature (1980-2015) of the vegetative period at Vallombrosa.
(Source: Thermopluviometric Station of Vallombrosa, 980 m a.s.l.)

Mediterranean climate is often not very well suited to the needs of silver fir. The long dry periods in summer decrease the trees' vitality, especially where adequate water reserves cannot accumulate during the winter. In addition, particular weather events, such as heavy snowfalls and cyclones put these conifers under stress (PUDDU ET AL. 2003). The situation is getting worse with the advance of the global climate disruption. As already shown in Fig. 18, mean annual air temperatures tend to go up especially since 1990 at Vallombrosa while annual rainfall remains unchanged. What is even more formidable is to have a look at the same parameters during the growing season (Fig. 19). The gradient of the temperature's trend line is steeper, and precipitation values have started to plummet at the same time. The amplitude of the graphs clearly

demonstrates that extreme droughts and heat-waves occur more frequently, sometimes in subsequent years. Such events aggravate any water deficit in the soil, thus reducing the capacity of the trees to photosynthesize and absorb nutrients. In default of plant defence mechanisms, pathogenic agents like *Heterobasidion* and *Armillaria* rapidly kill the enfeebled trees.

When assessing the possibility of intervention to limit the damage from root rot caused by *Heterobasidion annosum* s.l. and *Armillaria* spp., it is necessary to know the potential of the different species regarding their host spectrum, infection biology and ecological needs, considering their possible different behaviour with regard to the applied treatments. Bearing in mind that the extent of damages at Vallombrosa has crossed a threshold where it does not merely cause large economic loss, but threatens the stability of the ecosystem, it is advisable to apply practical measures to limit the spread of parasitic root decaying fungi. Since the area is protected, drastic operations like stump removal must not take place. Instead, preventive treatment on the stumps is recommended immediately after cutting to inhibit the airborne colonization of *Heterobasidion* and thus reduce the further extension of the rot through root contacts. In particular the utilization of antagonistic fungi of *H. abietinum* could provide an obstacle to the spread of the pathogen. Cuts should be done in summer when sporulation rate of *Heterobasidion* is low. In case of silver fir, lowering the rotation period to 100-120 years is advisable.

The long-term solution to restore the health of the forest should be based on respecting and supporting natural processes. That includes favouring tree species more adapted to the site, hence more resistant to diseases. Results of this work, in line with several other ones has shown that silver fir does not meet its ecological needs at Vallombrosa. Fir stands should be planted only in the most suitable soils of the area, Ultic Hapludalfs (GIU1). Creating mixed stands with broadleaves is a preference; as such forests are less susceptible to *Heterobasidion* (PUDDU ET AL. 2003) and *Armillaria* (GUILLAUMIN AND LEGRAND 2013) attacks.

5. Conclusions / Summary

Diploma thesis is based on data collection and personal experiences during Erasmus+ internship at the University of Florence (Italy). It deals with the pathological reasons and consequences of the storm that affected forest plantations in Tuscany. In particular, the goal of the work is to investigate the distribution of root rots and to delineate a comprehensive overview of the health condition of the Vallombrosa forest.

For the purpose of comparison with previous results, the study area was selected following FARINA ET AL. (1990) and the applied methodology was also similar. In order to illustrate the presence of *Heterobasidion annosum* s.l. and *Armillaria* species complexes, fungal and soil samples were collected from 52 uniformly scattered points. Visual assessment of trees was done according to CAPRETTI ET AL. (2009).

Analysis of samples was performed in the laboratories of the Department of Forest Protection and Wildlife Management and the Department of Forest Botany, Dendrology and Geobiocoenology at the Mendel University in Brno, Faculty of Forestry and Wood Technology. For identification of *Heterobasidion annosum* s.l., TSCP-PCR method was chosen (GONTHIER ET AL. 2003). The *Armillaria* specific primer pair based on conserved sequences within the ITS region (rDNA) was used for direct amplification of DNA from soil samples by nested PCR (LOCHMAN ET AL. 2004a). The individual species were determined by RFLPs analysis with restriction endonuclease *Hin*fI. The high percentages of positive samples prove the efficiency of the applied DNA-based identification methods.

Results of the analysis confirm the high presence of root destroying fungi in Vallombrosa forest. *Heterobasidion abietinum* was found on 30.8% of the points. Though this parasite is mostly related to silver fir, it is apparently spreading into beech stands. *Armillaria* spp. are ubiquitous; the most important species are *A. cepistipes*, *A. ostoyae* and *A. gallica* in order. Besides, there are unexpected findings of *A. mellea*. Distinct *Armillaria* species often coexist sympatrically in the same ecosystem, and are

considered to specialize themselves to different lifestyle and host species (PROSPERO ET AL. 2006). No ecological pattern has been found in the distribution of the pathogens.

The role of root rots must always be evaluated in relation with their dynamically changing environment. Reviewing the relevant literature has revealed an etiology in which soil properties and climatic circumstances are crucial elements, being able to predispose the trees to fungal infection and determine the disease outcome. Considering all these interconnected factors, the conclusion is that *Abies alba* and *Pinus* spp. are the most vulnerable tree species at Vallombrosa.

By now, silver fir has become the very symbol of the Vallombrosa forest (Fig. 20). In order to conserve this noble woody plant, the most important and urgent task is to improve its health condition and resistance. Silvicultural methods should focus on the establishment of mixed deciduous stands. Results of present work may help forest managers to decide which tree species are to be planted known their resistance to *Armillaria* species. However, presence of *Armillaria* DNA in soil may not precisely represent its real distribution in the forest (PROSPERO ET AL. 2006). More detailed studies such as analysing decayed wood samples are necessary to obtain a complete picture of *Armillaria* and *Heterobasidion* occurrence.

Figure 20: Fir trees surrounding the Abbey of Vallombrosa. (Photo by Paolo Capretti)

6. Souhrn

Diplomová práce je založena na sběru dat a osobních zkušenostech ze studentského pobytu Erasmus+ na univerzitě ve Florencii (Itálie). Práce se zabývá dopady a následky bouře, které postihly lesní porosty v Toskánsku. Cílem práce je zejména prozkoumat přítomnost kořenových hnilob a zpracovat ucelený přehled zdravotního stavu lesa Vallombrosa forest.

Výsledky byly porovnány s předchozími studiemi v oblasti (FARINA ET AL. 1990) za použití podobné metodiky. Pro ilustraci byla přítomnost kořenovníku *Heterobasidion annosum* s.l. a komplexu václavek *Armillaria* spp. šetřena v síti 52 rovnoměrně rozptýlených bodů, kde byly odebrány vzorky hub a půdy na další šetření. Vizuální vyhodnocení stromů bylo provedeno v souladu s CAPRETTI ET AL. (2009).

Analýza vzorků byla provedena v laboratořích Ústavu ochrany lesů a myslivosti, Lesnická a dřevařská fakulta. *Heterobasidion annosum* s.l. byl identifikován metodou TSCP-PCR (GONTHIER ET AL. 2003). Pro identifikaci václavek *Armillaria* by použit specifický pár primerů, založený na konzervativních sekvencích v rámci své oblasti (rDNA) použitý pro řízenou amplifikaci DNA z půdních vzorků pomocí nested PCR (LOCHMAN ET AL. 2004a). Jednotlivé druhy byly identifikovány analýzou RFLP s restrikční endonukleázou *Hin*fl. Vysoký podíl pozitivních vzorků prokáže účinnost aplikovaných identifikačních metod založených na DNA.

Výsledky potvrzují vysoké procento infekce primárně parazitickými dřevními houbami. Kořenovník jedlový *Heterobasidion abietinum* byl potvrzen na 30,8% bodů. Ačkoli výskyt této dřevní houby je primárně spojen s výskytem jedle bělokoré *Abies alba*, je zřejmé, že se šíří i do přilehlé bučiny. Václavky *Armillaria* spp. jsou všudypřítomné; nejvýznamnější jsou v pořadí *A. cepistipes*, *A. ostoyae* a *A. gallica*. Kromě toho byla překvapivě zaznamenána rovněž *A. mellea*. Různé druhy václavek často v ekosystému sympatricky koexistují a váží se na různé hostitele a mají odlišnou ekologickou funkci (PROSPERO ET AL. 2006).

Role kořenových hnilob musí být vždy hodnocena ve vazbě na dynamiku prostředí. Literární údaje potvrzují, že půdní vlastnosti a klimatické poměry jsou klíčovými faktory, které predisponují stromy k houbovým infekcím. S ohledem na tyto vzájemně propojené predispoziční faktory, jsou jedle bělokorá *Abies alba* a borovice *Pinus* spp. nejvíce ohroženými dřevinami ve Vallombrosa forest.

V současnosti je jedle bělokorá významným symbolem Vallombrosa forest (Obr. 20). Pro zachování této ušlechtilé dřeviny je nejdůležitějším a naléhavým úkolem zlepšit její zdravotní stav a odolnost. Pěstební postupy by se měly zaměřit na vytvoření smíšených listnatých porostů. Výsledky této práce mohou pomoci lesníkům rozhodnout, které dřeviny mají být vysazeny a jaká je jejich odolnosti vůči václavkám. Přítomnost DNA václavek v půdě nemusí však přesně reprezentovat jejich skutečnou distribuci v porostu (PROSPERO ET AL. 2006). Podrobnější studie, jako například analýza tlejících vzorků dřeva jsou nezbytné pro získání kompletního obrázku o výskytu václavek a kořenovníku.

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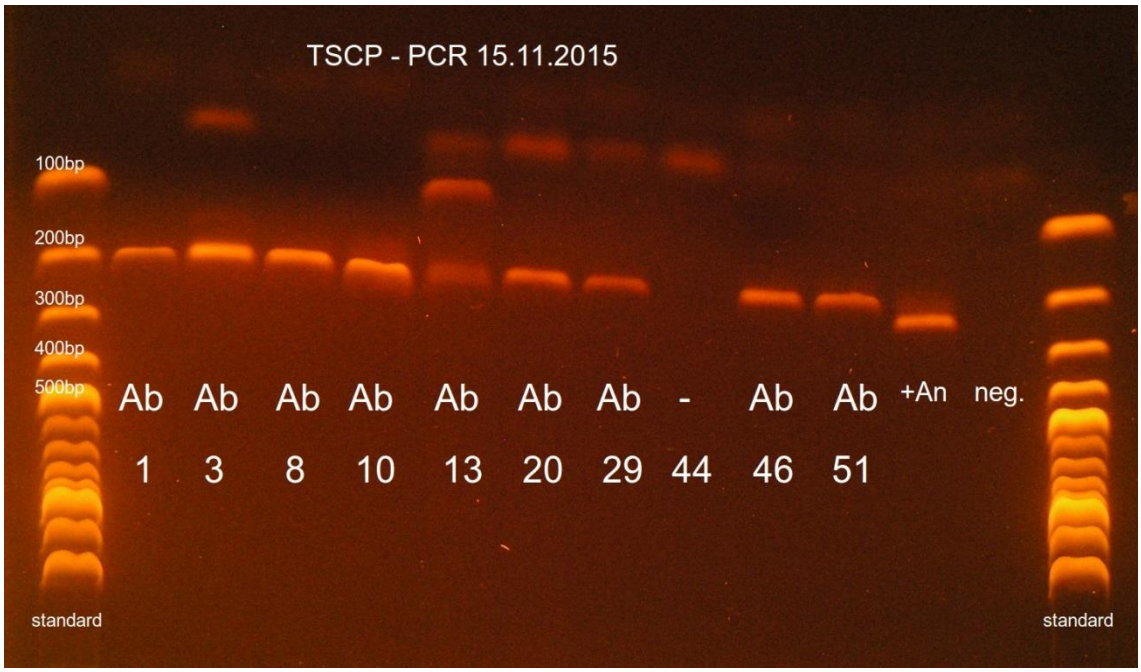
8. Appendix

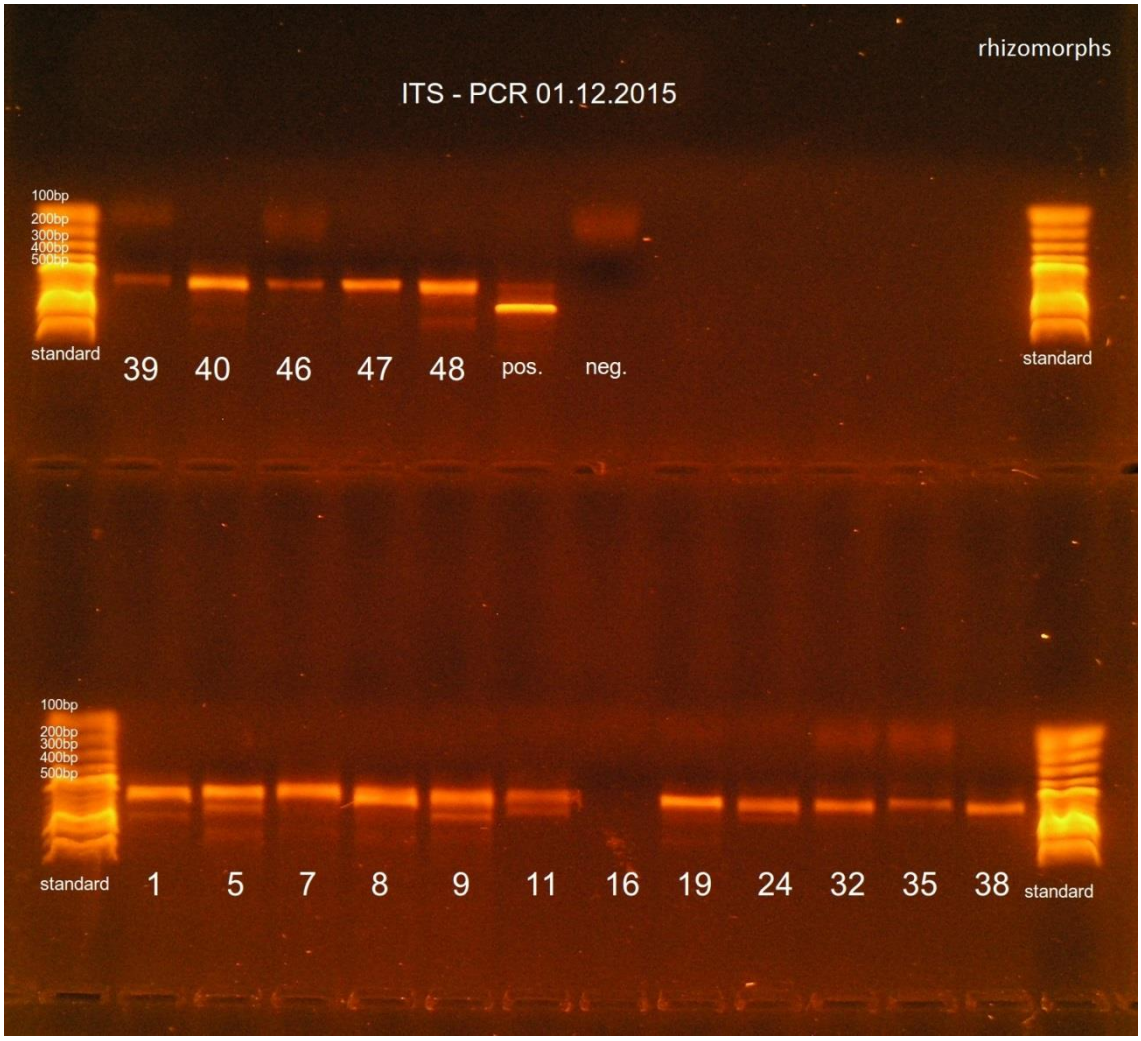
Annex 1: GPS data of sampling points.

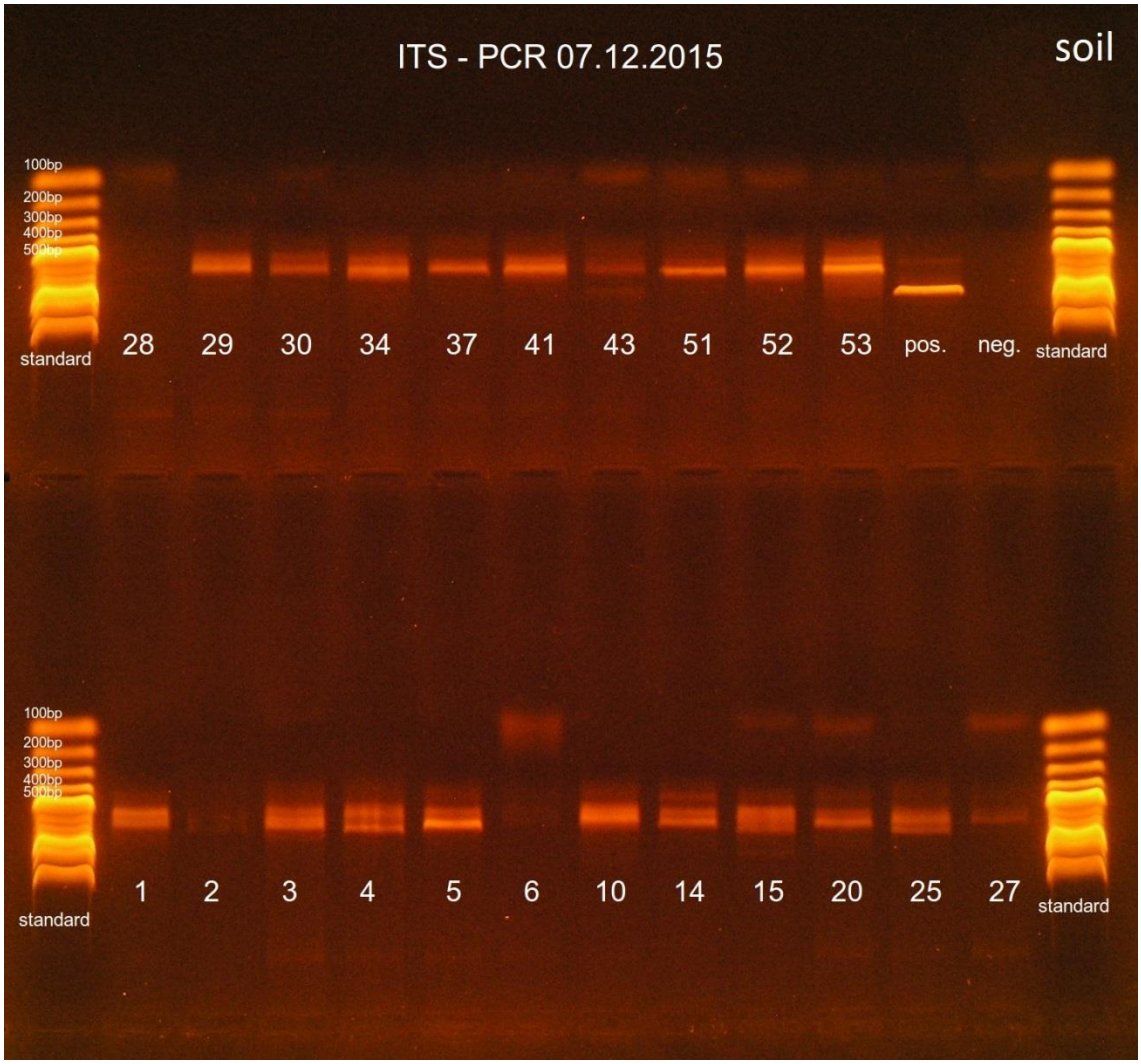
point	time	latitude	longitude	altitude	stand
P01	2015.07.28. 10:33:06	43.767771	11.582656	1103.5	5
P02	2015.07.24. 10:10:38	43.763331	11.570833	945.4	56
P03	2015.07.28. 09:34:17	43.763391	11.577143	1040.3	15
P04	2015.07.24. 07:38:53	43.757636	11.560083	877.0	40
P05	2015.07.24. 08:36:59	43.759528	11.565063	884.0	28
P06	2015.07.24. 09:26:24	43.758955	11.570807	923.2	85
P07	2015.07.28. 15:41:02	43.758498	11.575622	984.2	207
P08	2015.07.28. 12:16:24	43.758892	11.583232	1088.5	211
P09	2015.07.23. 10:34:55	43.754531	11.552586	765.1	35
P10	2015.07.23. 09:34:52	43.754473	11.558757	860.4	119
P11	2015.07.23. 15:37:47	43.754194	11.564658	998.7	192
P12	2015.07.23. 12:59:00	43.754371	11.571174	1027.3	93
P13	2015.07.23. 14:14:20	43.754023	11.575760	988.7	205
P14	2015.07.28. 13:38:56	43.754139	11.581992	1121.4	231
P15	2015.07.30. 09:54:23	43.750330	11.545788	671.0	
P16	2015.07.30. 08:33:12	43.750538	11.551410	795.2	140
P17	2015.07.17. 08:47:26	43.750197	11.557408	898.1	128
P18	2015.07.17. 09:35:32	43.750138	11.563753	978.4	199
P19	2015.07.15. 14:26:45	43.750023	11.569349	1122.2	256
P20	2015.07.15. 16:02:50	43.750025	11.575700	1062.3	248
P21	2015.07.28. 14:42:25	43.750092	11.582048	1157.7	396
P22	2015.07.14. 08:32:13	43.745948	11.534345	619.2	304
P23	2015.07.14. 09:34:22	43.746134	11.539079	649.1	200
P24	2015.07.14. 10:31:27	43.745816	11.545030	642.2	164
P25	2015.07.30. 13:51:25	43.745868	11.551279	767.2	280
P26	2015.07.17. 07:40:45	43.745907	11.557431	942.0	172
P27	2015.07.10. 09:29:06	43.745404	11.563475	1090.0	339
P28	2015.07.14. 14:24:44	43.745596	11.569340	1119.1	362
P29	2015.07.14. 15:41:14	43.745122	11.576195	1157.3	385
P30	2015.07.14. 16:37:37	43.745330	11.581971	1188.7	410
P31	2015.07.13. 17:09:44	43.741415	11.538799	768.8	310
P32	2015.07.13. 16:00:46	43.741309	11.545174	831.1	293
P33	2015.07.09. 14:48:44	43.741344	11.551353	790.6	288
P34	2015.07.29. 15:03:08	43.741266	11.556655	881.5	330
P35	2015.07.10. 10:21:17	43.741160	11.563036	1122.4	349
P36	2015.07.15. 09:31:33	43.741230	11.569198	1235.8	356
P37	2015.07.15. 08:00:51	43.740945	11.575698	1204.7	423
P38	2015.07.09. 16:25:28	43.736924	11.544659	879.4	316
P39	2015.07.09. 13:18:51	43.736829	11.550772	919.8	474
P40	2015.07.29. 13:55:34	43.736685	11.556670	926.8	460
P41	2015.07.10. 07:53:02	43.736767	11.562809	1057.7	521

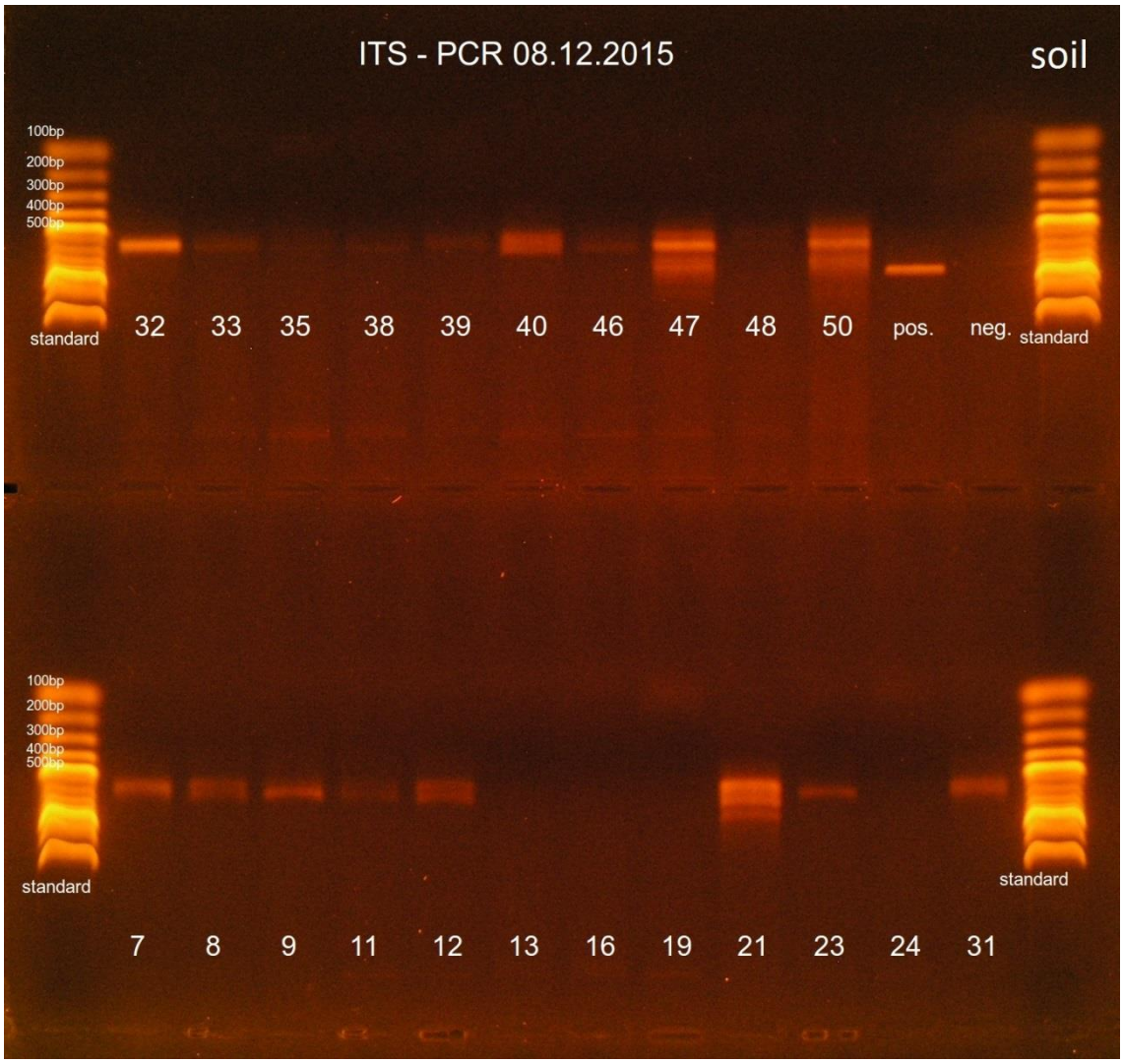
P42	2015.07.15. 08:49:31	43.736858	11.569136	1299.2	440
P43	2015.07.08. 15:47:56	43.732627	11.544651	985.4	483
P44	2015.07.08. 13:29:47	43.731847	11.551293	986.6	498
P45	2015.07.08. 13:00:00	43.726167	11.554000	976.0	
P46	2015.07.07. 15:05:36	43.732137	11.562747	1116.7	561
P47	2015.07.09. 09:43:43	43.732163	11.568893	1353.7	548
P48	2015.07.16. 08:11:47	43.728163	11.544618	1074.4	607
P49	2015.07.16. 09:34:14	43.728197	11.550885	1046.4	620
P50	2015.07.16. 10:29:16	43.728341	11.557243	1062.6	583
P51	2015.07.29. 08:06:40	43.728355	11.563144	1123.8	646
P52	2015.07.29. 09:54:38	43.727727	11.568806	1270.9	665
P53	2015.07.29. 08:53:34	43.723879	11.563445	1266.9	670

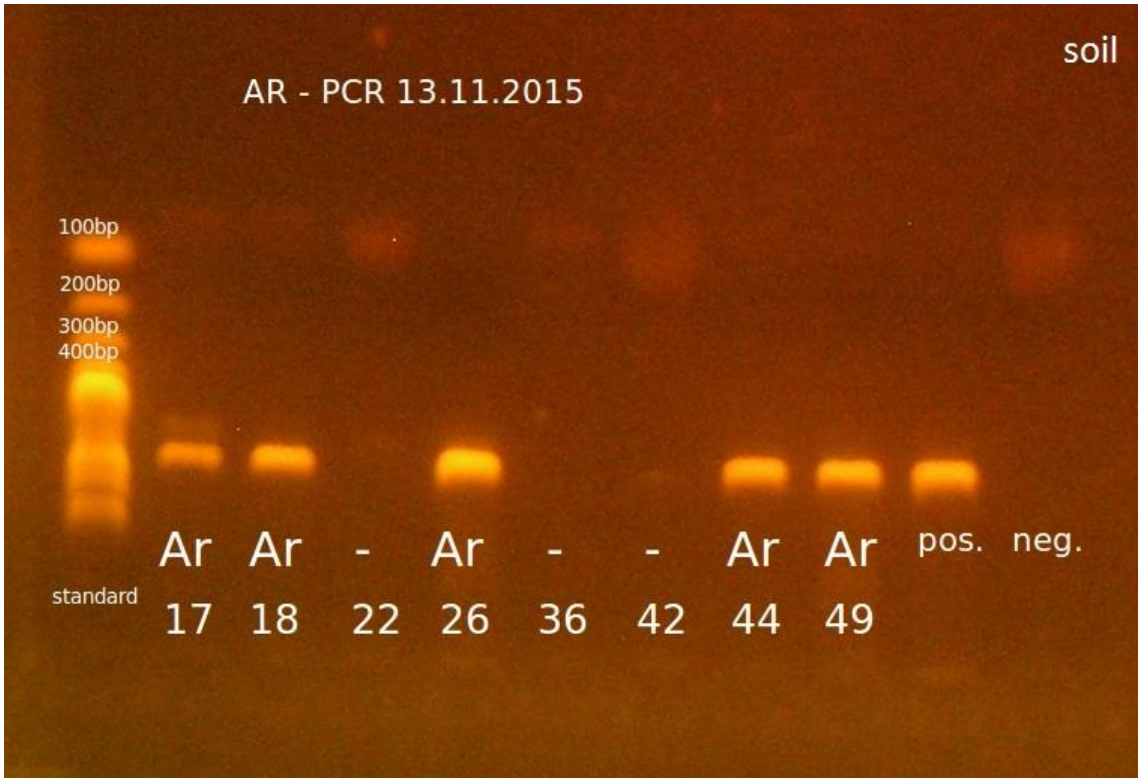
Annex 2: Results of gel electrophoresis under UV light.

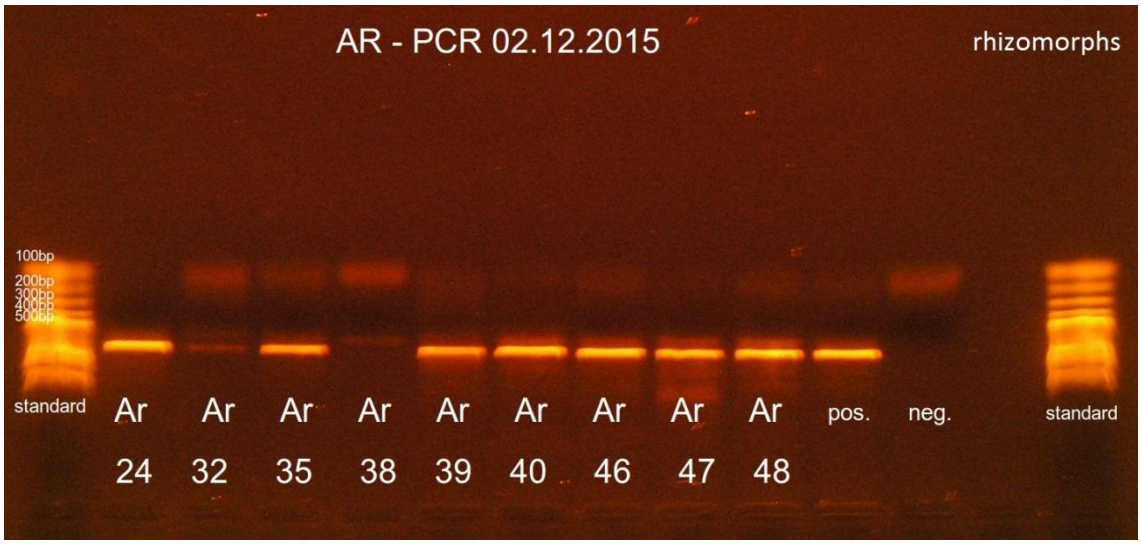


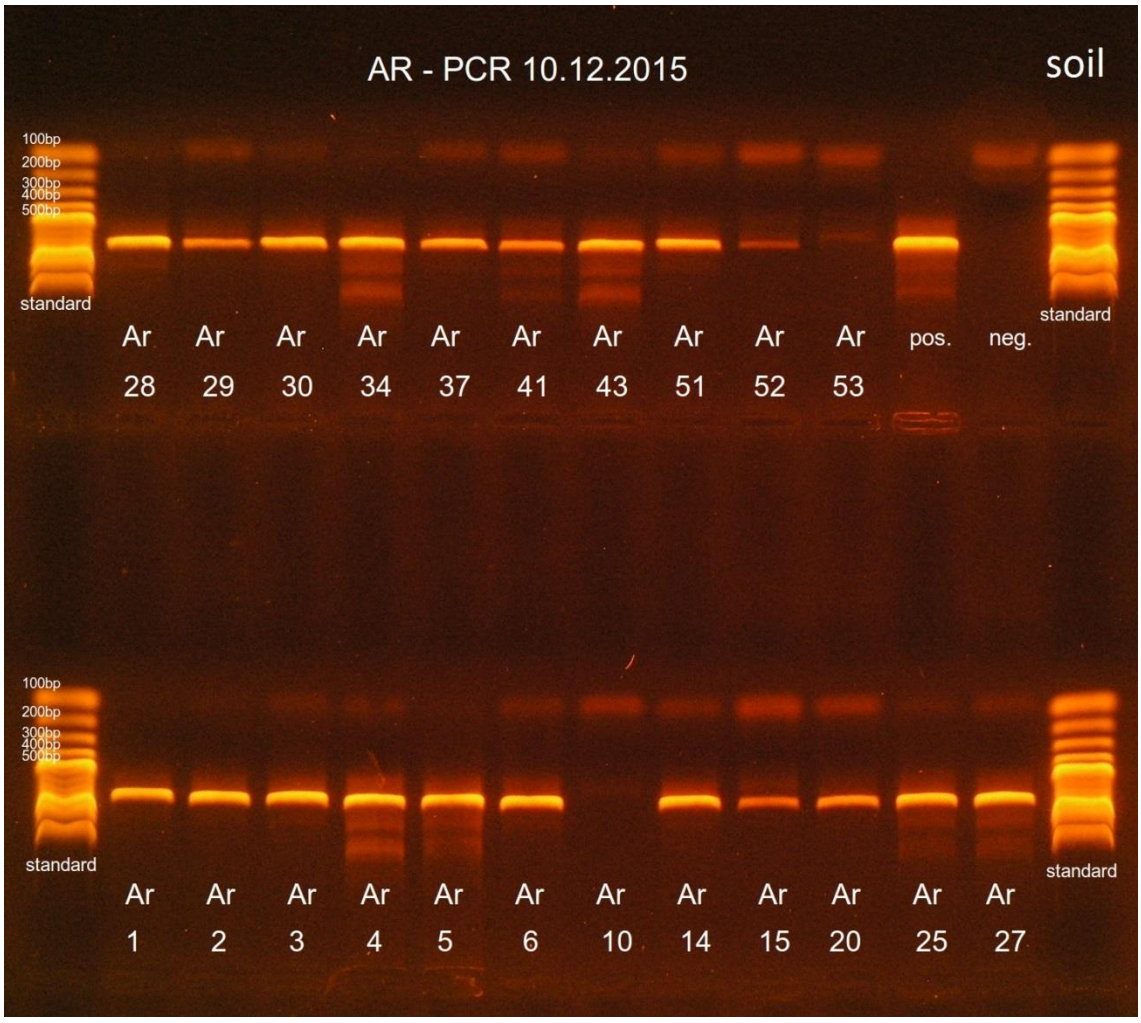

rhizomorphs
ITS - PCR 01.12.2015
100bp
200bp
300bp
400bp
500bp
standard
39
40
46
47
48
pos.
neg.
standard
100bp
200bp
300bp
400bp
500bp
standard
1
5
7
8
9
11
16
19
24
32
35
38
standard

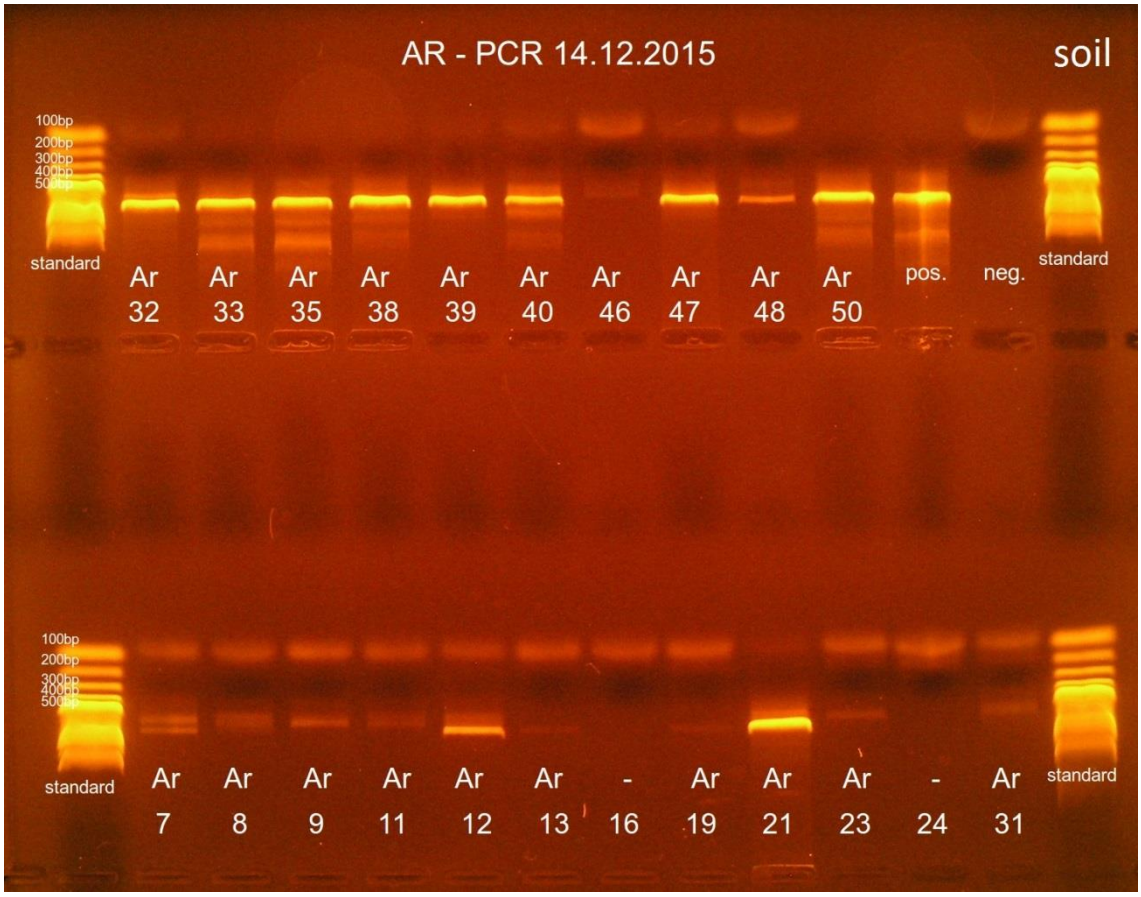

ITS - PCR 07.12.2015
soil
100bp
200bp
300bp
400bp
500bp
standard
28
29
30
34
37
41
43
51
52
53
pos.
neg.
standard
100bp
200bp
300bp
400bp
500bp
standard
1
2
3
4
5
6
10
14
15
20
25
27
standard

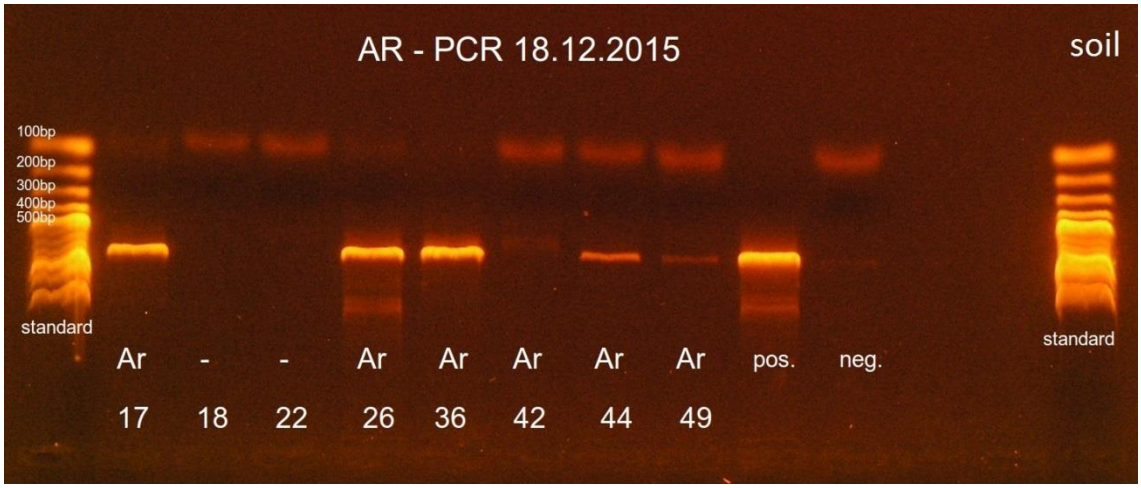

ITS - PCR 08.12.2015
soil
100bp
200bp
300bp
400bp
500bp
standard
32
33
35
38
39
40
46
47
48
50
pos.
neg.
standard
100bp
200bp
300bp
400bp
500bp
standard
7
8
9
11
12
13
16
19
21
23
24
31
standard

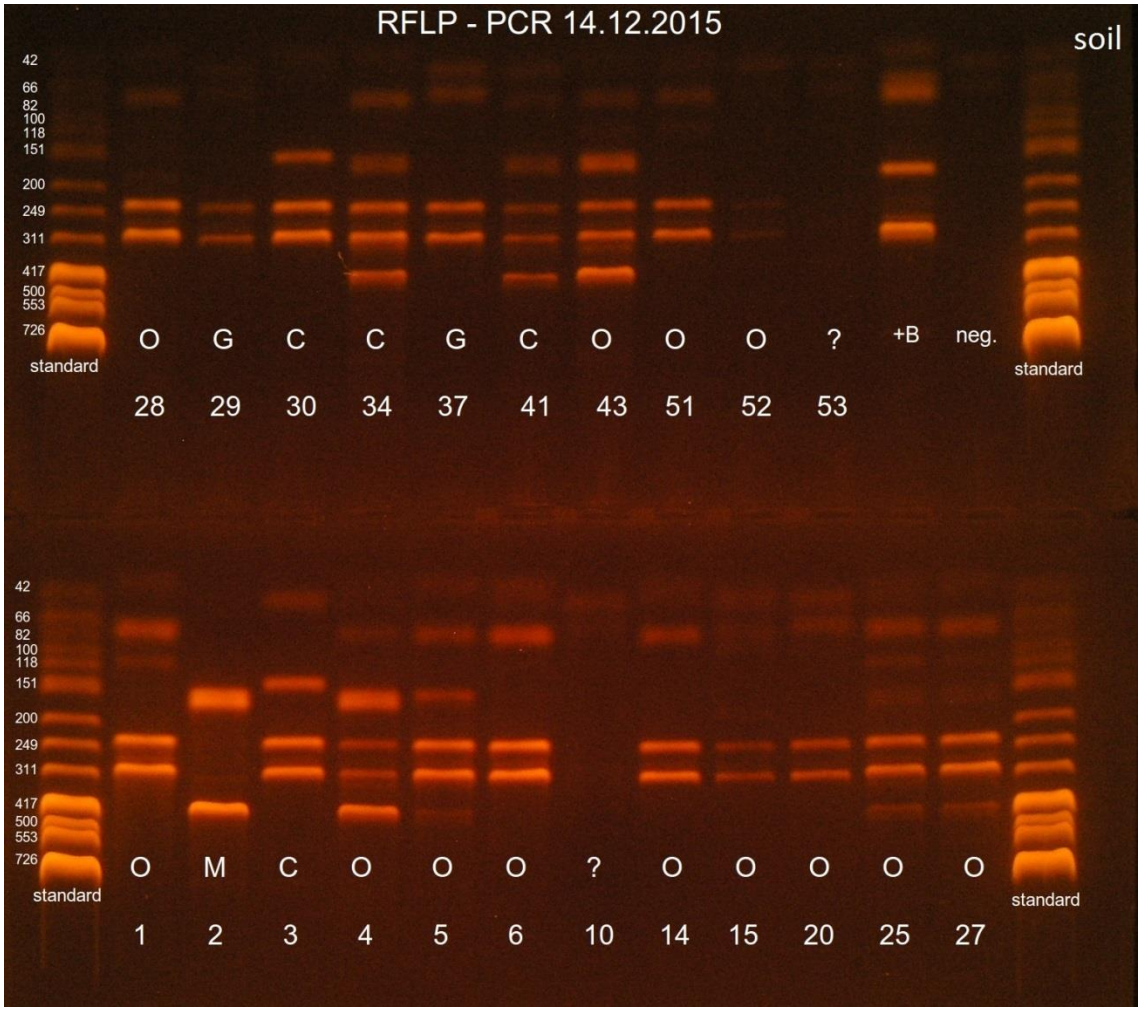

soil
AR - PCR 13.11.2015
100bp
200bp
300bp
400bp
standard
Ar 17
Ar 18
- 22
Ar 26
- 36
- 42
Ar 44
Ar 49
pos.
neg.

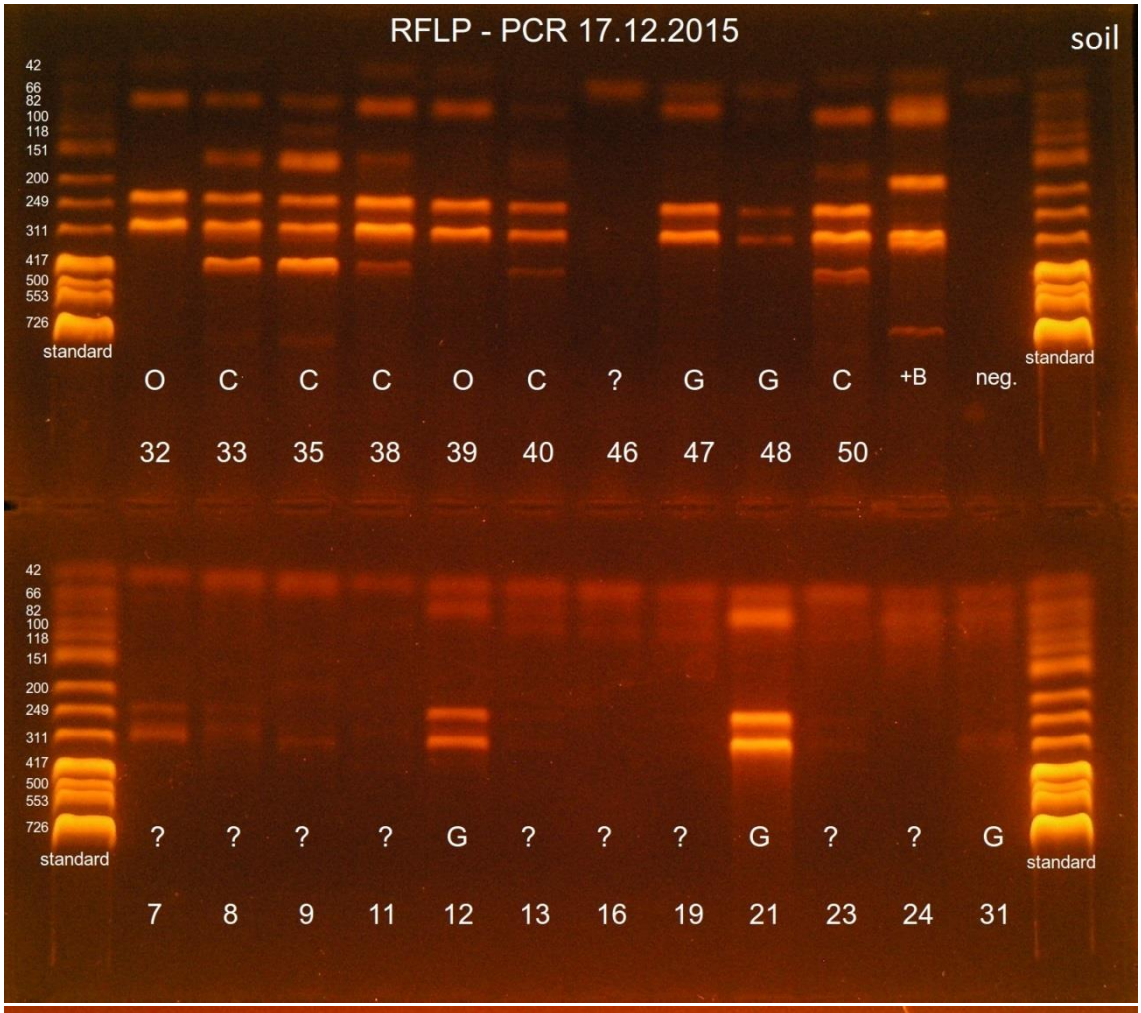

AR - PCR 02.12.2015
rhizomorphs
100bp
200bp
300bp
400bp
500bp
standard
Ar 24
Ar 32
Ar 35
Ar 38
Ar 39
Ar 40
Ar 46
Ar 47
Ar 48
pos.
neg.
standard

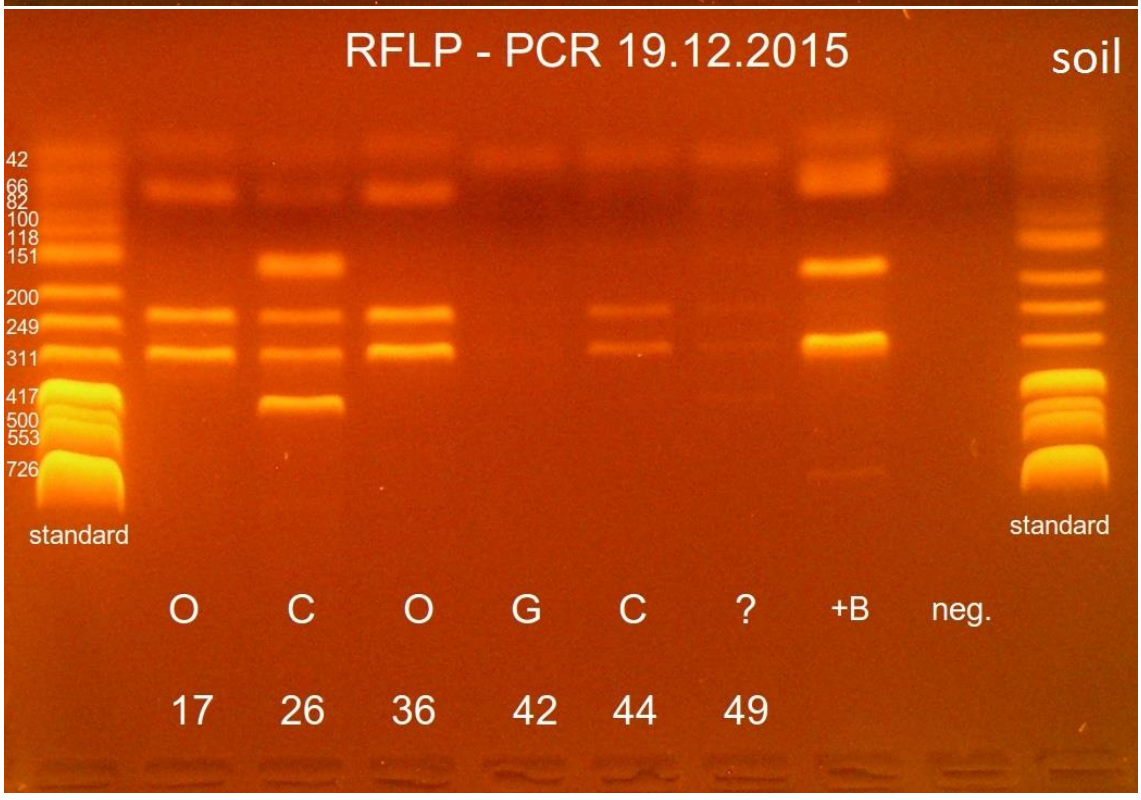

AR - PCR 10.12.2015
soil
100bp
200bp
300bp
400bp
500bp
standard
Ar 28
Ar 29
Ar 30
Ar 34
Ar 37
Ar 41
Ar 43
Ar 51
Ar 52
Ar 53
pos.
neg.
standard
100bp
200bp
300bp
400bp
500bp
standard
Ar 1
Ar 2
Ar 3
Ar 4
Ar 5
Ar 6
Ar 10
Ar 14
Ar 15
Ar 20
Ar 25
Ar 27
standard


AR - PCR 14.12.2015
soil
100bp
200bp
300bp
400bp
500bp
standard
Ar 32
Ar 33
Ar 35
Ar 38
Ar 39
Ar 40
Ar 46
Ar 47
Ar 48
Ar 50
pos.
neg.
standard
100bp
200bp
300bp
400bp
500bp
standard
Ar 7
Ar 8
Ar 9
Ar 11
Ar 12
Ar 13
- 16
Ar 19
Ar 21
Ar 23
- 24
Ar 31
standard


AR - PCR 18.12.2015
soil
100bp
200bp
300bp
400bp
500bp
standard
Ar 17
- 18
- 22
Ar 26
Ar 36
Ar 42
Ar 44
Ar 49
pos.
neg.
standard


RFLP - PCR 14.12.2015
soil
42
66
82
100
118
151
200
249
311
417
500
553
726
standard
O G C C G C O O O ? +B neg.
standard
28 29 30 34 37 41 43 51 52 53
42
66
82
100
118
151
200
249
311
417
500
553
726
standard
O M C O O O ? O O O O O
standard
1 2 3 4 5 6 10 14 15 20 25 27


RFLP - PCR 17.12.2015
soil
42
66
82
100
118
151
200
249
311
417
500
553
726
standard
O C C C O C ? G G C +B neg.
standard
32 33 35 38 39 40 46 47 48 50
42
66
82
100
118
151
200
249
311
417
500
553
726
standard
? ? ? ? G ? ? ? G ? ? G
standard
7 8 9 11 12 13 16 19 21 23 24 31


RFLP - PCR 19.12.2015
soil
42
66
82
100
118
151
200
249
311
417
500
553
726
standard
standard
O C O G C ? +B neg.
17 26 36 42 44 49

9. Acknowledgements

My deepest and sincere gratitude goes to my supervisors Prof. Paolo Capretti, for his continuous support and hospitality during my Erasmus+ stay in Florence, and prof. Dr. Ing. Libor Jankovský, for accepting my request for his supervision, his patience and trust in me.

I would like to express my warmest thanks to my consultants Ing. Petr Sedlák, Ph.D. and Ing. Tomáš Májek, for their amazing guidance, help and enthusiastic approach throughout my quest for knowledge.

I do not want to forget to say thanks to the following people who supported my work:

doc. RNDr. Michal Tomšovský, Ph.D.

Lorenzo Allighieri

Elisa Carrari

Prof. Davide Travaglini

Dr.ssa Francesca Bottalico

Prof. Donatella Paffetti

Prof. Giacomo Certini

My special thanks go to the International Visegrad Fund for granting me Intra-Visegrad Scholarship for 2 academic years of European Forestry Master's program. Without their financial support, it would not have been possible for me to devote myself to this work.

Last but not least, I wish to render thanks to Mgr. Ing. David Sís who constantly helped me with documents and every emerging issue during my studies.