Tartu University

Institute of Botany and Ecology

Chair of Mycology

Triin Suvi

Ectomycorrhizal fungal diversity of birch in Tagamõisa wooded meadow and the adjacent forest.

Master of Science Thesis

Supervisor: prof. Urmas Kõljalg

Tartu 2005

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1. Introduction

1.1 Mycorrhiza

Mycorrhiza is a term for mutualistic relationships between higher plant roots and fungi. Symbiotic fungi transfer different nutrients (P, N) from soil to plants and plants supply fungi with carbon fixed via photosynthesis (Smith & Read, 1997; Read & Perez-Moreno, 2003). Fungal partners also increase plant water uptake and protect their hosts against different pathogens. Although mycorrhiza is considered to be a mutualistic relationship, it is not always easy to detect the benefit gained from the association.

Ectomycorrhizal and arbuscular mycorrhizal fungi are considered obligate symbionts. Mycelium of some ectomycorrhizal fungi may grow faster when not associated with a host plant, but many mycorrhizal macrofungi lack the ability to produce fruiting bodies without being connected to a photosynthetically active plant (Jones & Smith, 2004). Therefore, fungal benefit gained from the symbiosis is quite well understood with the exception of the mycoheterotrophic association (Egger & Hibbett, 2004; Jones & Smith, 2004). Mycoheterotrophic plants are non-photosynthetic and they obtain carbon through the fungal partner that may be an ectomycorrhizal fungus gaining its carbon from green plants (McKendrick *et al.*, 2000). For green plants, benefits of the symbiosis are much less clear than benefits for fungal partners. Many studies have revealed increased plant growth and nutrient uptake when mycorrhizal (Finlay, 1989; Wu *et al.*, 1999; Perez-Moreno & Read, 2000; Tibbett & Sanders, 2002), but number of studies have, however, found negative effects or no influence on host plant biomass and nutrition (Danielson *et al.*, 1984). Therefore, are mycorrhizas really mutualistic symbioses? There are many different hypotheses to explain the coexistence of mycorrhizal plants and fungi (Egger & Hibbett, 2004; Jones & Smith, 2004). It seems that whether a plant exhibits a positive or a negative response to colonization depends on host plant and fungal species and on environmental conditions – competing plants, soil microbes, temperature, pH, moisture, nutrients, etc (Lehto, 1992; Baar & Elferink, 1996; Jones & Smith, 2004).

Arbuscular mycorrhiza and ectomycorrhiza are the most important mycorrhizal types in terms of distribution and economy. Arbuscular mycorrhiza played a key role in land colonization by higher plants (Brundrett, 2002). First terrestrial fungi colonized land before higher plants and

were probably associated with algae. Rhizoids and thalli of first land plants hosted arbuscules of possibly glomalean fungi. Nowadays, vast majority of terrestrial plants are associated with arbuscular mycorrhizal fungi (Smith & Read, 1997).

1.2 Ectomycorrhiza

Ectomycorrhiza developed later than arbuscular mycorrhiza and most probably many times independently. The oldest fossil evidence of ectomycorrhiza dates back to Eocene (LePage *et al*., 1997). Compared to arbuscular mycorrhiza, the number of potential hosts is much smaller – only 3% of phanerogams are ectomycorrhizal, which involve about 30 plant families (Smith & Read, 1997; Hibbett, 2002). Ectomycorrhizal habit has spread predominantly among woody plants, with the exception of some shrubs and herbaceous species, e.g. *Dryas* spp., *Helianthemum* spp. and *Polygonum viviparum* L. (Smith & Read, 1997). Despite the relatively small number of host plant species, ectomycorrhizal plants dominate vast areas in temperate and tropical ecosystems. Ectomycorrhizal Pinaceae and Fagaceae are the dominant trees in boreal forests of the northern hemisphere (Smith & Read, 1997); therefore ectomycorrhizal fungi possess great importance in these ecosystems (Read *et al*., 2004).

Although the number of ectomycorrhizal plant species is comparatively small, the number of fungi, which are able to form ectomycorrhiza, is remarkable – more than 6000 fungal species from phyla *Basidiomycota* and *Ascomycota* (Smith & Read, 1997). The majority of ectomycorrhizal fungi are basidiomycetes. New genera and even lineages of both ectomycorrhizal ascomycetes and basidiomycetes are discovered until present (Vralstad *et al*., 1998, Selosse *et al*., 2002;).

The majority of ectomycorrhizal fungal species possess a broad host range. Individual species may form ectomycorrhiza with different plant species simultaneously (Kennedy *et al*., 2003). This ability enables fungi to form networks, where one fungus is connected with several plants and one plant with different fungal species. Such networks allow nutrient flow between plants (Simard *et al*., 1997b; Simard *et al*., 1997c; He *et al*., 2004; Simard & Durall, 2004) and it may increase survival of seedlings and shaded plants (Simard *et al*., 1997a). However, several studies

have revealed no evidence for nutrient flow between ectomycorrhiza-connected plants (Wu *et al.,* 2001; Ek *et al*., 1996).

Many ectomycorrhizal fungal species possess narrow host range, specializing on a single plant genus (Bruns *et al*., 2002). In contrast, there are only a few plant genera that are highly specialized on certain ectomycorrhizal fungi. The genus *Alnus* is associated with approximately 20 species of highly specific ectomycorrhizal fungi (Pritsch *et al*., 1997). Nyctaginaceae spp. also possess narrow host range and some of these tropical species are associated only with Thelephoraceae (Haug *et al*., 2005).

Ectomycorrhizas possess three structural components: fungal mantel embedding the root tip, Hartig net between plant cortical cells, and emanating hyphae in the soil (Smith & Read, 1997). Emanating hyphae take up and transport nutrients and water from soil to a host plant. Some basidiomycetes form hyphal cords (rhizomorphs), which are very effective in nutrient transport. Exchange of nutrients, water and carbohydrates between plants and fungi occur through the Hartig net.

The features of hyphae and fungal mantel have been used for identification of fungal symbionts. As identification is solely based on anatomical and morphological characters of ectomycorrhiza, it is not very precise and usually does not allow detecting closely related species (Wurzburger *et al*., 2001). Different molecular methods have been more recently utilised to identify the mycobionts. These molecular techniques are mainly based on DNA and enable to determine fungal symbionts on a species or even on a genetic individual level (Horton, 2002; Landeweert *et al*., 2003). The most commonly used DNA region is ribosomal DNA (rDNA), which consists of rRNA genes and Internal Transcribed Spacers (ITS). ITS1 and ITS2 are transcribed with other parts of rRNA, but later these regions are cut off and they are not used in the ribosome. Probably therefore ITS1 and ITS 2 are highly variable and they enable identification of species or discover cryptic species.

Ectomycorrhiza facilitates growth, nutrient uptake and survival of young plants. Therefore it is essential for seedlings to become ectomycorrhizal as soon as possible. Pines cannot grow in exotic conditions without being associated with ectomycorrhizal fungi (Baar & Elferink, 1996; Bruns *et al*., 2002). Mature trees play a key role in inoculation of young plants' roots, because

their ectomycorrhizal roots are acting as inoculation source (Cline *et al*., 2005). Seedlings emerging in the vicinity of mature trees have more symbiotic fungi associated with their roots than single seedlings (Kranabetter, 1999).

Ectomycorrhiza protects plant roots against pathogens (Sylvia & Sinclair, 1983; Sen, 2001; Whipps, 2004) and increases resistance to drought (Garbaye, 2000). Ectomycorrhizal fungi bind toxic compounds with hyphae (Fomina *et al*., 2005) and hence help host plant to tolerate stress caused by heavy metals (Adriaensen *et al*., 2003). Therefore, ectomycorrhizal fungi are extremely important for plants growing in dry and polluted soils.

Ectomycorrhizal fungi affect root-associated bacterial communities. Fungi acidify the surrounding soil environment by excreting organic exudates, which reduce soil pH. Acidic substrates repress the growth of root pathogens (Whipps, 2004). Hyphae excrete carbohydrates that can be used by bacteria and other soil microorganisms for food (Heinosalo *et al*., 2004). Bacterial communities are, therefore, more diverse and abundant in the mycorhizosphere. Some fungal species need appropriate bacteria to form mycorrhiza (Jones & Smith, 2004). Bacteria may provide nutrients to fungi (Li *et al*., 1992) or stimulate the growth of certain fungal species and inhibit the others (Schelke & Peterson, 1996; Varese *et al*., 1996; Poole *et al*., 2001). Therefore, bacteria that stimulate ectomycorrhiza formation or enhance their nutrition are termed as helper bacteria.

Ectomycorrhizal fungi form communities like plants and animals. Ectomycorrhizal fungal communities are usually very species rich, containing a few dominant species and a long tail of rare species (Horton & Bruns, 2001; Izzo *et al*., 2005). Heterogeneous distribution of nutrients, disturbance, coexistence of different host plants and competition between fungi and other soil microbes are probably among the most important factors supporting high ectomycorrhizal fungal species richness (Bruns, 1995). Assessment of ectomycorrhizal fungal species richness is complicated due to patchy distribution of both root tips and fungal individuals.

Ectomycorrhizal fungal community studies based on sporocarp surveys failed to reveal the actual number and distribution of ectomycorrhizal fungi, because many species form inconspicuous resupinate or hypogeous fruit bodies. In addition, some species never form sporocarps. Correspondence between above- and below-ground views of species composition, spatial

frequency and abundance is usually very low (Gardes & Bruns, 1996; Jonsson *et al.*, 1999; Richard *et al.*, 2005). Therefore, it is essential to study below ground aspects to get more precise and trustworthy results.

Different ectomycorrhizal fungi colonize plants on different stages of succession. Early-stage fungi colonize young plants and seedlings, while late-stage fungi inhabit mature plants and more stable plant communities (Baar, 1996). Multi-stage fungi colonize both seedlings and mature trees. Still, seedlings that grow in the vicinity of mature trees are usually colonized by fungi that are associated with adults (Fleming, 1983; Kranabetter, 1999). Apparently, early-stage fungi are more important when mycorrhizal plants are pioneer colonists of a site (an old agricultural field) or in nurseries (Smith & Read, 1997).

Fungal communities are very sensitive to abrupt changes in the environment. Clear-cut and wildfire reduce the abundance of symbiotic fungi several-fold. Clear-cutting reduces the energy source of ectomycorrhizal fungi, leading to the loss of fungal inoculum (Jones *et al.*, 2003). Species composition is different and species richness lower in regenerating communities (Byrd *et al.*, 2000). Wildfires also reduce ectomycorrhizal fungal diversity and change species composition (Douglas *et al.*, 2005) depending on fire intensity. Low intensity fires have only a small impact (Jonsson *et al.*, 1999) compared with intensive wildfires that may burn the entire organic horizon together with ectomycorrhizal root tips and host trees (Erland & Taylor, 2002).

1.3 Wooded meadows

Wooded meadows are defined as sparse natural stands with a regularly mown herb layer (Kukk & Kull, 1997). Until the middle of the last century, wooded meadows were widespread in Europe, especially in countries around the Baltic Sea. Nowadays wooded meadows, like all semi-natural grasslands, have disappeared almost everywhere due to fundamental changes in land use (van Dijk, 2001).

Deciduous trees (*Quercus robur* L., *Betula* spp., *Tilia cordata* Mill.) and bushes (*Corylus avellana* L.) are the most common woody species in wooded meadows. Late summer mowing with scythe has been essential for maintenance of these ecosystems (Kukk & Kull, 1997). Grazing intensity is kept low, because animals may damage the soil surface. Wooded meadows

differ from parks by their natural (not planted) vegetation. Only plant species composition and appearance are influenced by humans in wooded meadows.

Wooded meadows are very rich in plant species: one of the world's highest plant community small-scale species richness has been recorded on an old regularly mown temperate meadow (Kull & Zobel, 1991). Shallow, neutral or alkaline infertile soils, long-term mowing (decrease competition), heterogeneity in moisture and light conditions and large local species pool are the main factors supporting high plant species richness (Kukk & Kull, 1997; Maron & Jefferies, 2001). Therefore, it is essential to maintain and protect these communities, because once abandoned, it is very difficult to restore them.

There are also many different bryophytes, animal and fungal species on wooded meadows, including numerous rare species (Kukk & Kull, 1997). For example, an ectomycorrhizal fungus *Boletus satanas* Lenz inhabits exclusively wooded meadows. Studies on fungal species composition on wooded meadows have been based only on fruiting bodies so far, but such surveys underestimate the actual number of fungal species. Therefore it is essential to study species composition on tree roots to get less biased view of the whole ectomycorrhizal fungal community.

1.4 The aims of the study

The main purposes of this study were to identify ectomycorrhizal fungal species using molecular and phylogenetic methods and to compare fungal diversity and community composition between the wooded meadow and neighbouring forest (the influence of land management). I also aimed to compare fungal diversity between upper and lower horizon, to describe ectomycorrhizal morphotypes on birch roots and to find appropriate morphological features to distinguish between birch and other tree species roots.

2. Materials and Methods

2.1 Study site

The field work was carried out in Tagamõisa wooded meadow and an adjacent forest, The Isle of Saaremaa, in August and September, 2003. Tagamõisa wooded meadow is included in a nature reserve, which was established in 1957 (Kukk & Kull, 1997). The nature reserve originally covered 130 ha of one of the best maintained wooded meadows in Saaremaa. During the Second World War the condition of wooded meadow deteriorated, because it was abandoned for ca 20 years. By the end of 1960s, Tagamõisa wooded meadow was covered with coppice that reduced herbaceous plant species richness, including protected species. The coppice was removed and the wooded meadow was mown regularly only for some years in the 1970s. The wooded meadow was also used as a cattle range for several years, which damaged the ground. Irregular mowing and inadequate land management decreased the value of Tagamõisa nature reserve. Therefore maintenance of wooded meadow as a nature reserve became questionable.

Botanical inventories in 1997 revealed that Tagamõisa wooded meadow has still high value as a nature reserve. There were up to 67 vascular plant species in one square meter-size plot making Tagamõisa wooded meadow one of the most species rich in Estonia. During the inventories many protected orchid species were found, for example *Dactylorhiza spp.* and *Platanthera spp*. (Kukk, 2000).

Tree layer is species rich in Tagamõisa wooded meadow, dominated by *Betula pubescens* Ehrh., *Q. robur*, *T. cordata*, *Fraxinus excelsior* L., *Populus tremula* L. and conifers. The age of the trees is approximately 30 - 80 years, but some of them are 140 years old (Kukk 2000). Bush layer includes *C. avellana.*

The adjacent forest is an old part of the wooded meadow, which has been unmanaged for decades. Therefore, the age of trees is different (both old and premature trees). Dominant tree species are *B. pubescens, Alnus glutinosa* (L.) Gaertn.*, P. tremula* and *T. cordata*. Bush layer consists mainly of *Salix* spp. and *C. avellana*. Herb layer in the forest is weakly developed and mainly dominated by *Carex* spp. Most of the forest area is flooded in the spring and therefore some pits comprise no herbaceous species.

Soils have developed on sea sand and limestone. The litter layer is approximately 2 cm in the wooded meadow and up to 3 cm in forest. The humus horizon is up to 15 cm thick in the forest. Soil humus layer comprises gravel in the wooded meadow. Forest soils are peaty due to floods in spring.

2.2 Sampling

Four plots (diameter 6 m) were established around a randomly selected mature birch trees both in the wooded meadow and adjacent forest. Five soil cores were randomly taken with spade or knife from each plot. Each soil core consisted of two samples (15 x 15 x 5 cm), including O-horizon (0 – 5 cm in depth) and A-horizon (15 – 20 cm). Total number of samples was 80.

In addition, three soil cores (5 x 5 x 5 cm) were taken from both horizons in each plot to determine the following soil parameters: pH, concentrations of P, K, Ca, Mg and organic matter. Soil samples, which were taken from the same plot and horizon were pooled and dried. Soil parameters were determined in the laboratory of plant biochemistry, Faculty of Agronomy, Estonian Agricultural University.

2.3 Processing of samples

Root samples were soaked in tap water to remove soil from the roots. All roots of ectomycorrhizal trees were separated and laid into Petri dishes, half-filled with tap water. Tree roots were sorted by species, using binoculare. All living birch roots were separated, cut into 2-3 cm fragments and placed into another Petri dish in water. Twelve fragments per sample were randomly chosen from the Petri dish and all ectomycorrhizal root tips were assigned to morphotypes, and counted. Morphotypes in one sample were coded. Therefore morphotypes possess different codes (further submorphotype) in different samples.

Morphological characters and taste were used to recognize birch roots. Morphotyping of root tips was based on the following characteristics: shape of root tip, colour and structure of fungal mantle, the presence and structure of cystidia, emanating hyphae and rizomorphs. One or more root tips from each morphotype was stored in FEA solution (90% formalin, 5% ethanol and 5% acetate) to prepare mantle squashes later in KOH.

2.4 DNA extraction and PCR

DNA was extracted from root tips and pure cultures. At least one young and fresh ectomycorrhizal root tip from each morphotype was selected under the binoculare and placed into an Eppendorf tube containing 300 µl 2% CTAB solution (0.2 g CTAB, 5.78 ml sterile distilled water, 1.0 ml 1M Tris-HCl, 2.8 ml 5M NaCl, 0.4 ml 0.5M EDTA) and stored at room temperature until further analyses.

DNA extraction followed Savolainen *et al.* (1995). 6 µl mercaptoethanol was added into each Eppendorf tube, followed by grinding using micropestles. Samples were kept at 65°C for 1 h. Then 300 µl chloroform was added and samples were centrifuged for 15 minutes (10 000 g). The liquid phase was removed and transferred into a clean Eppendorf tube. Then 600 µl isopropanol was added into each Eppendorf tube, followed by incubation at -20°C for at least 1 h to precipitate DNA. Samples were centrifuged for 15 minutes and liquid phase was discarded. 1 ml 70% ethanol was added to precipitate DNA and centrifuged for 15 minutes. Liquid phase was discarded and 30 µl sterile water was added to the DNA solution, and mixed gently with a pipette.

DNA purification was carried out using UltraClean^{TM} 15 Kit following manufacturer's instructions. 120 µl NaI and 6 µl glass milk (Ultra bind) were added into each Eppendorf tube, mixed and kept for 5 minutes at room temperature to facilitate DNA binding to silicon particles. Samples were centrifuged for 1 minute and liquid phase was discarded. 1 ml Ultra Wash was added to the samples. Samples were centrifuged for 15 seconds and liquid phase was discarded. Samples were additionally centrifuged for 5 seconds and liquid droplets were removed from Eppendorf tubes by pipetting. 200 µl sterile water was added and Eppendorf tubes were shaken against each other so that DNA could be separated from silicon particles. Then, samples were centrifuged for 1 minute to precipitate glass milk. Liquid phase was transferred into clean Eppendorf tube and kept at 4°C until further analyses.

PCR was carried out using puReTaq Ready-To-Go^{TM} PCR Beads in 500 µl Eppendorf tubes. Ready-To-Go^{TM} PCR Beads contained tiny balls which consisted of Taq polymerase, buffers and nucleotides: 2.5 units of puRe Taq polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 nM each dNTP, stabilizers, including BSA (Amersham Pharmacia Biotech.). 0.5 µl

two different 20 nM primers and 24 µl purified DNA were added to the beads. Techne Genius (Techne Inc.) machine was used for amplification. The first cycle was at 95°C for 3 minutes and was followed by 35 cycles: at 95°C for 30 seconds, at 55°C for 30 seconds and at 72°C for 1 minute (during each cycle the last part was 2 seconds longer), after 35 cycles there was a final cycle at 72°C for 10 minutes.

Many different primers and their combinations were used for amplification (figure 1, table 1). Mainly two fungus-specific primers, ITS1F and LR21, were used. These primers amplify the end of 18S rDNA, ITS1, 5.8S rDNA, ITS2 and the beginning of 28S rDNA (figure 1). If PCR reaction failed with these primers, other primers were applied in different combinations

2 µl PCR product was mixed with 1 µl bromophenol blue and run in agarose gel (0.4 g agarose, 40 ml TBA buffer, 40 µl ethidium bromide). The presence of PCR product was visualized under UV light. 1 µl Exo-SAP mixture (Amersham Biosciences) was added to the samples that contained amplified DNA (positive samples). Exo-SAP contains two different enzymes, Exonuclease I and Shrimp Alkaline Phosphatase that degrade primers and redundant nucleotides. Techne Genius machine was used for PCR product purification process. The process was initiated at 37°C (20 minutes) and the inactivation of enzymes at 80°C (15 minutes).

DNA was diluted with 100 µl sterile distilled water and DNA content was measured using GeneQuant pro RNA/DNA Calculator. DNA content in the solution was measured compared to distilled water. The machine also enabled to measure the purity of solution from proteins and humic substances.

2.5 Sequencing

Sequencing was done in the Botanical Institute of the Göteborg University (Sweden) by Dr. Ellen Larsson. Sequencing was carried out using Beckman Coulter machine (Perkin-Elmer Corporation, Applied Biosystem Division). 10 ng DNA solution was set to the holes of micro plates and brought to 10 µl using distilled water. 2 µl primer and 8.0 µl DTCS “Quick Start Master Mix” were added and mixed. Samples were inserted to the PCR machine. Amplification

Table 1. Primers used for PCR and sequencing

Primer	Sequence	Reference
ITS1	TCCGTAGGTGAACCTGCGG	White et al., 1990
ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes ja Bruns, 1993
ITS3	GCATCGATGAAGAACGCAGC	White et al., 1990
ITS4	TCCTCCGCTTATTGATATGC	White et al., 1990
ITS4B	CAGGAGACTTGTACACGGTCCAG	Gardes ja Bruns, 1993
LR21	ACTTCAAGCGTTTCCCTTT	Hopple ja Vilgalys, 1999
LR5	TCCTGAGGGAAACTTCG	Hopple ja Vilgalys, 1999
LR7	TACTACCACCAAGATCT	Hopple ja Vilgalys, 1999
CTB6	GCATATCAATAAGCGGAGG	unknown
TW14	GCTATCCTGAGGGAAACTTC	unknown

process contained 30 cycles: 20 seconds at 96°C, 20 seconds at 50°C, 4 minutes at 60°C. Then 4 µl Stop solution (1.5 M NaO-acetate, 50 mM EDTA) was added into each hole. The mixture was centrifuged and washed four times with cold 95% ethanol and then vacuum dried for 40 minutes. Then 40 µl "Sample Loading" buffer was added and samples were covered with mineral oil (Sigma) and inserted to the sequencing machine. Primer ITS3 was used for sequencing. This primer amplified ITS2 and the beginning of 28S rDNA region (up to 350 nucleotides). For genera *Inocybe* and *Tomentella* additional primer ITS1 or ITS4 was used in order to obtain the sequence for ITS1 region for better species identification. Raw chromatogram files generated by Beckman Coulter machine were sent to Tartu for the further analyses.

The quality of sequences was checked by the author using computer program Sequencer ver 4.2 (GeneCodes Corp.). Errors of the sequences were manually corrected if possible. When sequence quality was very poor, an extra PCR reaction was performed and PCR product sequenced. All sequences (sequence subtypes) that resembled >98% to each other were merged into one sequence type that represents one fungal species and morphotype.

To detect the fungal species, National Center for Biotechnology Information (NCBI), UNITE and EMBL Nucleotide Sequence Database were used. NCBI and UNITE use BLAST algorithm (Altschul *et al*., 1997) and EMBL Nucleotide Sequence Database uses FASTA algorithm

Figure 1. Map of primers used for PCR and sequencing in ITS regions of ribosomal DNA.

(Pearson, 1994) to detect local similarity between query and database sequences. FASTA algorithm was used when similarity between query and database sequence was low, because otherwise the search with BLAST algorithm indicated similarity only for small parts of sequences. In general, ITS regions vary inside the species up to 3% and between the species at least 3.5%. Therefore 97% of ITS2 sequence identity was selected as species recognition criterion.

2.6 Phylogenetic analyses

Due to limitations of the public databases where ITS sequences of many fungal groups are missing, a number of taxa could not be resolved beyond family. For more exact species identification phylogenetic analysis were used. *Inocybe*, *Tomentella* and *Sebacina* were the most problematical genera. Analyses were performed with ITS2 region for *Sebacina* spp. and with ITS1 and ITS2 for *Tomentella* spp. and *Inocybe* spp. Additional sequences were downloaded from NCBI and UNITE database. From every sequence type the sequence of the most frequent and abundant sequence subtype was selected for the analyses. Additional sequences used to identify *Inocybe* species are not published (E. Larsson, unpublished data). Phylogenetic analysis for *Inocybe* was performed by Ellen Larsson (University of Göteborg, Sweden). The selected model HKY85 was implemented during Neighbour Joining distance analyses with Paup 4.0d81 (Swofford, 2003).

Alignment of *Thelephora/Tomentella* sequences was performed using MAFFT ver. 5.6 (Katoh et *al.*, 2002) and then manually improved. The data matrix contained 186 ITS sequences and 674 characters. Fifty-six ITS sequences were obtained from mycorrhizal root tips and 130 sequences from fruitbodies of resupinate thelephoroid fungi. Sequence types 55 and 17 were represented with two sequences. Selection of molecular evolution models was accomplished using Modeltest ver 3.6 (Posada & Crandall, 1998), which found two equally appropriate models. Hierarchical likelihood ratio tests (hLRT) selected transitional model (TIM) with proportion of invariable sites

(I) and rate variation among sites (G). The Akaike information criterion (AIC) selected nearly the same model (TIMef+I+G). The only difference between TIM and TIMef models is that TIM uses unequal base frequencies and TIMef equal frequencies. Selected models were implemented during neighbour joining (NJ) distance analyses with Paup 4.0d81.

Additional *Sebacina* sequences obtained from fruit bodies and ectomycorrhizal root tips were downloaded from NCBI database and aligned manually. Final data matrix contained 45 sequences and 273 characters. Parsimony analyses were performed with Paup 4.0d81. The number of replicates of the heuristic search was 1000 and gaps were treated as the fifth character. Parsimony analyses were selected, because both Neighbour Joining and Maximum Likelihood resulted in poorly resolved phylograms.

2.7 Statistical analyses

Shannon-Wiener function (Krebs, 1999) was used to measure the diversity of ectomycorrhizal fungi in different plots.

$$H' = -\sum_{i=1}^{S} (p_i)(\log_2 p_i)$$

were p_i = proprtion of species i in the sample and S = the number of species.

Mixed ANOVA (analyses of variance) was used to study the effects of plot, horizon, plant community and the interaction of plot and community on species richness of sample, fragment and plot, diversity index, number of root tips and soil parameters. Mixed ANOVA was essential, because fixed factors (horizon, plant community, horizon*community) and random (plot) factors were included in the analyses. As mixed ANOVA produces output only for fixed parameters, Covtest was included to uncover the significance of random factors. Computer program SAS (SAS System for Windows 8.02) was used to perform the analyses at significance level $\alpha = 0.05$.

Soil parameters (excluding pH) were transformed using log function in order to obtain normal distribution of residuals, the assumptions of multi-way ANOVA.

2.8 Ordination

Two different ordination analyses were used to study gradients in the fungal communities. Canonical correspondence analysis (CCA) was used to determine the influence of environmental variables on fungal community structure. Monte Carlo test was used to test the significance of axes. Hypothesis tested was: no structure in the main matrix and therefore no linear relationship between matrices. The number of permutations (runs) was 1000. Detrended correspondence analysis (DCA) was used to detect the influence of horizon, plot and plant community on the structure of ectomycorrhizal fungal community. Analyses were performed with Program PC-ORD ver. 4.01 (McCune & Mefford, 1999).

3. Results

3.1 Identification of roots

Tree roots were difficult to distinguish in Tagamõisa wooded meadow and in forest. Some tree species had specific root morphology that enabled to identify them unambiguously. Ash roots were thicker, paler and non-ectomycorrhizal. Spruce roots were also easy to recognize due to greater thickness, smell and taste of resin. Aspen roots were very fine, pale brown or yellowish and with acrid taste.

Linden, birch, hazel and alder roots were more difficult to distinguish due to reddish colour and polygonal epidermis cells. Linden roots had a mild taste, specific smell and turned red when cut. Hazel roots were slightly paler than other tree roots and the surface of roots was covered with specifically shaped net, which consisted of torn cortex cells. Alder and birch roots were most difficult to distinguish, because usually alder roots had no nodules in Tagamõisa. Alder roots were slightly darker than birch roots and they had bitter taste and strong smell. Birch roots had no taste or smell. Alder roots were more easy to broke than birch roots, which were more elastic.

3.2 Soil parameters

The majority of soil parameters differed significantly between plant communities (Table 2) and soil horizons (table 3); pH was higher in the wooded meadow and in lower soil horizon, whereas all other soil parameters displayed higher values in the forest and upper soil horizon. Plot and interaction between horizon and community did not significantly affect environmental variables. All soil parameters were autocorrelated.

3.3 Morphotypes and fungal species

Of 10 796 ectomycorrhizal root tips studied, 58 and 51 fungal species were detected from the forest and wooded meadow, respectively (table 4). Only 16 morphotypes were found in common for two plant communities. Forty-two fungal species inhabited only forest and 35 species occurred only in the wooded meadow plots. Thirty species were found only form the upper soil horizon and 15 species from the lower soil horizon. Forty-eight species were in common for both

Table 2. Effect of plant community on soil parameters based on mixed ANOVA.

Soil parameter	Wooded meadow vs forest				
	Mean ± SE, Mead.	Mean ± SE, Forest	df	F-value	P-value
pH	6.53 ± 0.18	5.65 ± 0.17	1	11.14	0.0157
P	14.55 ± 4.66	42.33 ± 7.76	1	16.34	0.0068
K	83.97 ± 30.51	209.93 ± 44.93	1	22.11	0.0033
Ca	4636.88 ± 1394.11	10369.25 ± 1391.01	1	11.86	0.0137
Mg	337.74 ± 101.39	859.44 ± 116.59	1	14.71	0.0086
Organic matter	15.7 ± 5.67	48.21 ± 8.04	1	14.54	0.0088

Table 3. Effect of soil horizon on soil parameters based on mixed ANOVA.

Soil parameter	Upper vs lower horizon				
	Mean ± SE, lower	Mean ± SE, upper	df	F-value	P-value
pH	6.4 ± 0.21	5.77 ± 0.21	1	81.52	0.0001
P	16.57 ± 4.72	40.31 ± 8.64	1	21.58	0.0035
K	64.76 ± 17.68	229.14 ± 42.9	1	32.25	0.0013
Ca	6310.13 ± 1818.19	8696 ± 1585.31	1	2.52	0.1632
Mg	457 ±143.51	740.18 ± 130.35	1	5.43	0.0587
Organic matter	20.54 ± 6.85	43.36 ± 9.39	1	15.36	0.0078

horizons. Six ectomycorrhizal fungal species were found only on one root tip and 12 on a single root fragment and 37 in a single plot (table 4).

Morphotypes formed by fungal species of the same family were often remarkably different. Only ectomycorrhizas formed by Sebacinaceae spp. were quite similar to each other (plate 1), possessing yellow or brownish pseudoparenchymatous or plectenhymatous mantle and abundant emanating hyphae (no clamps). Thelephoraceae spp. formed ectomycorrhizas that possessed very different morphology (Plate 2, 3, 4) - distinct colour and structure of fungal mantle and hyphae (with or without clamps, cystidia or rhizomorphs). Cortinariaceae spp. formed mycorrhizas with different morphology (plate 5): distinct colour and structure of mantle, emanating hyphae, cystidia and rhizomorphs present or not. Ectomycorrhizas formed by Russulaceae were yellow or

Table 4. The number of colonized root tips in upper and lower horizon and the number of plots where certain sequence type was found in the forest and wooded meadow.

	Wooded meadow			Forest		
Sequence type	colonised root tips, upper horizon	colonised root tips, lower horizon	Meadow plots	colonised root tips, upper horizon	colonised root tips, lower horizon	forest plots
Cenococcum gephilum	767	129	4	658	749	4
Thelephoraceae 1	132	40	4	10	8	2
Thelephoraceae 5	0	0	0	12	3	1
Thelephoraceae 6	0	0	0	55	0	2
Thelephoraceae 8	2	7	2	15	0	1
Thelephoraceae 9	39	0	2	0	0	0
Thelephoraceae 10	0	0	0	1	4	1
Thelephoraceae 11	0	0	0	4	57	2
Thelephoraceae 12	8	0	1	0	0	0
Thelephoraceae 13	0	0	0	0	2	1
Thelephoraceae 14	0	0	0	8	9	2
Thelephoraceae 17	20	0	2	92	8	1
Thelephoraceae 18	16	0	1	0	0	0
Thelephoraceae 20	2	11	1	0	0	0
Thelephoraceae 21	25	14	2	0	0	0
Thelephoraceae 23	6	16	1	0	0	0
Thelephoraceae 25	6	0	1	9	0	1
Thelephoraceae 26	0	0	0	13	122	1
Thelephoraceae 30	5	0	1	0	0	0
Thelephoraceae 36	0	0	0	0	12	1
Thelephoraceae 38	0	107	1	0	0	0
Thelephoraceae 39	45	0	1	0	0	0
Thelephoraceae 40	6	3	2	0	0	0
Thelephoraceae 41	33	13	1	0	0	0
Thelephoraceae 42	0	5	1	0	0	0
Thelephoraceae 43	10	1	1	0	0	0
Thelephoraceae 44	0	1	1	0	0	0
Thelephoraceae 45	10	0	1	0	0	0
Thelephoraceae 46	14	0	2	0	0	0
Thelephoraceae 47	0	0	0	63	4	1
Thelephoraceae 48	0	0	0	12	0	2
Thelephoraceae 49	0	0	0	3	1	2
Thelephoraceae 50	0	0	0	64	20	1
Thelephoraceae 51	0	0	0	3	28	2
Thelephoraceae 52	0	0	0	4	77	1
Thelephoraceae 53	0	0	0	22	0	1
Thelephoraceae 54	0	0	0	0	11	1
Thelephoraceae 55	0	0	0	13	15	1
Thelephoraceae 56	0	0	0	5	0	1
Thelephoraceae 57	0	0	0	1	0	1
*Inocybe*1	7	0	1	0	1	1
Inocybe 2	0	0	0	0	1	1
Inocybe 3	0	0	0	0	25	1
Inocybe 4	3	4	1	19	31	2

Inocybe 5	4	13	1	0	13	1
Inocybe 6	0	1	1	0	0	0
Inocybe 7	60	19	1	182	565	4
Inocybe 11	2	5	2	72	23	2
Inocybe 16	0	0	0	19	17	1
Inocybe 17	0	0	0	3	0	1
Sebacina 3	0	0	0	0	2	1
Sebacina 4	0	0	0	87	58	3
Sebacina 5	0	0	0	187	26	2
Sebacina 7	8	0	1	142	32	2
Sebacina 8	0	1	1	161	18	2
Sebacina 9	0	0	0	17	0	1
Sebacina 13	21	2	1	0	0	0
Sebacina 14	5	0	1	0	0	0
Sebacina 15	152	0	1	0	0	0
Sebacina 16	0	0	0	1	5	1
Sebacina 17	0	0	0	13	0	1
Sebacina 18	0	0	0	66	0	1
*Genea*1	25	18	4	0	0	0
Pezizales 1	91	0	3	0	0	0
Pezizales 3	6	90	4	2	0	1
Pezizales 4	21	1	3	0	0	0
Pezizales 6	0	21	1	0	0	0
Wilcoxina	11	94	3	0	0	0
Tuber 1	0	0	0	10	20	1
Tuber 2	0	0	0	4	8	1
Hebeloma 2	0	0	0	17	0	1
Hebeloma 3	0	3	1	0	0	0
Hebeloma 4	0	0	0	15	2	2
Hebeloma 5	0	0	0	58	0	1
Hebeloma 6	0	0	0	10	0	1
Hebeloma 7	0	0	1	0	0	0
Alnicola 2	0	0	0	20	33	1
Cortinarius 1	0	0	0	1	0	1
Cortinarius 6	64	0	0	145	113	2
Cortinarius 7	81	1	1	0	0	0
Cortinarius 8	178	553	1	0	0	0
Lactarius pubescens	0	0	3	794	19	4
Lactarius torminosus	127	25	0	178	0	2
Lactarius evosmus	311	904	2	0	0	0
Boletus luridusr	42	7	4	0	0	0
Leccinum 1	26	0	3	168	5	3
Leccinum 2	72	170	1	0	0	0
Melanogaster 2	156	0	1	0	0	0
Paxillus 3	9	0	2	166	19	3
Amphinema byssoides	6	5	1	0	0	0
Unknown 3	0	0	2	0	0	0
Unknown 4	0	0	0	0	7	1
Unknown 5	0	2	0	1	0	1

Table 5. Identified ectomycorrhizal taxa.

Phyla	Order	Family	Genus
Basidiomycota	Thelephorales	Thelephoraceae	*Thelephora, Tomentella, Tomentellopsis*
	Boletales	Boletaceae	*Boletus, Leccinum*
		Paxillaceae	*Paxillus*
	Cortinariales	Cortinariaceae	*Cortinarius, Hebeloma, Inocybe, Alnicola*
	Russulales	Russulaceae	*Lactarius*
	Hymenogastrales	Melanogastraceae	*Melanogaster*
	Aphyllophorales	Atheliaceae	*Amphinema*
	Auriculariales	Sebacinaceae	*Sebacina*
Ascomycota	Pezizales	Tuberaceae	*Tuber*
		Pyronemataceae	*Genea, Wilcoxina*
		Pezizaceae	*Peziza*
	Unknown	unknown	*Cenococcum*

orange, with lactifers (plate 6). Emanating hyphae were always absent and cystidia were present or absent.

Nine and 81 identified fungal species belonged to phyla Ascomycota and Basidiomycota, respectively. Three ectomycorrhizal morphotypes (Unknown 3, 4 and 5) remained unidentified, due to failure in PCR reaction. Identified ectomycorrhizal species belonged to 19 different genera (table 5). Several genera were restricted only to one community. *Boletus, Melanogaster, Amphinema, Genea* and *Wilcoxina* occurred only in the wooded meadow, whereas *Alnicola* and *Tuber* were found only in the forest. *Tomentella* was the most species rich genus (38 spp.), followed by *Sebacina* (12 spp.) and *Inocybe* (10 spp.). *Cenococcum geophilum, Lactarius pubescens* and Thelephoraceae 1 were the most frequent species. *C. geophilum*, *L. pubescens* and *Boletus luridus* colonized the highest propotion of root tips (table 4).

Thirty ectomycorrhizal fungal species were identified based on sequence databases (table 6). Additional species were detected for genera *Inocybe, Tomentella* and *Sebacina* based on phylogenetic analyses.

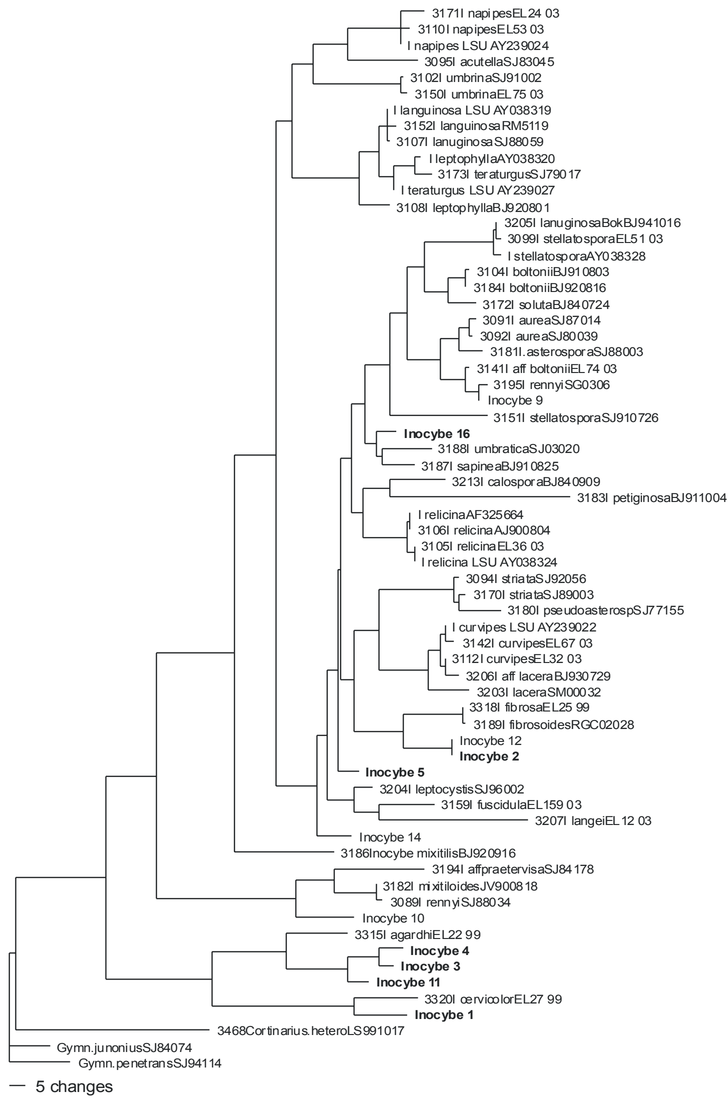

Figure 2. Phylogenetic affinities of ectomycorrhizal sequence types from Tagamõisa to identified Inocybe species based on Neighbour-joining analysis with model HKY85. Names in bold indicate sequence types from Tagamõisa.

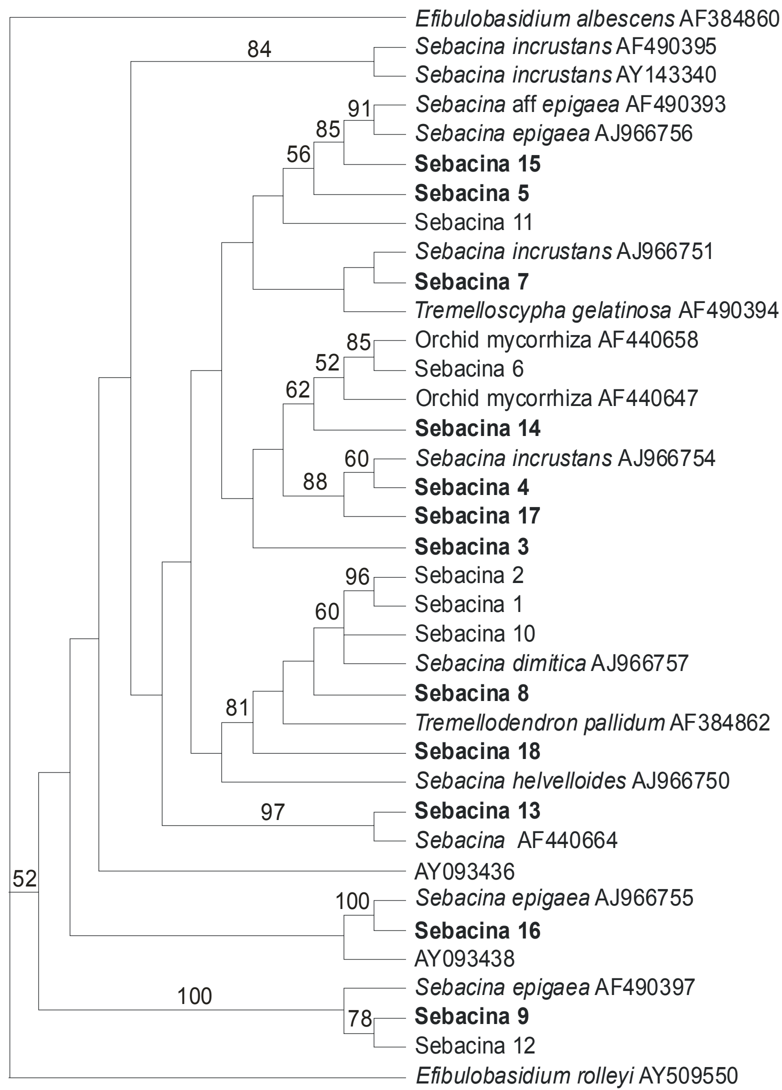

Figure 4. Phylogenetic affinities of ectomycorrhizal sequence types from Tagamõisa to Sebacinaceae spp. based on bootstrap analysis. Bootstrap values above 50% are shown. Names in bold indicate sequence types from Tagamõisa.

Table 6. Molecular identification of ITS2 sequence types according to different sequence databases. <100% similar sequence subtypes are separately queried against sequence databases.

Sequence type	Best match	Sequence subtype	Accession Number	similarity %
Cenococcum gephilum Fr.				
Thelephoraceae 1	*Tomentella coerulaea* (Bres.) Höhn.*	**L151**, TS072, TS077, TS108, TS153, TS223, TS239, TS245	UDB000266	92.3
	Tomentella coerulaea *	TS116	UDB000266	92.8
Thelephoraceae 5	*Tomentella bryophila* (Pers.) M.J. Larssen *	**L191**	UDB000253	93.3
	Tomentella bryophila *	TS500, TS504, TS533	UDB000253	95.9
Thelephoraceae 6	*Tomentella bryophila* *	**L193**, TS350, TS468, TS485	UDB000253	91.8
Thelephoraceae 8	*Tomentella pilosa* (Burt.) Bourdot & Galzin	**L203a**, TS173, TS225, TS398	AJ421252	100
Thelephoraceae 9	*Tomentella viridula* Bourdot & Galzin	**L206**, TS182, TS272	AF272914	96.9
Thelephoraceae 10	*Tomentella atramentaria* Rostr. *	**L225**	UDB000235	91.2
	Tomentella atramentaria *	TS379	UDB000235	91.6
Thelephoraceae 11	*Tomentella cinerascens* (P.Karst.)Hoehn. & Litsch. *	**L260**, TS357, TS460	UDB000232	98.7
Thelephoraceae 12	*Tomentella badia* (Link) Stalpers *	**L267**	UDB000238	92.9
	Tomentella badia *	TS289	UDB000238	93
Thelephoraceae 13	*Tomentella lapidum* (Pers.) Stalpers	**L272**, TS391	AF272941	90.1
Thelephoraceae 14	*Thelephora anthocephala* (Bull.: Fr.) Pers.	**L280**, TS365, TS451, TS427	AF272927	95
Thelephoraceae 17	*Tomentella lilacinogrisea* Wakef. *	**L339**, TS141, TS275, TS516, TS546	UDB000272	99.6
Thelephoraceae 18	*Tomentella umbrinospora* MJ Larsen	**L360**, TS251	AF272920	99.5
Thelephoraceae 20	*Thelephora caryophyllea* Schaeff.) Fr. *	**L377**, TS125	UDB000119	92
Thelephoraceae 21	*Tomentella umbrinospora* Larssen.*	**L378**, TS156, TS177a	UDB000233	94.5
Thelephoraceae 23	*Tomentella bryophila* *	**L369**, TS283	UDB000253	92.4
Thelephoraceae 25	*Tomentella lapida* (Pers.)Stalpers *	**L415**, TS111, TS486	UDB000250	97.3
Thelephoraceae 26	*Tomentella viridula* Bourdot & Galzin	**L429**	AF272914	99.5
Thelephoraceae 30	*Tomentella punicea* (Alb.& Schw.: Fr.) Schröt. *	**L473b**, TS093	UDB000959	99.8
Thelephoraceae 36	*Tomentella bryophila* *	**L551**, TS526, TS536, TS538, TS495, TS496	UDB000253	93.3
Thelephoraceae 38	*Thelephora caryophyllea* *	**TS071**, TS096	UDB000119	94.7
Thelephoraceae 39	*Tomentella lateritia* Pat.	**TS100**	AF272926	88.8
Thelephoraceae 40	*Tomentella atramentaria*	**TS152**	AF272904	89.8

Thelephoraceae 41	*Tomentella lapida* *	**TS197**, TS226	UDB000250	96.4
Thelephoraceae 42	*Tomentella viridula*	**TS210**	AF272914	93.3
Thelephoraceae 43	*Tomentella bryophila* *	**TS221**	UDB000253	91.5
Thelephoraceae 44	*Tomentella lapida*	**TS242**	AF272941	90.6
Thelephoraceae 45	*Tomentella ramosissima* (Berk. & Curtis) Waekf.	**TS271**	TRU83480	96.9
Thelephoraceae 46	*Tomentella lapida* *	**TS279**	UDB000249	96.9
Thelephoraceae 47	*Tomentella subclavigera* Litsch.	**TS309**, TS337, TS551	AF272939	93.6
Thelephoraceae 48	*Tomentella atramentaria* *	**TS318**, TS503	UDB000955	97.3
Thelephoraceae 49	*Tomentella lateritia* *	**TS328**, TS426	UDB000963	92.9
Thelephoraceae 50	*Tomentella bryophila* *	**TS340**	UDB000253	91.8
Thelephoraceae 51	*Thelephora anthocephala* (Bull.: Fr.) Pers.	**TS341**, TS364	AF272927	99.6
	Thelephora anthocephala	TS479, TS481	AF272927	99.6
Thelephoraceae 52	*Tomentella ellisii* (Sacc.) Jülich & Stalpers *	**TS452**	UDB000219	97.7
Thelephoraceae 53	*Tomentella subclavigera*	**TS470**, TS471, TS575	AF272939	92.9
Thelephoraceae 54	*Tomentella botryoides* (Schwein.) Bourdot & Galzin *	**TS515**	UDB000255	94.8
Thelephoraceae 55	*Tomentella lapida*	**TS521**, TS545, TS597, TS547	AF272941	91.6
Thelephoraceae 56	*Tomentella terrestris* Berk. & Br.)MJLarsen	**TS512**	AF272901	99.1
Thelephoraceae 57	*Tomentellopsis echinospora* (Ellis) Hjortstam	**TS502**	AJ410758	100
*Inocybe*1	*Inocybe quetiodor* °	**L167**, TS080	EL115_04	100
Inocybe 2	*Inocybe flocculosa* (Berk.) Sacc.	**L183**, TS508	AY228534	66.8
Inocybe 3	*Inocybe rimosa* (Bull:Fr.) Gill. *	**L195**, TS506, TS540, TS541	UDB000103	94.0
Inocybe 4	*Inocybe rimosa* *	**L201**	UDB000103	88.3
	Inocybe rimosa *	TS494	UDB000103	90.4
	Inocybe rimosa *	TS118, TS380, TS390	UDB000103	90.4
Inocybe 5	*Inocybe nitidiuscula* (Britz.) Sacc.	**L234**, TS115, TS491, TS493	INI534934	77.0
Inocybe 6	*Inocybe maculta* Boud.	**L243**, TS113	AJ534933	72.4
Inocybe 7	*Inocybe maculata* °	**L251**, TS076, TS445, TS439, TS344, TS441, TS482, TS449, TS360, TS430, TS444	AJ534933	100
Inocybe 11	*Inocybe rimosa* *	**L424**, TS168, TS264, TS446, TS492, TS421, TS447	UDB000103	86.8
Inocybe 16	*Inocybe godeyi* Gill. *	**TS347**, TS363	UDB000101	91.2
Inocybe 17	*Cortinarius subtortus* (Pers.: Fr.) Fr.	**TS543**	AY174859	65.1
Sebacina 3	*Sebacina epigaea* (Berk. & Br.) Neuh. *	**L192**, TS527	UDB000977	88.2
Sebacina 4	*Sebacina epigaea* *	**L194**	UDB000975	95.5
	Sebacina epigaea *	TS306, TS346	UDB000975	95
	Sebacina epigaea *	TS539	UDB000975	95.5
Sebacina 5	*Sebacina epigaea* *	**L199**, TS369, TS370, TS518	UDB000977	90.7
Sebacina 7	*Sebacina incrustans* (Pers. ex Fr.) Tul. *	**L271**, TS143, TS375, TS322	UDB000979	89.4
Sebacina 8	*Sebacina dimitica* Oberw. *	**L278**, TS133, TS297,	UDB000974	93.4

		TS478, TS490		
Sebacina 9	*Sebacina epigaea*	**L407**, TS386	AF490397	86.5
Sebacina 13	*Sebacina helvelloides* (Schwein.) Burt *	**TS204**	UDB000972	89.1
Sebacina 14	*Tremellodendron schweinitzii* (Pk.) Atk.	**TS206**	AY296258	86.3
Sebacina 15	*Sebacina epigaea* *	**TS246**, TS288	UDB000977	92.8
Sebacina 16	*Sebacina incrustans* (Pers.: Fr.) Tul.	**TS442**, TS462	AF490395	68.7
Sebacina 17	*Sebacina epigaea* *	**TS487**	UDB000975	65.1
Sebacina 18	*Tremellodendron pallidum* (Schwein) Burt.	**TS5213**	AF384862	95.2
*Genea*1				
Pezizales 1				
Pezizales 3				
Pezizales 4				
Pezizales 6	*Peziza michelii* (Boud.) Dennis. *	**L473**, TS286	UDB000986	100
Wilcoxina	*Wilcoxina mikolae* (Yang & Wilcox) Yang & Korf	**L343**, TS089, TS285	AY219841	80.4
	Wilcoxina mikolae	TS104	AY219841	80
Tuber 1	*Tuber rapaeodorum* Tul.	**L546**, TS342	AJ557525	100
Tuber 2	*Tuber rufum* Pico.	**L330**, TS343	AF106892	65.5
Hebeloma 2	*Hebeloma hiemale* Bres.	**L282**, TS489	AF124669	100
Hebeloma 3	*Hebeloma ammophilum* Bohus.	**TS253**	AY308585	100
Hebeloma 4	*Hebeloma nigellum* Bruchet	**TS467**	AY311524	99.5
Hebeloma 5	*Hebeloma incarnatulum* Smith.	**L208**	AF124684	100
	Hebeloma velutipes Bruchet.	TS472	AF123676	100
Hebeloma 6	*Hebeloma aestivale* Petersen & Vesterh.	**TS514**	TRU83480	98.6
Hebeloma 7	*Hebeloma cavipes* Huijsm.	**TS158**	AF124670	95.3
Alnicola 2	*Alnicola bohemica* (Vel.) Kühn. & Mre.	**TS440**	AF124712	83.0
Cortinarius 1	*Cortinarius dionyseae* Henry	**TS524**	AY174813	90.0
Cortinarius 6	*Cortinarius pulchellus* Lange	**L398**, TS294, TS301, TS338	AY083192	91.0
Cortinarius 7	*Cortinarus saturninus* (Fr.) Fr.	**TS142**	AY083189	98.3
Cortinarius 8	*Cortinarius atrocoeruleus* (Moser) Moser	**TS184**	AY083178	97.8
Lactarius pubescens	*Lactarius pubescens* Fr.	**L180k**, TS419	AY336958	100
	Lactarius pubescens	TS078, TS240, TS324	AY336958	99.2
	Lactarius tesquorum	TS146, TS196	AY336955	100
	Lactarius pubescens	TS123, TS395	AY336958	99.6
	Lactarius pubescens	TS122, TS126	AY336958	99.6
Lactarius torminosus	*Lactarius torminosus* (Schaeff.: Fr.) Pers.	**L273**, TS465, TS476, TS554	AY336959	98.1
Lactarius evosmus	*Lactarius evosmus* Kühn. & Romagn. *	**L357**, TS154, TS170, TS183, TS207	UDB000983	100
Boletus luridus	*Boletus luridus* Schaeff: Fr.	**L161**	AY278766	99.6
	Boletus luridus	**TS070**, TS145	AY278766	100
Leccinum 1	*Leccinum rigidipes* Orton	**L353a**, TS202	AF454584	100
Leccinum 2	*Leccinum variicolor* Watl.	**L442**	AF139706	98.8
Melanogaster 2	*Melanogaster variegatus* (Vittad.) Tul.	**TS088**, TS095	AJ555534	99.6

Paxillus 3	*Paxillus involutus* Fr.	**TS073**, TS499	AY585921	100
Amphinema byssoides	*Amphinema byssoides* (Pers. : Fr.) Erikss.	**L299**, TS079	AY838271	100
Unknown 3				
Unknown 4				
Unknown 5				

Identifications based on UNITE or other database are marked with asterisks and circles, respectively. Identifications without marks are based on NCBI database.
Sequences in bold are obtained from the most frequent and abundant submorphotype.

Only two *Inocybe* species were identified based on databases (table 6)- *I. quetiodor* (*Inocybe* 1) and *I. maculata* (*Inocybe* 7) (table 6). Phylogenetic analysis did not enable to identify additional species (figure 2), but revealed that *Inocybe* 1 is closely related to *Inocybe cervicolor. Inocybe* sp11, sp3 and sp4 are closely related to each other and to *Inocybe agardhi*. Also *Inocybe* 12 and 2 are closely related species and related to *I. fibrosa* and *I. fibrosoides*. Some conspecific fruit body sequences of *Inocybe* spp. were situated in unrelated branches on the phylogenetic tree, possibly representing misidentified taxa.

Phylogenetic analysis for Thelephoraceae confirmed identifications based on sequence databases – 12 ectomycorrhizal fungi were identified on a species level (table 6, figure 3). Phylogenetic analyses revealed that *Tomentella* and *Thelephora* were paraphyletic. One additional species was identified based on phylogenetic alalysis (figure) - Thelephoraceae 46 is probably *T. ferruginea* (Pers.) Pat.

No *Sebacina* species were identified based on sequence databases due to lack of fruit body sequences. Phylogenetic analysis for Sebacina revealed no additional identified species (figure 4). Also on this tree sequences obtained from same species situated in different places. Phylogram reveal that Sebacinaceae 14 is related with species that were isolated from orchid roots.

3.4 Structure of ectomycorrhizal fungal community

The mean species richness per plot, per sample and per fragment were significantly higher in the upper horizon ($F_{1,15}$=10.36, p=0.018; $F_{1,15}$= 22.5, p= 0.003; $F_{1,15}$=10.89, p=0.016, respectively) Species richness was not significantly influenced by community and plot. The number of root tips per root fragment was not significantly affected by soil horizon, plot and community. Diversity

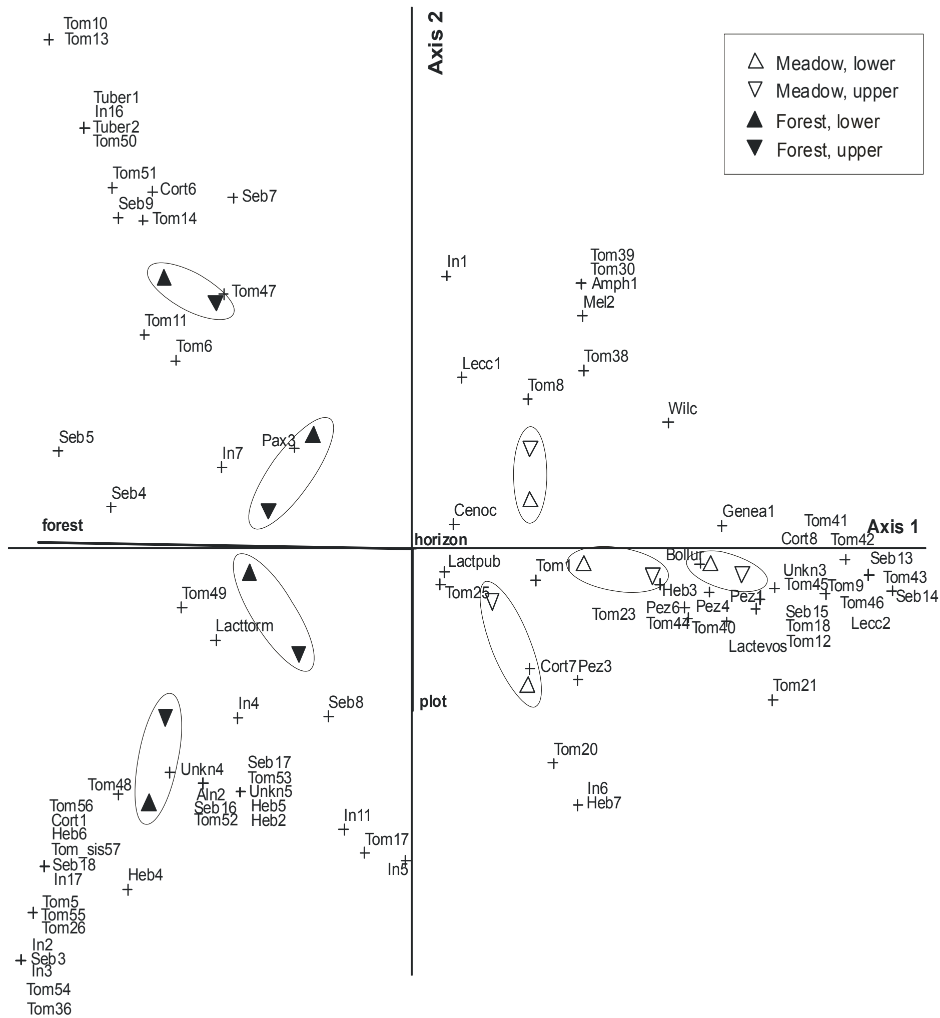

Figure 5. Detrended correspondence analysis based on resiprocal averaging of plots, and species frequency (crosses). Samples were pooled by plot and horizon. Different horizons of plots are indicated with trangles (see legend.)Different soil horizons of the same plot are encircled. Tom, Tom_sis (Thelephoraceae), Seb (*Sebacina*), In *(Inocybe*), Pez *(Pezizales*), Lecc *(Leccinum*), Cort *(Cortinarius*), Mel *(Melanogaster*), Aln *(Alnicola*), Heb *(Hebeloma*), Pax *(Paxillus*),Wilc *(Wilcoxina*), Amph *(Amphinema*), Cenoc *(Cenococcum geophilum*), Lactpub *(Lactarius pubescens*), Lactevos *(Lactarius evosmus*), Bollur (*Boletus luridus*), Unkn (Unknown).

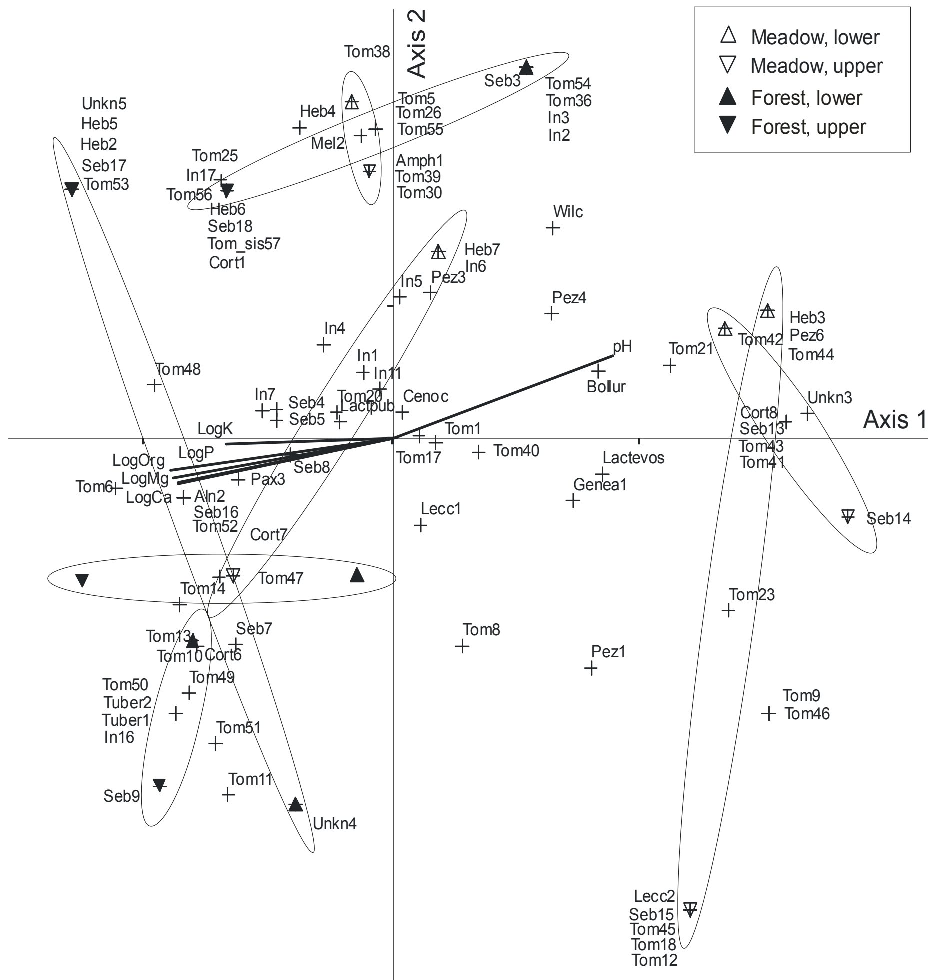

Figure 6. Canonical correspondence analysis indicating the relationship between species and soil variables. Samples were pooled by plot and horizon. Different horizons of plots are indicated with trangles (see legend), species with crosses and effects of soil parameters with arrows. Different horizons of the same plots are encircled. Tom, Tom_sis (Thelephoraceae), Seb (*Sebacina*), In (*Inocybe*), Pez (*Pezizales*), Lecc (*Leccinum*), Cort (*Cortinarius*), Mel (*Melanogaster*), Aln (*Alnicola*), Heb (*Hebeloma*), Pax (*Paxillus*)Wilc (*Wilcoxina*), Amph (*Amphinema*), Cenoc (*Cenococcum geophilum*), Lactpub (*Lactarius pubescens*), Lactevos (*Lactarius evosmus*), Bollur (*Boletus luridus*), Unkn (Unknown).

index was significantly higher in the upper soil horizon ($F_{1,15}$= 7.98, p= 0.03), but it was not affected by community and plot.

Spatial distribution of ectomycorrhizal fungal species was strongly affected by community and plot, whereas influence of horizon was neglible (figure 5). Axis 1 described 35.3% and Axis 2 described 14.3% of total variation with eigenvalues of 0.678 and 0.437, respectively.

Spatial distribution of fungal species was slightly influenced by soil parameters (figure 6). Axis 1 (eigenvalue 0.544, p= 0.001) and axis 2 (eigenvalue 0.443, p= 0.012) described 11.1% and 9.0% of total species variation, respectively. Here, P-values indicate the significance of the proportion of randomised runs with an eigenvalue greater than or equal to the observed eigenvalue.

4. Discussion

Wooded meadows are species rich plant communities. Mowing is probably the main factor supporting high species richness. Mowing reduces competition between plants and enables more different species to coexist (Kull & Zobel, 1991). Moderately fertile calcareous soil may be responsible for increased species richness. Fertilization decreases species richness, because high fertility favours fast growing plant species, which attain dominance and displace slow-growing species (Kull & Zobel, 1991). In this study I found high ectomycorrhizal fungal species richness in Tagamõisa wooded meadow (51 species). Very often only 20-40 or even less ectomycorrhizal species have been detected inhabiting certain plant community (Heslin *et al*., 1992; Horton & Bruns, 2001) although sampling was often carried out in several months or years.

While removal of trees is usually positive factor for plant species (more light) it is negative factor for ectomycorrhizal fungi. Removal of host plants decreases the number of ectomycorrhizal fungi (Byrd *et al*., 2000). Although wooded meadows are more sparsely covered with trees, the number of ectomycorrhizal tree species is still high, which enables ectomycorrhizal fungi with different host preference to coexist.

Wooded meadows are very rich in econiches. Groups of trees and shrubs vary with open areas creating different light and moisture conditions. Also herb and moss layer is mosaic - among vascular plants there are patches with mosses that increase the number of niches. Therefore fungal species with different moisture demands can inhabit wooded meadows. O'Dell *et al*. (1999) showed that different fungi prefer different moisture conditions when associated with the same host plant, hence great variety of econiches increases fungal species richness.

Calcareous soil is the third important factor affecting plant species richness. Species richness depends on species pool, the consortium of species that may potentially grow in a certain community (Zobel 1992; Pärtel & Zobel, 1999). The impact of soil pH on species richness depends on whether the species pool has evolved on soils of high or low pH (Pärtel, 2002). Probably the nearest species pool originates from high pH soil, as calcareous soils are more plant species rich in Estonia. After glacial plant species predominantly recolonized continental land from south. Calcareous soils dominate the Mediterreanean region, the major refugium. The species pool hypothesis may apply to fungi similarly, because several studies have revealed

smaller ectomycorrhizal fungal diversity in conifer forests (Horton & Bruns, 2001) where soil pH is lower.

Ectomycorrhizal fungal species composition was different between the forest and wooded meadow according to DCA. The proportion of common species was small compared to unique species. The fungal community in different horizons of the same plot was more similar than between plots. Almost half of the species were found only in a single plot, which explains the difference of fungal community between the plots. Similar distribution patterns were observed by Izzo *et al.* (2005) with 71% of ectomycorrhizal species occurring only once. Horizon had no impact on species composition, although soil parameters differed significantly between horizons. There were more species common to both horizons than species found in a single horizon. As soil parameters and species composition were different between forest and wooded meadow one might conclude that differences in fungal communities are due to soil parameters. Baar and Elferink (1996) found that chemical composition of the soils affects significantly ectomycorrhizal development. However, CCA demonstrated that differences in spatial distribution of fungal species in Tagamõisa are not derived from soil parameters. Rosling *et al.* (2003) also found no relationship between soil parameters and ectomycorrhizal fungal species composition.

In addition to measured soil parameters, several other factors differ between forests and wooded meadows. The herb layer is continuous and very species rich in Tagamõisa wooded meadow, while forest herb layer contains mainly *Carex* species and herb cover is low. Litter layer is thicker in forest than in wooded meadow. Herb layer is removed from the meadow by mowing, which inhibits the development of thick litter layer. Both grasses and litter affect fungal communities via allelopathy, competition and differential quality (Rose *et al*., 1983; Baar & Vries, 1995; Hashimoto & Hyakumachi, 1998). The water regime differs between the forest and wooded meadow. Forest floor is flooded in spring, but not in wooded meadow. Decreased organic content and its moisture retaining capacity, and exposure to the sun make soil of the wooded meadow more susceptible to drought, which also affects fungal communities. It remains unknown, which of these factors most affects the ectomycorrhizal fungal species composition in Tagamõisa.

I assumed that species richness is higher in the forest and upper horizon due to greater host plant root density, but species richness was significantly higher only in the upper soil horizon with no

difference between the meadow and forest and between plots. Organic soil contains more fungal species and tree roots (Gardes & Bruns, 1996; Dickie *et al*., 2002; Tedersoo *et al*., 2003; Hirose *et al*., 2004) and the species composition is often different in deep soils (Dickie *et al*., 2002; Rosling *et al*., 2003). In this study, fungal species composition did not differ between upper and lower horizon.

Nutrient concentrations were lower in the wooded meadow than forest. Nutrient-poor ecosystems usually comprise more ectomycorrhizal species than nutrient-rich ecosystems (Douglas *et al*., 2005). Accordingly, wooded meadows should comprise more species than forests, but I found no difference in species richness between the two communities. The wooded meadow was slightly less species rich probably due to sparse distribution of host plants, which may cause sparse distribution of roots in soil (personal observations; not tested).

Cenococcum geophilum was the most abundant ectomycorrhizal fungal species in wooded meadow and forest. *C. geophilum* is an ascomycete lacking sexual structures (LoBuglio *et al*., 1996). It reproduces via mycelium and sclerotia, but it is still the most common and widespread ectomycorrhizal fungus in the world. It inhabits both young and mature stands and is associated with different host species (Richard *et al*., 2005). *C. geophilum* is absent from communities where large-scale disturbance level has been low for a long time (300 years; Douglas *et al*., 2005). Many studies have revealed *C. geophilum* as a dominant species (Kranabetter, 1999; Izzo *et al*., 2005) and there is evidence that this fungus forms large genets (LoBuglio & Taylor, 2002). This may explain why *C. geophilum* is so abundant. Conversely, a small volume of soil may contain many genetically different *C. geophilum* strains (Jany *et al*., 2002; Wu *et al*., 2005). *C. geophilum* and its ectomycorrhizas were suggested to survive severe drought (Piggott, 1982). Therefore trees associated with *C. geophilum* may benefit from increased drought resistance.

Seabcina was the second most species rich genus in this study. The family Sebacinaceae formerly belonged to Auriculariales, which included mostly saprotrophic heterobasidiomycetes with longitudinally septate basidia (Hansen & Knudsen, 1997). Therefore, Sebacinaceae spp. were considered to be saprotrophic or parasitic fungi for a long time (Selosse *et al*., 2002). These fungi form soft or tough resupinate, gelatinous, waxy or coriaceous fruit bodies (Hansen & Knudsen, 1997). Recent molecular phylogenetic studies have revealed that Sebacinaceae does not belong to Auriculariales (Weiss & Oberwinkler, 2001). Therefore, Weiss *et al*. (2004) suggested creating a

new order, Sebacinales, to separate root-associated Sebacinaceae from saprobic or parasitic Auriculariales. Sebacinaceae spp. form different types of mycorrhizas, including orchid mycorrhiza, ectomycorrhiza, ericoid mycorrhiza (Weiss *et al.*, 2004). Some species form both ecto- and orchid mycorrhiza that enable orchids to derive photosynthetically fixed carbon from trees via mycorrhizal fungi (Selosse *et al.*, 2002; McKendrick *et al.*, 2002). *Sebacina* 14 detected from Tagamõisa was closely related to a fungus isolated from orchid roots, supporting this theory. Several studies have revealed Sebacinaceae as ectomycorrhizal fungi with different tree and shrub species (Glen *et al.*, 2002; Selosse *et al.*, 2002; Urban *et al.*, 2003), including *Carpinus betulus* L.*, C. avellana, T. cordata, Fagus sylvatica* L.*, Eucalyptus marginata* Donn ex SM.*, Picea abies* (L.) Karst.*, Q. robur*. Therefore, sebacinoid species probably possess a broad host range. *Sebacina* spp. forming ectomycorrhiza with *B. pubescens* was reported for the first time in this study.

The most species rich genus was Tomentella, which belong to Thelephoraceae. Thelephoraceae spp. were also for the long time considered saprobes, because they form resupinate fruit bodies under dead branches or litter (Kõljalg *et al.*, 2000). The ability to form ectomycorrhiza was discovered lately, but it seems that they are common ectomycorrhiza formers in different plant communities (Kõljalg *et al.*, 2000; Tedersoo *et al.*, 2003; Haug *et al.*, 2005). In this study almost half of the species belonged to Thelephoraceae. Unlike Sebacinaceae spp., Thelephoraceae spp. form ectomycorrhizas with different morphology, but the majority of them possess melanized hyphae (Kõljalg *et al.*, 2000), which complicates identification of different species. Most of Thelephoraceae spp. were rare, occurring in a few samples. Thelephoraceae sp1 was the only species found from most of the samples, but it colonized only 1.77% of total root tips. Presumably most Thelephoraceae spp. possess clumped distribution, which makes them difficult to detect. Therefore, they are usually reported as rare species (Kõljalg *et al.,* 2000).

Many species belonged to genus *Inocybe* (Cortinariaceae). Unlike Thelephoraceae and *Sebacina, Inocybe* species form stipitate fruit bodies. The majority of *Inocybe* spp. possess small bell-shaped cap (Leisner & Kalamees, 1987). Macroscopical and microscopical features of fruit bodies are invariable and therefore the systematics is complex and little studied. Public sequence databases contained only a few sequences of *Inocybe* spp. and therefore only one species was identified based on NCBI data (*I. maculata*). Another species (*I. quetiodor*) was identified based

on personal database of E. Larsson. I found nine different species (sequence types) by comparing sequences with each other. Phylogenetic analyses did not enable to identify any additional species. Seven species were found only from one to three plots. Therefore, it is possible that *Inocybe* spp. possess also quite clumped distribution and the actual number of species represented in wooded meadow and forest is probably greater than nine. According to Leisner and Kalamees (1987) there are 71 *Inocybe* species in Estonia, but presumably the number of species is much higher.

I used morphotyping and molecular methods to detect ectomycorrhizal fungal species. Separation into morphotypes was supported by sequencing, but different morphotypes sometimes gave identical sequences and some morphotypes comprised ectomycorrhizas formed by different species. Therefore, identification of fungal species based only on morphological features may lead to over- or underestimation of actual species richness. Molecular methods (PCR and sequencing) allowed me to distinguish between different fungal taxa, but it was problematical to identify ectomycorrhizal fungi on a species level, because databases contained too few ITS sequences. Thelephoraceae spp., *Inocybe spp.* and *Sebacina spp.* were the most difficult to identify. Phylogenetic analyses were slightly more effective to identify species or their relations with other identified species. Although, phylogenetic trees revealed that identification of *Inocybe* spp. and *Sebacina* spp. based on morphological and anatomical features of fruit bodies is insufficient, because some fruit body sequences were situated in unrelated branches on the phylogenetic tree.

Identification of tree roots based on morphological features was quite effective to distinguish between roots of different plant species, but it was also very time consuming. Several other DNA-based methods are used for more precise and rapid identification (Brunner *et al*., 2001; Kennedy *et al*., 2003).

Tagamõisa wooded meadow and adjacent forest are very rich in ectomycorrhizal fungal species probably due to high diversity of host plants, calcareous soil and heterogeneity of environmental variables. Ectomycorrhizal species composition is different between the two plant communities, indicating the influence of land management. Wooded meadows are unique ecosystems and additional studies are needed to make conclusions for fungal species richness in Estonian wooded meadows in general.

Summary

The aims of this study were to describe and identify ectomycorrhizal morphotypes on birch in a wooded meadow and neighbouring forest using molecular methods. The additional purposes were to compare fungal species richness between upper and lower soil horizons and plant communities, and to study the distribution of fungal species according to soil parameters. The field work took place in Tagamõisa wooded meadow, The Isle of Saaremaa, in August and September, 2003. Four plots were established around individual downy birch (*Betula pubescens*) trees in the wooded meadow and adjacent forest. Five root samples were taken around each tree. Every sample contained two subsamples: one from upper and the other from lower soil horizon. Plant community and plot were the main factors affecting spatial distribution of fungal species. Both horizon and soil parameters explained negligible variance of the fungal community. The most species rich genera were *Tomentella*, *Sebacina* and *Inocybe*. *Cenococcum geophilum*, *Lactarius pubescens* and *Thelephoraceae* 1 were the most frequent species. The ectomycorrhizal fungal community is diverse in Tagamõisa wooded meadow and adjacent forest probably due to high diversity of host plants, calcareous soil and heterogeneity of environmental variables.

Kokkuvõte

Töö eesmärgiks oli kirjeldada kase juurtel olevad ektomükoriisa morfotüübid ning määrata seensümbiontide liigid kasutades molekulaarseid meetodeid, võrrelda ektomükoriissete seente liigirikkust erinevate mulla horisontide ja taimekoosluste vahel. Uuriti ka liikide ruumilist jaotumist taimekoosluse, proovipaiga, mulla horisondi ja mullaparameetrite järgi. Uurimustöö viidi läbi Saaremaal, Tagamõisa puisniidul, 2003. aasta augustis ja septembris. Puisniidult ja metsast valiti välja 4 kaske. Iga kase ümbert võeti viis proovi kahest erinevast mullahorisondist. Kokku kirjeldati kase juurtelt 93 ektomükoriisa morfotüüpi, millest 51 kirjeldati puiniidult ja 58 metsast. Dominantliigid olid *Cenococcum geophilum*, *Thelephoraceae* 1, *Lactarius pubescens*, mis esinesid peaaegu kõikides proovipaikades. Kõige rohkem esines liike perekondadest *Sebacina*, *Inocybe* ja sugukonnast lehternahkiselised (*Thelephoraceae*). Liigirikkus oli suurem ülemises mullakihis. Seeneliikide ruumilist jaotmist mõjutas kõige tugevamini taimekooslus ja proovipaik. Tagamõisa puisniit ja mets on ektomükoriisaseente poolest väga liigirikkad, mida arvatavasti põhjustab lubjarikas muld, peremeestaimede ning keskkonnatingimuste mitmekesisus.

Acknowledgements

I thank my supervisor prof. Urmas Kõljalg. I also thank Leho Tedersoo for all his help and support and Ellen Larsson for her help in sequencing and phylogeny analyses. I am grateful to workgroup who helped me during the field work.

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Databases

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