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# Ectomycorrhizal fungal communities of pedunculate and sessile oak seedlings from bare-root forest nurseries

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**Abstract** In this study, we present the detailed molecular investigation of the ectomycorrhizal (ECM) community of *Quercus petraea* and *Quercus robur* seedlings grown in bare-root forest nurseries. In all tested oak samples, mycorrhizal colonization was nearly 100%. Morphological observation and molecular investigations (sequencing of fungal ITS rDNA) revealed a total of 23 mycorrhizal taxa. The most frequent and abundant fungal taxa were *Hebeloma sacchariolens*, *Tuber* sp., and *Peziza* sp.; from the detected fungal taxa, 20 were noted for *Q. petraea* and 23 for *Q. robur*. Depending on the nursery, the species richness of identified ECM fungal taxa for both oak species ranged from six to 11 taxa. The mean species richness for all nurseries was 5.36 and 5.82 taxa per *Q. petraea* and *Q. robur* sample, respectively. According to the analysis of similarity, ECM fungal communities were similar for *Q. petraea* and *Q. robur* ($R$=0.019; $p$=0.151). On the other hand, detected fungal communities were significantly different between nurseries ($R$=0.927; $p$<0.0001). Using the Spearman rank correlation, it was determined that the ectomycorrhizal diversity (in terms of richness, the Shannon diversity, evenness, and Simpson dominance indices) is significantly related to the soil parameters of each nursery. We conclude that individual nursery may be considered as separate ecological niches that strongly discriminate diversity of ECM fungi.

**Keywords** Ectomycorrhiza · Quercus petraea · Quercus rubra · Diversity

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## Introduction

Pedunculate oak (*Quercus robur* L.) and sessile oak (*Q. petraea* [Matt.] Liebl.) are among the major managed tree species in Europe. The geographic range of European deciduous and mixed temperate oak forests extends from the western edge of Europe (northern Portugal and Spain) to the Ural Mountains and adjoins the taiga from the south (Jalas and Suominen 1976). In Poland, oak woodlands (with maple, sycamore maple, elm, and ash) cover 6.2% of forest area, with oaks considered the most ecologically and economically important woody plant. Furthermore, the significance of native oaks for Polish forestry is expected to rise. The Polish National Forest Strategy predicts a growth of forest cover from 28.5% to 33% by year 2050 with the primary focus which is the increased contribution of deciduous tree species (from 22% to 33%). Oaks are critically dependent on ectomycorrhizal (ECM) fungi for optimal development under natural conditions (Trappe 1962; Meyer 1973). Mycorrhizas increase root absorptive area, enhance nutrient acquisition, facilitate water uptake, and enhance tolerance to adverse soil conditions and reduce the effects of soil-borne pathogens on tree roots (Smith and Read 2008). Studies on ECM communities of pedunculate and sessile oaks in European ecosystems are not very numerous and until now, have focused on surveying old forest stands based on the observations of fruiting bodies or superficial morphotyping (Dominik 1951; Pachlewski and Gągalska 1953; Voiry 1981; Driessche and Pierart 1995; Causin et al. 1996; Kovacs et al. 2000). As the level of sophistication in sampling and identifying ECM fungi has increased over time, some papers have provided more detailed descriptions of oak morphotypes and DNA information or taxonomic identification (Agerer 1987–2008; Valentine et al. 2004; Mosca et al. 2007; Urban et al. 2008).

Recently, a large variety of ectomycorrhizal species colonized oak roots in reforested areas and mature stands of North American oak species have been shown (Morris et al. 2008; Walker et al. 2008; Moser et al. 2009). However, much less information exists about ECM fungi associated with oaks during the early stages of seedling development in natural stands and nurseries (Garbaye et al. 1986; Southworth et al. 2009). Ectomycorrhizal fungi, naturally colonizing seedlings in nurseries, are indispensable for the establishment and survival of young trees for several years after outplanting as they increase the capacity of tree seedlings to capture resources quickly and are necessary to resist pest and pathogens and survive climatic stress (Perry et al. 1987). The natural regeneration of oaks is rather scarce because of insufficient area for mature oak stands and irregular acorn production, which may range from bumper acorn crops in some years to poor or no crops in others. Therefore, for establishment of new stands, oak seedlings are grown for 2–3 years in forest nurseries. In Polish bare-root nurseries, oak seedlings constitute the majority of production of deciduous tree species (almost 270 million seedlings per year) (Zajączkowski 2008). Nursery seedlings are mainly used for reforestation and also afforestation of post-agricultural areas where no ECM inoculum is usually present. In the latter case, the importance of a high diversity of ECM fungi established in nurseries is especially pronounced. Recently, the usefulness of PCR-based methods for identifying mycorrhizal associates of different tree species produced in forest nurseries was demonstrated (Menkis et al. 2005; Iwański et al. 2006; Rudawska et al. 2006; Trocha et al. 2006; Aučina et al. 2007; Leski et al. 2008), but none of the previous studies has focused on ECM fungal communities of pedunculate and sessile oak seedlings.

In this study, we present the detailed molecular investigation of the ECM community of two species of European oaks grown in bare-root forest nurseries. We further asked if the identities of the mycorrhizal fungi varied across different nurseries. We hypothesized that in nursery conditions, ECM community structure of oaks can be different than in cases of conifer tree species, grown in these same circumstances. We used this knowledge to explore the ecology of ECM fungi in the context of pioneer ECM communities under the influence of the abiotic soil characteristic of forest bare-root nurseries.

## Materials and methods

### Nurseries and seedling sampling

We tested five bare-root forest nurseries belonging to the forest districts in northwestern Poland: Lopuchówko (L; 52°35′ N, 17°05′ E), Tuchola (T; 53°30′ N, 17°51′ E), Susz (S; 53°44′ N, 19°33′ E), Osie (O; 53°35′ N, 18°20′ E) and Runowo (R; 53°19′ N, 17°27′ E). The seedlings (2-year-old) of both *Q. petraea* and *Q. robur* were harvested in August (L, S, and O) and September (T and R) 2007. Tested nurseries are located in regions with a mean annual temperature ranging from 7.4°C to 8.1°C and a mean annual precipitation ranging from 510 mm to 605 mm. Chosen nurseries are all large provincial nurseries ranging in size from 5 to 10 ha and separated into several compartments with four to five standard nursery seedbeds in each. Soils in which seedlings were grown are fine sandy loam with poorly developed horizons and low organic matter content. Oak seedlings were all precision-seeded by machine and fertilized following a schedule designed to satisfy their nutrient requirements based on a soil analysis of each nursery.

A total of 66 samples (330 seedlings) were analyzed. Depending on the seedbed's size from each nursery, five to nine samples composed of five pedunculate or sessile oak seedlings were randomly sampled (for more details see Table 4). The seedlings, *Q. petraea* and *Q. robur*, which were taken for analysis from each nursery were all grown on the same nursery compartment on neighboring seedbeds. Seedlings were removed from the nursery together with an adjacent soil (approximately 2,500 $cm^3$) and transported to the laboratory in plastic bags.

In addition, five soil cores from each forest nursery compartment were taken, thoroughly sieved, and dried for chemical analyses. The pH of soil samples was determined by mixing 20 ml of soil substrate with 40 ml of deionized water or 0.5 M KCl. After 1 h, the pH was measured with a calibrated pH meter equipped with a glass electrode. Total N and C contents were measured using the elemental combustion system CHNS-O (Constech Analytical Technologies Inc., Valencia, USA). The Mg, K, and P concentrations were measured by an ionic chromatograph Dionex 100 and a Varian BQ 20 atomic absorption spectrophotometer with a granite cuvette.

### Ectomycorrhizal assessment

The root system of each sample (five seedlings) was rinsed on a sieve under tap water in order to remove soil particles. Lateral roots were excised from the main root and mixed in a container filled with distilled water. The large number of root tips present on each seedling (several thousand per seedling) made assessing of morphotype abundance by counting all root tips too time consuming. Therefore, the clean roots were cut into approximately 1.5 cm long sections and placed into a Petri dish filled with water. Sections were randomly selected and the numbers of all active root tips colonized by each morphotype were counted. Three subsamples were counted until approximately

300 root tips. Observations of root samples were conducted under a dissecting microscope at 10× to 60× magnification. Ectomycorrhizas were separated into morphotypes based on macroscopic observations (type of ramification, color of a mantle, presence of rhizomorphs, extramatrical hyphae, and cystidia) and referred to a database used in our Laboratory of Mycorrhizal Research at the Institute of Dendrology (Rudawska et al. 2001, 2006; Iwański et al. 2006). Earlier descriptions published by Agerer (1987–2008) and Ingleby et al. (1990) were also useful. From each nursery, 15–20 single, cleaned root tips of each morphotype from each sample, were placed in Eppendorf-tubes in cetyltrimethyl ammonium bromide (CTAB) buffer and stored at room temperature until processing for DNA analysis. No attempt was made to relate morphotypes between oak species, samples, and nurseries until molecular analysis was complete; thus, each morphotype was treated separately in a subsequent molecular typing and pooled for abundance calculation only after the molecular analysis indicated that morphotypes are identical.

## Molecular identification

Four to six single ectomycorrhizal root tips from each morphotype (from each sample and from each nursery) were separately subjected to DNA analysis. Ectomycorrhizal fungi were identified using sequencing of the PCR amplified internal transcribed spacer of rDNA (ITS rDNA). DNA was extracted using the miniprep method developed by Gardes and Bruns (1996). DNA was amplified following the protocol of Henrion et al. (1994) as modified by Kåren et al. (1997). The reagents of the PCR reaction and their final concentrations were as follows: 20 mM Tris–HCl (pH 8.4); 50 mM KCl, 2.5 mM MgCl2, 0.05% W-1 (Qiagen); 200 mM ultra pure dATP, dCTP, dGTP, and dTTP (Qiagen); 0.2 mM of the two primers (ITS1F and ITS4; IBB PAN, Poland); and 1.75 units Taq DNA polymerase (Qiagen). The PCR amplification sequence consisted of a first step at 94ºC for 10 min followed by 35 cycles of 40 s at 92ºC, 40 s at 57ºC, and 80 s at 72ºC using a T3 thermocycler (Biometra). To optimize PCR amplification, 1:10, 1:20, 1:40, 1:60, and 1:100 dilutions of extracted templates were tested before the sample was presumed to be not amplified. Presence of a PCR product was tested by separated PCR product on 1.5% agarose gel electrophoresis (10 V/cm), stained with 0.5% ethidium bromide, and recorded on black and white Polaroid film. Overall successful amplification rate reached 82%. During molecular analysis, double banded ITS products were obtained in less than 1% of analyzed mycorrhizas and were not subjected to further study. Samples for which a definite PCR product was found were sequenced in the forward and reverse directions with ITS1 and ITS4 primers at the Laboratory of Molecular Biology of Adam Mickiewicz University (Poznan). Three to four consensus sequences for each morphoptype (from each sample and from each nursery) were constructed after manual editing of ambiguous readings. Obtained sequences were compared with published sequences in GenBank or UNITE databases using the BLAST tool. A positive identification of a mycorrhizal species was confirmed if they shared ≥98% ITS region sequence identity with the reference sequence. From each nursery, the best representative of each unique ITS sequence type was deposited in GenBank (GQ154473-GQ154520).

## Data analysis

The diversity of the ectomycorrhizas on the seedlings was expressed as the number of identified ECM species (species richness). The relative abundance of each morphotype (number of root tips of each morphotype/total number of mycorrhizas) was calculated separately for each sample. Species richness, Shannon diversity index (*H*'), evenness, and Simpson dominance index were calculated using PAST 1.89. To assess the sufficiency of the sampling effort, a species accumulation curve (Mao Tau), and first-order jackknife estimator of true species richness were determined with the EstimateS program version 8.0 (Colwell 2006) using 100 randomized runs without sample replacement. Comparison of ECM fungal composition was calculated using the Bray–Curtis (BC) dissimilarity coefficient (Bray and Curtis 1957), which is one of the most robust coefficients for the analysis of taxonomic composition data. Bray–Curtis coefficients were used in the analysis of similarity (ANOSIM) (Clarke 1993) to determine whether the ECM fungal communities differed between oak species and tested forest nurseries. Data were standardized and square root transformed prior to analysis. The advantages of the ANOSIM test are that it does not assume any underlying distribution to the data and avoids using the BC index to directly compare sets of communities. Instead, it is a nonparametric test based only on the rank order of the matrix values. Analysis of similarity produces an *R* statistic that is an absolute measure of distance between groups. Large positive *R* (up to one) implies dissimilarity between groups. Nonmetric multidimensional scaling (NMDS) was used to provide a visual summary of the pattern of BC values. Both ANOSIM and NMDS were performed using PAST 1.8 (Hammer et al. 2001).

Analysis of variance (ANOVA) with Tukey's test was used to compare the soil parameters and ecological indices between oak species and nurseries. Relationships between species richness, diversity, evenness, dominance, and soil variables were examined using the Spearman rank correlation.

## Results

### Soil parameters

The measured soil parameters (pH, Mg, K, P, C and N, and C/N ratio) exhibited a high degree of variation among the tested nurseries (Table 1). The pH (in $H_2O$ and KCl) was significantly lower in the Lopuchówko nursery (L). Content of Mg, C, and N was highest in the Tuchola nursery (T) reaching 6.18%, 4.58%, and 0.39% respectively, whereas, the lowest values of Mg, K, and P occurred in the Osie nursery (O) which were 2.40%, 3.85%, and 3.80%, respectively. The highest potassium content was noted in L (10.50%) and differed significantly from other tested nurseries.

### Mycorrhizal communities

In all tested oak samples, mycorrhizal colonization was nearly 100%. Very small proportions of the root tips appeared dark and less turgid and were omitted in the analysis. Morphological observation and molecular investigations revealed a total of 23 fungal taxa (Table 2 and Fig. 1). Of these, four were assigned to order or family level (*Pezizales* 1, *Pezizales* 2, *Pezizales* 3, and *Tricholomataceae*), six to genus (*Alnicola* sp., *Inocybe* sp., *Hebeloma* sp., *Peziza* sp., *Tomentella* sp., and *Tuber* sp.) and 11 to species (*Amphinema byssoides*, *Cenococcum geophilum*, *Hebeloma sacchariolens*, *Inocybe curvipes*, *Laccaria proxima*, *Laccaria tortilis*, *Paxillus involutus*, *Phialocephala fortinii*, *Rhizoscyphus ericae*, *Scleroderma aerolatum*, and *Scleroderma verrucosum*). Based on ITS sequence analysis, one morphotype was designated as a member of basidiomycota. Only one ECM type could not be amplified, however, based on its unique morphological features, it was recognized as separate unidentified species. Despite some differences in the sequences obtained from Tuber type mycorrhizas that originated from different nurseries, phylogenetic analysis of ITS sequences placed these mycorrhizas in one clade (phylogenetic tree not presented).

From detected 23 ECM fungal taxa, 20 were noted for *Q. petraea* and 23 for *Q. robur*. A species area curve (Sobs Mao Tau) revealed an asymptotic pattern in species number with an increase in the number of samples for *Q. petraea* seedlings (Fig. 2). In the case of *Q. robur* seedlings, the species area curve tended to asymptote. The first-order jackknife estimator of ECM richness was 21.9 for *Q. petraea* and 28.8 for *Q. robur*. Hence, the observed number of ECM taxa was above 90% of the estimated richness for *Q. petraea* and nearly, 80% for *Q. robur*.

The mean relative abundance of each fungal taxon in particular nurseries is shown in Table 3. Only *Tuber* sp. and *H. sacchariolens* were observed in all nurseries. The relative abundance of these taxa ranged between 5.17% and 27.46% for *Tuber* sp. and between 0.13% and 27.71% for *H. sacchariolens*. Other frequent taxa, noted in four nurseries, were *P. involutus, Peziza* sp., and *R. ericae*. In three nurseries, *C. geophilum*, *I. curvipes*, and *S. verrucosum* were detected. Depending on the nursery and oak species different ECM fungal taxa dominated in observed communities. In L, the dominant taxa were *H. sacchariolens* for *Q. petraea* (27.71%) and *L. tortilis* for *Q. robur* (41.19%). *Peziza* sp. was most abundant species in T, S, and R for *Q. robur* (51.40%, 34.49%, and 45.28%, respectively) and in R for *Q. petraea* (33.64%). *S. verrucosum* was the most abundant ECM fungal species on *Q. petraea* seedlings in T (35.89%) and *Tuber* sp. was most abundant in S and O on *Q. petraea* and *Q. robur* (23.74% and 27.46%, respectively). Comparison of the mean relative abundances of ECM fungal species from all nurseries for both oak species is presented in Fig. 3.

The overall species richness of identified ECM fungal taxa for both oak species was variable and ranged from six to 11 taxa depending on the nursery (Tables 3 and 4). The highest mean species richness per sample was noted in L (7.33 for *Q. petraea* and 6.86 for *Q. robur*) and the

**Table 1** Soil parameters for the examined forest bare-root nurseries (values are means ± SD; $n=5$)

| Soil parameter | Forest nursery | | | | |
|---|---|---|---|---|---|
| | Lopuchówko | Tuchola | Susz | Osie | Runowo |
| pH $_{H2O}$ | 4.79±0.25 a* | 6.88±0.21b | 6.99±0.41b | 6.22±0.71b | 6.55±0.46b |
| pH $_{KCl}$ | 4.21±0.35a | 6.11±0.38b | 6.06±0.37b | 5.75±0.58b | 6.00±0.39b |
| Mg (%) | 3.29±0.28b | 6.18±0.31a | 3.20±0.35b | 2.40±0.26c | 2.12±0.43c |
| K (%) | 10.50±1.12a | 7.30±1.21b | 7.47±1.18b | 3.85±0.98c | 7.57±0.81b |
| P (%) | 6.60±1.12b | 8.50±1.18ab | 11.51±2.15a | 3.80±0.78c | 6.45±1.35b |
| C (%) | 1.26±0.21c | 4.58±0.49a | 2.82±0.59b | 2.56±0.41b | 1.88±0.39bc |
| N (%) | 0.09±0.02b | 0.39±0.10a | 0.15±0.06b | 0.12±0.02b | 0.11±0.03b |
| C/N | 15.50± | 13.50± | 19.00± | 20.98± | 17.37±0.95 |

* Letters indicate significant differences between nurseries at $p<0.05$ (Tukey's test)

**Table 2** Identification of ectomycorrhizas associated with *Q. petraea* and *Q. robur* seedlings in forest bare-root nurseries (L—Lopuchowko, O—Osie, T—Tuchola, R—Runowo, and S—Susz)

| Fungal taxa | Nursery | Accession number[a] | Best match sequence/ accession number | E-value | Sequence similarity (%) |
|---|---|---|---|---|---|
| *Agaricales* | L | GQ154515 | Uncultured Tricholomataceae DQO93734 | 0.0 | 99 |
| *Alnicola* sp. | O and S | GQ154473–GQ154474 | *Alnicola macrocarpa* AY900107 | 0.0 | 91 |
| *Amphinema byssoides* | T | GQ154475 | *Amphinema byssoides* EF493272 | e-104 | 100 |
| Basidiomycota | O | GQ154476 | Uncultured cf. Tricholoma sp. AY254876 | e-113 | 94 |
| *Cenococcum geophilum* | S, O, and R | GQ154477–GQ154479 | *Cenococcum geophilum* EU427331 | 0.0 | 98 |
| *Hebeloma sacchariolens* | L, O, R, S, and T | GQ154480–GQ154484 | *Hebeloma sacchariolens* AY312985 | e-146-0.0 | 98-99 |
| *Hebeloma* sp. | S | GQ154485 | *Hebeloma helodes* AY311514 | 0.0 | 91 |
| *Inocybe curvipes* | L, O, and R | GQ154490–GQ154492 | *Inocybe curviceps* UDB000616 | 0.0 | 98–99 |
| *Inocybe* sp. | T | GQ154493 | *Inocybe calida* AM88290 | 0.0 | 97 |
| *Laccaria proxima* | O | GQ154494 | *Laccaria proxima* DQ499639 | 0.0 | 99 |
| *Laccaria tortilis* | L | GQ154495 | *Laccaria tortilis* UDB001589 | 0.0 | 100 |
| *Paxillus involutus* | L, O, R, and T | GQ154496–GQ154499 | *Paxillus involutus* EU346879 | 0.0 | 100 |
| *Peziza* sp. | O, R, S, and T | GQ154500–GQ154503 | *Peziza ostracoderma* EU819461 | 0.0 | 97 |
| *Pezizales* 1 | O | GQ154504 | Uncultured Pezizales DQ469743 | 0.0 | 96 |
| *Pezizales* 2 | O and R | GQ154505–GQ154506 | Uncultured ectomycorrhiza (Pezizaceae) DQ974750 | 0.0 | 95 |
| *Pezizales* 3 | R and S | GQ154507–GQ154508 | Uncultured ectomycorrhiza (Pezizales) FJ210743 | 0.0 | 97 |
| *Phialocephala fortinii* | T | GQ154509 | *Phialocephala fortinii* EF446148 | 0.0 | 99 |
| *Rhizoscyphus ericae* | L, O, R, S | GQ154486–GQ154489 | *Rhizoscyphus ericae* AM084704 | 0.0 | 98 |
| *Scleroderma areolatum* | R | GQ154510 | *Scleroderma areolatum* EU819518 | 0.0 | 100 |
| *Scleroderma verrucosum* | L, S, and T | GQ154511–GQ154513 | *Scleroderma verrucosum* UDB000044 | 0.0 | 99-100 |
| *Tomentella* sp. | R | GQ154514 | Uncultured ectomycorrhiza (Tomentella) AJ879644 | 0.0 | 99 |
| *Tuber* sp. | L | GQ154516 | uncultured fungus EU554698 | 0.0 | 100 |
| | O | GQ154517 | Uncultured mycorrhiza (Tuber) FJ348400 | 0.0 | 100 |
| | R and S | GQ154518–GQ154519 | *Tuber* sp. AM900418 | 0.0 | 99 |
| | T | GQ154520 | Uncultured fungus DQ508796 | 0.0 | 100 |
| Unidentified | O and R | Not sequenced | - | - | - |

[a] If fungal taxon was detected in more than one nursery, ITS sequences were submitted separately from each nursery

lowest in O (3.17 for *Q. petraea*) and S and R (5.00 for *Q. robur*). Statistical analysis based on the ANOVA showed that oak species did not have a significant effect on ECM species richness (Table 4). The mean species richness for all nurseries was 5.36 and 5.82 taxa per sample for *Q. petraea* and *Q. robur*, respectively. Shannon diversity, evenness, and dominance coefficients did not differ significantly between both oak species. The highest diversity index was noted for *Q. petraea* and *Q. robur* in L (1.73 and 1.55, respectively). Evenness index was highest in L for *Q. petraea* (0.87) and in O for *Q. robur* (0.83). The highest species dominance was characteristic for S (0.43 and 0.52 for *Q. petraea* and *Q. robur*, respectively).

According to ANOSIM analysis, ECM fungal communities were similar for *Q. petraea* and *Q. robur* ($R$=0.019; $p$=0.151). On the other hand, detected fungal communities were significantly different between nurseries ($R$=0.927; $p$<0.0001). Nonmetric multidimensional scaling ordination of the ECM fungal assemblages consistently separated tested nurseries (Fig. 4). Spearman's rank correlation showed that the species richness and Shannon diversity index were negatively correlated with nursery soil pH and the level of C and N. Potassium content in the nursery soil positively influenced species richness and diversity. Evenness of ECM fungal communities was negatively correlated with pH and P and K content. Positive, significant correlation was noted between dominance index and soil pH, P, C, and N (Table 5).

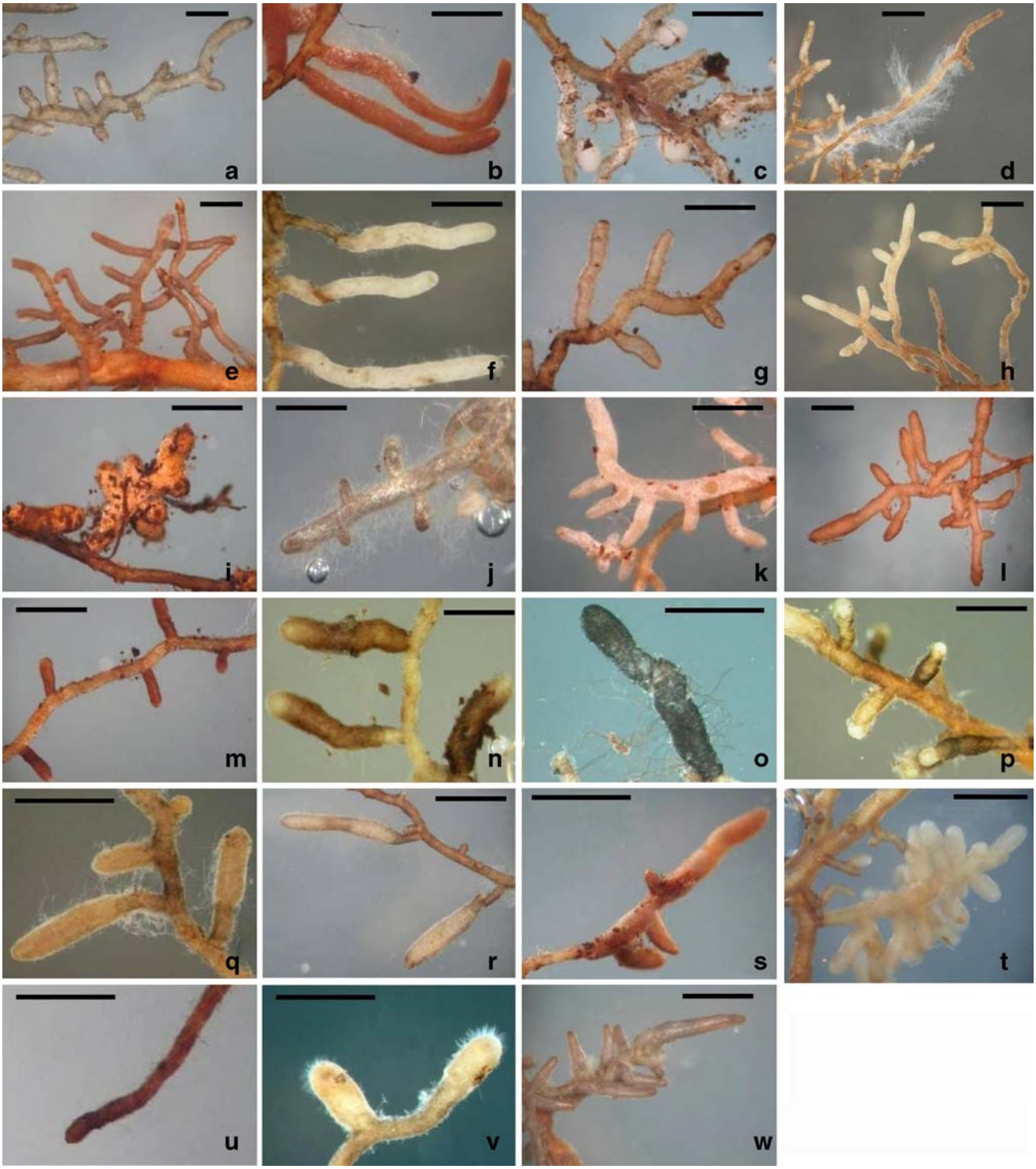


**Fig. 1** Plan views of mycorrhizas observed on *Q. petraea* and *Q. robur* seedlings from bare-root forest nurseries—*Alnicola* sp. (**a**); *Amphinema byssoides* (**b**); *Hebeloma sacchariolens* (**c**); *Hebeloma* sp. (**d**); *Inocybe curvipes* (**e**); *Inocybe* sp. (**f**); *Laccaria proxima* (**g**); *Laccaria tortilis* (**h**); *Paxillus involutus* (**i**); *Scleroderma areolatum* (**j**); *Scleroderma verrucosum* (**k**); *Agaricales* (**l**); Basidiomycota (**m**); *Tomentella* sp. (**n**); *Cenococcum geophilum* (**o**); *Rhizoscyphus ericae* (**p**); *Peziza* sp. (**r**); *Pezizales* 1 (**s**); *Pezizales* 2 (**t**); *Pezizales* 3 (**u**); *Phialocephala fortinii* (**w**); *Tuber* sp. (**x**); and unidentified (**y**). bars=1 mm

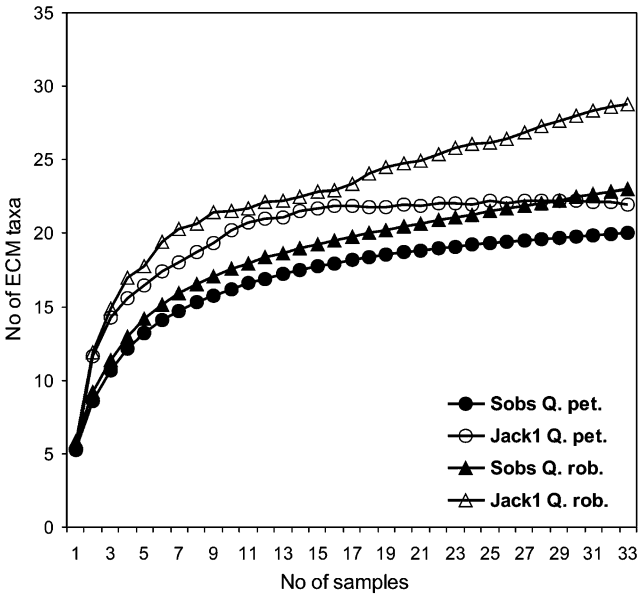


**Fig. 2** Ectomycorrhizal species richness estimation curves for *Q. petraea* (Q. pet.) and *Q. robur* seedlings (Q. rob.). Sobs—species observed, Jack1—first-order Jacknife richness estimator

## Discussion

To our knowledge, this is the first investigation to provide a detailed assessment of the ECM diversity of *Q. robur* and *Q. petraea* seedlings in bare-root nursery conditions. Twenty-three taxa contributed to the mycorrhizal community structure of two-year-old pedunculate and sessile oak seedlings. This is a richer diversity than reported in previous studies for coniferous species (Scots pine, Norway spruce, and European larch) (Rudawska et al. 2001, 2006; Menkis et al. 2005; Iwański et al. 2006; Trocha et al. 2006; Leski et al. 2008) and deciduous trees (e.g., small-leaved lime or garry oak) (Timonen and Kauppinen 2008; Southworth et al. 2009) in nursery conditions. At the same time, this is a much lower diversity compared with the 75 ECM types on out-planted seedlings of two oak species (*Q. rubra* and *Q. prinus*) grown in mixed forests in Appalachian Mountains (Walker et al. 2005). However, the species' accumulation curve for both tested oak species and the jackknife species estimate (Fig. 2) indicate that we had sampled sufficiently and observed species richness to truly reflect the potential of oak seedlings

**Table 3** Relative abundance of mycorrhizal fungal taxa associated with *Q. petraea* and *Q. robur* seedlings from five forest bare-root nurseries

| Mycorrhizas fungal taxa | Nuresry/oak species | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Lopuchówko | | Osie | | Tuchola | | Runowo | | Susz | |
| | *Q. pet.* | *Q. rob.* | *Q. pet.* | *Q. rob.* | *Q. pet.* | *Q. rob.* | *Q. pet.* | *Q. rob.* | *Q. pet.* | *Q. rob.* |
| *Peziza* sp. | | | 10.92 | | 33.32 | 51.40 | 33.64 | 45.28 | 21.40 | 34.49 |
| *Tuber* sp. | 5.42 | 8.01 | | 27.46 | 5.17 | 9.85 | 20.43 | | 23.74 | 15.05 |
| *I. curviceps* | 10.52 | 7.57 | 16.81 | | | | 31.96 | 37.37 | | |
| *R. ericae* | 21.25 | 16.84 | 10.67 | 13.03 | | | | 1.93 | 0.90 | 0.45 |
| *L. proxima* | | | 25.80 | 24.15 | | | | | | |
| *H. sacchariolens* | 27.71 | 17.20 | 0.13 | 7.92 | 1.59 | 5.86 | 0.91 | 1.01 | 1.28 | 1.39 |
| *L. tortilis* | 22.80 | 41.19 | | | | | | | | |
| *P. involutus* | 6.01 | 6.02 | 20.13 | 1.71 | 7.06 | 8.27 | 1.30 | 1.14 | | |
| *S. verrucosum* | 6.12 | 2.15 | | | 35.89 | 10.01 | | | 5.27 | |
| *Hebeloma* sp. | | | | | | | | | 4.62 | 44.37 |
| *Pezizales* 1 | | | 10.68 | 15.92 | | | | | | |
| *C. geophilum* | | | 4.61 | | | | 3.25 | 11.04 | 12.56 | 4.25 |
| *P. fortinii* | | | | | 16.98 | 12.01 | | | | |
| *Pezizales* 3 | | | | | | | 1.23 | 1.37 | 18.41 | |
| *Alnicola* sp. | | | | 4.66 | | | | | 11.82 | |
| Basidiomycota | | | | 5.16 | | | | | 21.40 | 34.49 |
| *Pezizales* 2 | | | 0.20 | | | | 6.71 | 0.55 | 23.74 | 15.05 |
| *Agaricales* | 0.18 | 1.03 | | | | | | | | |
| *A. byssoides* | | | | | | 1.30 | | | 0.90 | 0.45 |
| *Inocybe* sp. | | | | | | 1.30 | | | | |
| *S. areolatum* | | | | | | | 0.43 | 0.16 | | |
| Unidentified | | | 0.05 | | | | 0.06 | 0.05 | 0.05 | |
| Tomentella sp. | | | | | | | 0.06 | 0.10 | | |

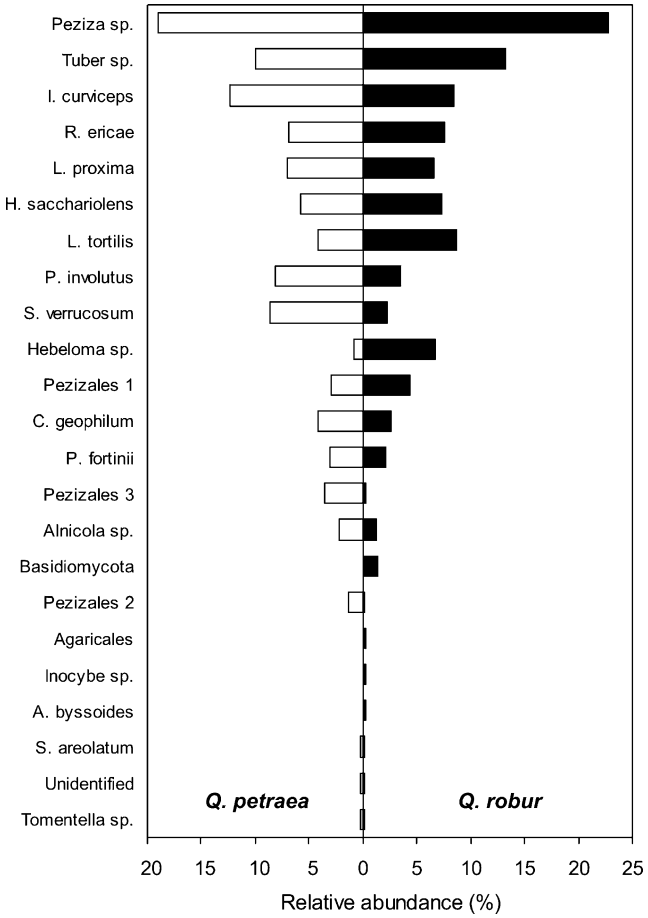


**Fig. 3** Mean relative abundance of mycorrhizal fungal taxa associated with *Q. petraea* (*open columns*) and *Q. robur* (*closed columns*) seedlings in forest bare-root nurseries. Taxa in rank order

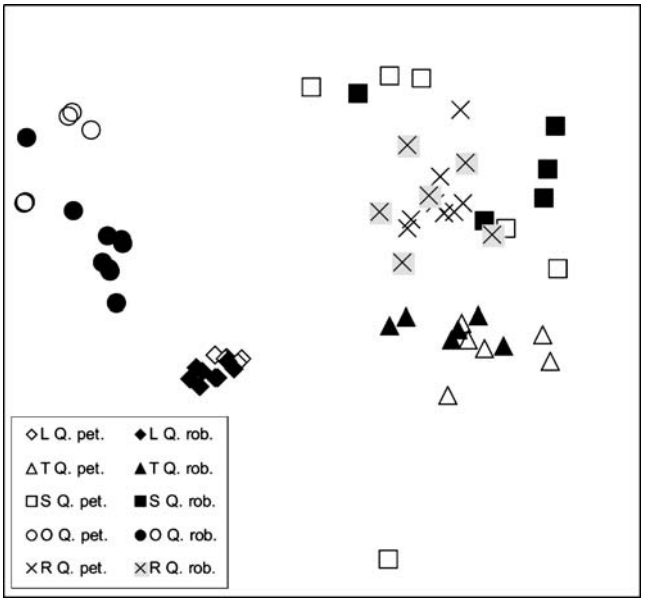


**Fig. 4** Nonmetric multidimensional scaling ordination of ECM fungal communities on *Q. petraea* (Q. pet) and *Q. robur* (Q. rob) seedlings from five tested forest nurseries (L—Lopuchowko, T—Tuchola, S—Susz, O—Osie, and R—Runowo)

to form mycorrhiza in nursery conditions. The average value of ECM species richness per nursery sample amounted to 5.4 for *Q. petraea* and 5.8 for *Q. robur* and was obviously higher than for Norway spruce and European larch studies (2.7 and 2.3, respectively) conducted in comparable locations and using the same nursery practices (Rudawska et al. 2006; Leski et al. 2008). Therefore, we presume that observed differences may be connected with the higher photosynthetic activity of oak leaves than needles of spruce and larch. In general, the leaves of deciduous species have higher rates of light-saturated photosynthesis than do leaves of conifers when the rates are expressed on a leaf dry weight basis (Pallardy 2007). The lower input of carbon from young seedlings of coniferous trees may be a factor limiting the availability of a wider fungal spectrum capable of colonizing roots. The relationship between greater diversity of ECM fungi and additional energy (e.g., carbohydrates) as a result

**Table 4** Species richness, Shannon diversity, evenness, and Simpson dominance indices of ectomycorrhizal communities associated with *Q. petrea* and *Q. robur* seedlings in five forest bare-root nurseries

| | Nuresry/oak species | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lopuchowko | | Tuchola | | Susz | | Osie | | Runowo | | Sum[a]/mean[b] | |
| | *Q. pet.* | *Q. rob.* | *Q. pet.* | *Q. rob.* | *Q. pet.* | *Q. rob.* | *Q. pet.* | *Q. rob.* | *Q. pet.* | *Q. rob.* | *Q. pet.* | *Q. rob.* |
| Number of samples | 6.00 | 7.00 | 6.00 | 6.00 | 6.00 | 5.00 | 6.00 | 9.00 | 9.00 | 6.00 | 33.00 | 33.00 |
| Total species richness | 8.00 | 8.00 | 6.00 | 8.00 | 9.00 | 6.00 | 10.00 | 8.00 | 11.00 | 11.00 | 20.00 | 23.00 |
| Average species richness | 7.33 | 6.86 | 4.00 | 5.17 | 5.17 | 5.00 | 3.17 | 6.44 | 6.56 | 5.00 | 5.36 | 5.82 |
| Diversity | 1.73 | 1.55 | 1.17 | 1.28 | 1.08 | 0.88 | 0.98 | 1.54 | 1.29 | 1.09 | 1.25 | 1.31 |
| Evenness | 0.87 | 0.81 | 0.85 | 0.78 | 0.67 | 0.56 | 0.86 | 0.83 | 0.70 | 0.73 | 0.78 | 0.76 |
| Dominance | 0.20 | 0.27 | 0.36 | 0.36 | 0.43 | 0.52 | 0.42 | 0.26 | 0.34 | 0.40 | 0.35 | 0.35 |

[a] Sum is given for number of samples and total species richness

[b] Mean is given for average species richness, diversity, evenness, and dominance

**Table 5** Spearman's rank correlation of species richness and diversity indices of soil parameters in forest nurseries

| | $H_2O$ | KCl | Mg | K | P | C | N | C/N |
|---|---|---|---|---|---|---|---|---|
| Richness | −0.62*** | −0.58*** | 0.01 | 0.38** | −0.15 | −0.52*** | −0.51*** | −0.09 |
| Diversity | −0.71*** | −0.67*** | 0.13 | 0.26*** | −0.33*** | −0.46*** | −0.46*** | −0.17 |
| Evenness | −0.25* | −0.24* | 0.16*** | −0.09*** | −0.37*** | −0.10 | −0.09 | −0.11 |
| Dominance | 0.67*** | 0.63*** | −0.10 | −0.21*** | 0.35*** | 0.43*** | 0.43*** | 0.14 |

*Significant correlation at $p \leq 0.05$

**Significant correlation at $p \leq 0.01$

***Significant correlation at $p \leq 0.001$

of enhanced net photosynthetic rate supplied by seedlings were also suggested by Simard et al. (1997).

Our investigation did not show significant differences between ECM fungal compositions on pedunculate and sessile oak seedlings. This result confirmed the conclusions drawn by Walker et al. (2005) and Ishida et al. (2007) who argued that closely related (e.g., congeneric) hosts have a similar ECM fungal composition. A recent study of oaks from different subgenera, however, shows evidence that ECM community assemblages vary with host species (Morris et al. 2008; Cavender-Bares et al. 2009). It is likely that genetic, physiological, and ecological differences between the host species act in concert to influence their associated ECM communities (Morris et al. 2008). Our results, thus, show that our two native oaks, as closely related species, bear comparable ECM fungal communities. This finding was further confirmed by Shannon diversity, evenness, and Simpson dominance indices, which were unaffected by oak species (Table 4).

In the nurseries we tested, the taxa identified belonged to the asco- and basidiomycota, and no dominance for either fungal group was found. The most frequent fungal taxon was *Tuber* sp., which was found in each tested nursery and most samples. *Tuber* spp. were shown to be a common component of fungal communities in forest nursery conditions (Ursic and Peterson 1997; Menkis et al. 2005; Iwański et al. 2006; Rudawska et al. 2006; Trocha et al. 2006; Aučina et al. 2007; Leski et al. 2008; Southworth et al. 2009). Based on the BLAST search, *Tuber* sp. from nursery T showed a high homology (96%) to mycorrhizas found on Scots pine seedlings in post-agriculture areas in Poland (EU379679). *Tuber* sp. from the nursery O revealed 100% similarity with uncultured ectomycorrhiza *Tuber* (FJ348400) identified by Southworth et al. (2009) on *Q. garryana* seedlings from forest nurseries in Washington, D.C., USA. Both Polish native oaks are potential host plants for different species of truffles, for example *T. melanosporum*, *T. aestivum*, or *T. borchii* (Bencivegna 1999). Fruit bodies of truffles are detected in the immediate vicinity of mature oaks in Poland (Ławrynowicz 1988; Hilszczańska et al. 2008) but have not been found up to now in nursery conditions. Presumably, in forest nurseries fungi from the *Tuber* genus exist in a vegetative state (anamorphs), which requires less energy and time compared with ascomata production. The anamorphic stage in fungi development was recently described for small white truffles by Urban et al. (2004). A large group of recognized mycorrhizas belonged to *Pezizales*. Recent studies revealed a high diversity of this group (Tedersoo et al. 2003; Izzo et al. 2005), however, few morphotypes were recognized to a species level (Tedersoo et al. 2006). *Peziza* sp., from our studies, had a high sequence identity (97%) to *P. ostracoderma* (EU819461) and appeared in a high abundance (around 20% for both oaks). The rest of *Pezizales* were detected rarely, in one or two nurseries with low abundance. Exact recognition of *Pezizales* 1, *Pezizales* 2, and *Pezizales* 3 was impossible because limited availability of ITS sequences from identified voucher specimens. Unidentified *Pezizales* were also detected in forest nurseries by Trocha et al. (2006) and Leski et al. (2008) on coniferous tree seedlings. The presence of ECM pezizalean (ascomycota), a group of fungi with a notorious preference for disturbed sites and base-rich soils (Tedersoo et al. 2006), which are well-represented belowground in various ECM communities (Fujimura et al. 2005; Tedersoo et al. 2006), may be due to the strongly transformed forest nursery soils (Van den Driessche 1984). *Cenococcum geophilum* has been found on both tested oaks in three nurseries, with low frequency and abundance not higher than 13%. *C. geophilum* often dominates roots in ecosystems with extreme environmental conditions such as drought (LoBuglio 1999). This situation was not observed in analyzed nurseries because all nurseries are equipped with an irrigation system that precludes the occurrence of drought periods. *C. geophilum* is a widespread, multihost ectomycorrhizal fungus, common and abundant in mineral soil, the forest floor, logs and stumps, probably indicating wide environmental tolerances and a lack of specificity for substrate (LoBuglio 1999). Recently, *C. geophilum* was noted as a ECM symbiont of different oak species on seedlings and mature trees (Valentine et al. 2004; Richard et al. 2005; Walker et al. 2005, 2008; Mosca et al. 2007; Morris et al. 2008), however, was not found by Southworth et al. (2009) on nursery seedlings of

*Q. garryana*. Another ascomycota found in our studies was the morphotype identified as a *Rhizoscyphus ericae* aggregate. Phylogenetic relationships and species separation within this group are not clear (Vrålstad et al. 2000, 2002).

In tested nurseries, species regarded as early stage fungi (R-selected, ruderal) often occurred. To this group belong taxa from the *Inocybe*, *Hebeloma*, or *Laccaria* genus which are characterized by rapid growth, tolerance to disturbance, and low competitive abilities. Presence of these ECM taxa may be also related to a high production of spores, which spread easily on open areas of nursery bed-soil and closely echoes the fruiting bodies and mycorrhizas survey undertaken on one- to five-year-old *Q. petraea* and *O. robur* nursery seedlings (Garbaye et al. 1986). Multistage *Paxillus involutus* and *Scleroderma* spp. often appeared as a component of ECM communities of bare-root nurseries. Both taxa may spread not only by spores but also by ectomycorrhizal networks. In this case, the seedling root system is colonized by rhizomorphs that extend from mature trees growing in adjacent forests. The influence of surrounding forests on young trees was described by Dickie et al. (2002) for red oak seedlings.

Our research is the first report concerning the coexistence of *Alnicola* sp. with oaks in nursery conditions. Previous studies have shown the close compatibility of *Alnicola* sp. with *Alnus* and *Salix*. However, only once have the fruiting bodies of *Alnicola* sp. been shown to be associated with sessile oak (Moreau et al. 2006). *Alnicola* sp. identified in two nurseries from our studies was similar to the *Alnicola macrocarpa* fruiting bodies (AY900107) found in vicinity of *Salix aurita* and *Salix caprea* (Moreau et al. 2006). A possible explanation of the presence *Alnicola* sp. on nursery oaks from our studies is the direct vicinity of alder and oak nursery bed-soil in nursery S and the high density of mature alder trees in the neighborhood of nursery O. Obtained results indicate that host-specificity of *Alnicola* sp. is not as narrow as previously assumed.

Profound differences were found among tested nurseries in terms of species composition and relative abundance. From 23 recognized fungal taxa, 11 were detected in a single nursery or even in a single sample. This is in accordance with studies carried out by Walker et al. (2008) on *Q. rubra* and *Q. prinus* seedlings where 42 from 73 mycorrhizal types were isolated from only one research plot. Some works revealed that species of ECM fungi differ in their ability to exploit soil nutrients, and this functional diversity might explain their distribution among different ecological niches (Bruns 1995; Dickie et al. 2002; Tedersoo et al. 2003). Individual nurseries may be considered as separate ecological niches in terms of organic matter content, mineral soil, or dead woody debris that strongly discriminate the functional diversity of ECM fungi (Aučina et al. 2007). This is highlighted by the results of this study that show that ectomycorrhizal structure (in terms of richness, the Shannon diversity, evenness, and Simpson dominance indices) is significantly related to the soil parameters of each nursery. Most probably, this is due to the replacement of some fungal species by others, which suit better to the particular environmental conditions of that given nursery.

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