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Fungi in Roots of Nursery Grown *Pinus sylvestris*: Ectomycorrhizal Colonisation, Genetic Diversity and Spatial Distribution

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Abstract The aims of this study were to investigate patterns of ectomycorrhizal (ECM) colonisation and community structure on nursery grown seedlings of *Pinus sylvestris*, spatial distribution of ECMs in the nursery plot and genetic diversity of commonly isolated ECM basidiomycete *Hebeloma cavipes*. One hundred seedlings were sampled in 225 m^2 area using a systematic grid design. For each seedling, 20 individual root tips were randomly collected, morphotyped, and surface sterilised for fungal isolation in pure culture. Results showed that ECM community was comprised of nine distinct morphotypes among which *Thelephora terrestris* (39.7%), *Hebeloma* sp. (17.8%) and *Suillus luteus* (6.1%) were the most abundant. Spatial distribution of ECMs in the nursery plot was determined by their relative abundance: even in common ECMs and random in rare ones. Fungal isolation yielded 606 pure cultures, representing 71 distinct taxa. The most commonly isolated fungi were the ascomycetes *Neonectria macrodidyma* (20.3%), *Phialocephala fortinii* (13.5%), *Neonectria radicicola* (6.3%) and the ECM basidiomycete *H. cavipes* (4.5%). Intraspecific genetic diversity within 27 *H. cavipes* isolates was studied using two methods: restriction digestion of the amplified intergenic spacer of nuclear ribosomal DNA and genealogical concordance of five genetic markers. Five and eight genotypes were revealed by each respective method, but both of those were largely consistent, in particular, in determining the largest genotype (A) composed of 18 isolates. Mapping positions for each *H. cavipes* isolate and genotype in the field showed that isolates of the A genotype covered a large part of the nursery plot. This suggests that *H. cavipes* is largely disseminated by vegetative means of local genotypes and that nursery cultivation practices are likely to contribute to the dissemination of this species in the forest nursery soils.

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Introduction

In forest nurseries, roots of tree seedlings are associated with diverse communities of fungi [25, 36, 38]. Beneficial components of those communities are the ectomycorrhizal (ECM) fungi, which contribute to vigour of tree seedlings in the nursery [24, 50] and subsequently enhance their establishment and growth following outplanting in the field [13, 19, 28, 30, 37, 42, 43]. ECM fungi support plants with mineral nutrients and water [48], and protect roots against unfavourable abiotic and biotic stress factors [7, 9, 40, 42, 57]. Such positive impacts are likely to be most pronounced on poor fertility planting sites under harsh environmental conditions [48]. Sandy dunes at the Baltic Sea coast can be a good example for such sites. Here, the establishment of tree seedlings is usually problematic and several years of extensive replanting are often required, in particular on areas devastated by forest fires (V. Kolokšanskis, Curonian Spit National Park, personal communication). As the absence of living tree roots and reduced ECM inoculum are characteristic for soil on post-fire sites [11], success of outplanted seedlings is therefore much dependant on fungi pre-colonising their roots in the nursery.

In intensive nursery cultivation, the environment is usually modified by fertilisation, irrigation, mechanical and chemical weed, and pest control. However, those management practices either adversely affect beneficial mycoflora of seedling roots [25, 29, 41, 58], or promote

colonisation of roots by particular ECM species. One example is the basidiomycete *Thelephora terrestris*, which appears to be the most common ECM fungus in the forest nurseries worldwide [34]. Although this species is well adapted to environmental conditions of the nursery, it often fails to support seedling establishment in the field [20, 31, 35]. Consequently, nursery management that promotes root colonisation by ECM species more adapted to field conditions would be desirable. It is known, however, that ECM colonisation of seedling roots vary considerably in forest nurseries [12, 22, 36], while in forest ecosystems practically all fine roots of trees are ECM [52].

To date, several studies on fungal communities associated with seedling roots have been conducted in forest nurseries with the primary aim to identify abundance and diversity of ECM species [22, 25, 36, 46, 54], but little is known about their spatial distribution and genetic diversity. Such information can be of practical importance allowing further optimisation of ECM management practices in the forest nurseries. Furthermore, origin of fungal symbiont may also have a significant impact on seedling performance in the field [10, 56].

Insights into such questions can be gained by using molecular fingerprinting, which allow to assess genetic diversity and relatedness between different individuals of the species. Although a number of such methods have been developed, in fungi, no single method has established as a dominant and each method has its own advantages and limitations [49]. In this study, we compared two different polymerase chain reaction (PCR)-based molecular fingerprinting methods including restriction digestion of amplified fragments (PCR-RFLP) and genealogical concordance of several genetic markers. The first method was considered as a relatively simple and fast where intergenic spacer (IGS) of nuclear ribosomal DNA was targeted by PCR-RFLP as this region was shown to be informative for identification of diversity within certain ECM species [15]. Another method incorporated DNA sequence information of five genetic markers. Koufopanou et al. [27] have shown that compatibility of different gene genealogies can be used to study population genetics. Genealogies constructed from different parts of clonal genomes will be identical, while those from parts of organisms which undergone recombination will be different.

The aims of the present study were to investigate patterns of ECM colonisation and community structure on nursery grown seedlings of *Pinus sylvestris*. Moreover, the data is provided on culturable fungal species, spatial distribution of ECMs in the nursery, and on genetic diversity of *Hebeloma cavipes*, a commonly isolated ECM fungus. Taken together, we assessed quality of the seedling material in respect to associated fungi and possible effects of the nursery practices on those associations.

Materials and Methods

Study Site and Sampling

The study site comprised a forest nursery situated in the vicinity of the Baltic Sea coast in western Lithuania (N 56° 01′, E 21°14′). This nursery is owned by Kretinga Forest Enterprise, and produces seedling material using standardised bare-root cultivation in beds. The nursery is the only supplier of *P. sylvestris* seedlings to Curonian Spit National Park, and this tree species is the most common tree species used for afforestation of coastal dunes of the Baltic Sea in Lithuania. Within the area, mean annual precipitation is ca. 720 mm and the length of the growing season is ca. 195 days. Temperatures average ca. 14°C during the growth season. The site is characterised by sandy soils and was derived in the past from the forest land corresponding to *vaccinio-myrtilliosa* forest type. Two-year old seedlings of *P. sylvestris* were sampled after the growing season in October 2007. The sampling area was 225 m^2 in size and included four adjacent beds each 1.5 m wide and 37.5 m long. In total, 100 seedlings were sampled using a systematic grid design at a spacing of 1.5×1.5 m. This was achieved by sampling 25 seedlings in the middle of each bed at the regular interval of 1.5 m. Seedlings were gently excavated to preserve fine roots, individually labelled, packed into plastic bags, transported to the laboratory, and kept at 4°C for a maximum period of 2 weeks.

Assessment of Fine Roots

Fine roots were sampled and assessed following description by Menkis et al. [36]. Briefly, each root system was washed with tap water and 20 individual root tips from each plant were randomly collected from different parts of the root system using forceps. Sampled roots were assessed for ECM colonisation using macro- and microscopic features. In the presence of ECM colonisation, ECM roots were identified following the guidelines of the atlas of ectomycorrhizae [1]. Only these mycorrhizas which matched descriptions were given taxonomic names. Mycorrhizas which did not match any of available descriptions, were grouped accordingly to morphological characters, and given a descriptive name (Table 1).

Isolation of Fungi into the Pure Culture

The isolation of fungal cultures was attempted from 2,000 individual root tips. Before isolation, root tips were placed in 10×20 mm net bags (mesh size 0.2×0.2 mm), sterilised in 33% hydrogen peroxide for 30 s, and then rinsed three times in sterile deionised water. About ten tips per each

Table 1 Frequency of ectomycorrhizal morphotypes on root tips of *Pinus sylvestris* seedlings bare-root cultivated in forest nursery

Morphotypes	Colonisation (%)	
	Plants (100)	Root tips (2000)
Hebeloma sp.	54.0	17.8
Piceirhiza bicolorata	4.0	0.3
Suillus luteus	23.0	6.1
Suillus sp.	7.0	1.0
Thelephora terrestris	92.0	39.7
Unidentified no. 1 (whitish)	1.0	0.3
Unidentified no. 2 (matt yellow)	1.0	0.2
Unidentified no. 3 (brown)	2.0	0.1
Wilcoxina sp.	6.0	0.8
All	100	66.1

9 cm diameter Petri dish were plated onto modified Melin Norkrans medium [32] and incubated at 21°C in the dark. Dishes were checked daily and for each newly emerging mycelium, the date of outgrowth was recorded and those were immediately transferred to new dishes with fresh agar media. Isolated cultures were examined under a microscope (Carl Zeiss Axioplan, Oberkochen, Germany) equipped with 10× ocular and 25× long distance objective magnification and grouped into mycelial morphotypes.

DNA Work and Species Identification

For species identification, the internal transcribed spacer of the fungal ribosomal DNA (ITS rDNA) was sequenced for representatives of each mycelial morphotype using primers ITS1F and ITS4 [59]. Extraction of DNA and amplification followed established methods described by Rosling et al. [45]. Sequencing was performed by Macrogen Inc., Seoul, Korea, utilising ABI 3730 XL automated sequencers (Applied Biosystems, Foster City, CA, USA). Raw sequence data were analysed using the SeqMan version 5.01 software from DNASTAR package (DNASTAR, Madison, WI, USA) and BioEdit version 7.0.5.2 [16]. Databases at GenBank [3], UNITE [26] and at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala were used to determine the identity of the sequences.

Figure 2 NJ PAUP generated tree from ITS rDNA sequences of fungi isolated from fine roots of *Pinus sylvestris* seedlings. Bootstrap branch support values of 1,000 replicates are indicated near the branches. The topology was rooted using midpoint option

Assessment of Intraspecific Diversity Within Isolates of *H. cavipes*

Two different methods were compared to assess intraspecific diversity of the ECM fungus *H. cavipes*. First, we used PCR-RFLP of IGS rDNA. Amplification of the interior and more variable IGS2 region, situated between the 5S and 18S genes of rDNA, was done with the primers 5SRNAP and invSR1R. Description of those primers is available at http://www.lutzonilab.net/primers/index.shtml. Double digestion of amplified PCR products was done using restriction enzymes HinfI and HaeIII (Fermentas Life Sciences, Germany) according to the manufacturer's recommendations. Restriction fragments were separated by electrophoresis on 1% agarose gels (Agarose D1, Conda, Spain) in 1× SB buffer [6] for 3 h at 150 V. The gels were stained with ethidium bromide and obtained images were analysed in Quantity One version 4.6.3 (Bio-Rad laboratories, CA, USA) software. This software was also used to estimate Dice's similarity coefficient between the isolates and to generate topology using UPGAMA (unweighted pair group method using arithmetic averages) method. The UPGAMA method was selected, as indicated by the manufacture, for giving the most plausible clusters and being affected the least by outlier's samples.

In the second method, the intraspecific diversity of *H. cavipes* was studied using genealogical concordance of five genetic markers: ammonium-metabolising glutamate dehydrogenase (GDHA), elongation factor 1 alfa (EF1a), ITS rDNA, large subunit of nuclear ribosomal DNA (nLSU) and small subunit of mitochondrial ribosomal DNA (mtSSU). GDHA,

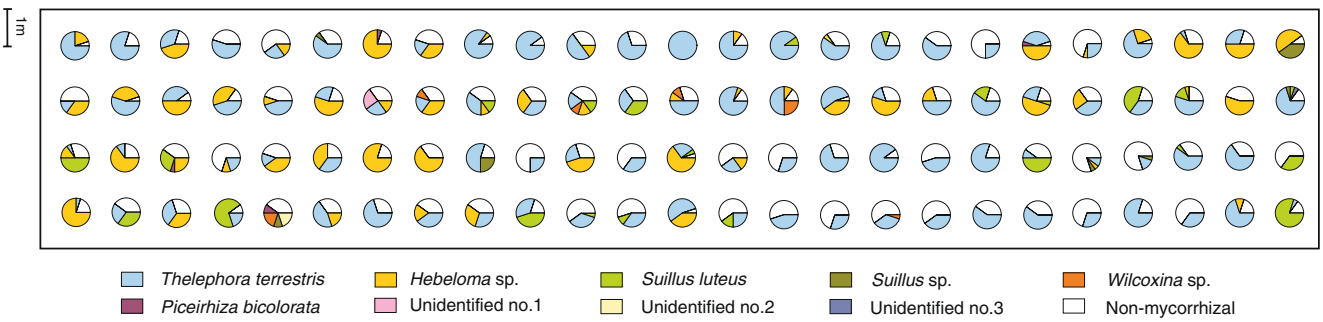
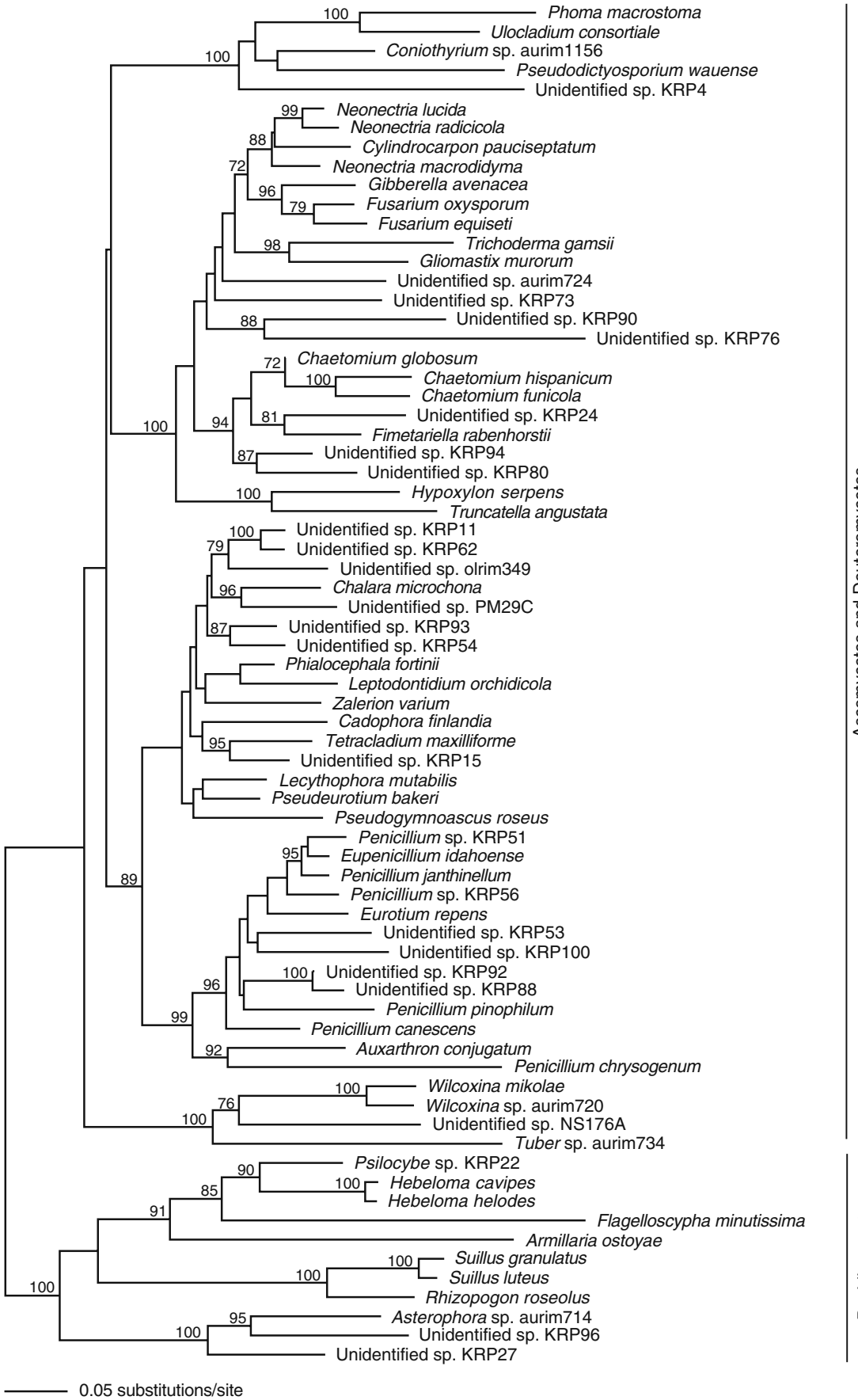


Figure 1 Abundance, diversity and spatial distribution of ECM morphotypes on fine roots of *Pinus sylvestris* seedlings in confined forest nursery plot. Each *circle* represents one seedling


Phoma macrostoma
Ulocladium consortiale
Coniothyrium sp. aurim1156
Pseudodictyosporium wauense
Unidentified sp. KRP4
Neonectria lucida
Neonectria radicicola
Cylindrocarpon pauciseptatum
Neonectria macrodidyma
Gibberella avenacea
Fusarium oxysporum
Fusarium equiseti
Trichoderma gamsii
Gliomastix murorum
Unidentified sp. aurim724
Unidentified sp. KRP73
Unidentified sp. KRP90
Unidentified sp. KRP76
Chaetomium globosum
Chaetomium hispanicum
Chaetomium funicola
Unidentified sp. KRP24
Fimetariella rabenhorstii
Unidentified sp. KRP94
Unidentified sp. KRP80
Hypoxylon serpens
Truncatella angustata
Unidentified sp. KRP11
Unidentified sp. KRP62
Unidentified sp. olrim349
Chalara microchona
Unidentified sp. PM29C
Unidentified sp. KRP93
Unidentified sp. KRP54
Phialocephala fortinii
Leptodontidium orchidicola
Zalerion varium
Cadophora finlandia
Tetracladium maxilliforme
Unidentified sp. KRP15
Lecythophora mutabilis
Pseudeurotium bakeri
Pseudogymnoascus roseus
Penicillium sp. KRP51
Eupenicillium idahoense
Penicillium janthinellum
Penicillium sp. KRP56
Eurotium repens
Unidentified sp. KRP53
Unidentified sp. KRP100
Unidentified sp. KRP92
Unidentified sp. KRP88
Penicillium pinophilum
Penicillium canescens
Auxarthron conjugatum
Penicillium chrysogenum
Wilcoxina mikolae
Wilcoxina sp. aurim720
Unidentified sp. NS176A
Tuber sp. aurim734
Psilocybe sp. KRP22
Hebeloma cavipes
Hebeloma helodes
Flagelloscypha minutissima
Armillaria ostoyae
Suillus granulatus
Suillus luteus
Rhizopogon roseolus
Asterophora sp. aurim714
Unidentified sp. KRP96
Unidentified sp. KRP27
Ascomycetes and Deuteromycetes
Basidiomycetes
0.05 substitutions/site

EF1a and ITS rDNA were amplified and sequenced using their specific primers [4, 14, 55]. nLSU and mtSSU were amplified and sequenced using respective primer pairs LR0R and LR7, and NS19 and NS6. For those, primer information is available at http://www.lutzonilab.net/primers/index.shtml.

Cluster Analyses

Cluster analyses were used in order to (1) determine phylogenetic relationships between root inhabiting fungi using ITS rDNA sequence information and (2) study intraspecific genetic diversity in *H. cavipes* using a combination of five genetic marker dataset. Sequence alignment for each genetic marker was constructed using the Clustal W algorithm of BioEdit and adjusted manually. Analyses were carried out in PAUP 4.0b10 [51]. A neighbour-joining (NJ) similarity tree of ITS rDNA sequences of isolated fungi was constructed using the Hasegawa Kishino Yano (HKY) model [18]. All characters were of equal weight and unordered. Bootstrap analysis consisted of 1,000 replicates.

Topology of the combined five genetic marker dataset was generated using UPGMA algorithm and the HKY distance model. Bootstrap analysis consisted of 1,000 replicates. To determine if the GDHA, EF1a, ITS rDNA, nLSU and mtSSU datasets were in significant conflict, the partition homogeneity test option in PAUP was used between the loci in all possible pair-wise combinations, using 100 replicates and the heuristic general search option. The null hypothesis of congruence was rejected if $p<0.001$.

Statistical Analyses

Nonparametric Mann-Whitney test in Minitab [39] was used to determine if the time required for mycelial outgrowth from surface sterilised roots differed significantly when compared asco- and deuteromycetes vs. basidiomycetes. Clark–Evans nearest neighbour method [8] was used to estimate whether spatial distribution of ECM morphotypes in the confined nursery plot was random ($R=1$), even ($R>1$) or clustered ($R<1$). The statistical test of significance of the nearest neighbour statistic provides degree to which the distribution of individuals on a given area differs from that of a random distribution, and is estimated as the ratio of expected and observed mean value of the nearest neighbour distances.

Results

Mycorrhizal Colonisation of Roots

In total, 2,000 individual fine roots were morphotyped and 1,321 (66.1%) of them were found to be ECM. The overall ECM community was comprised of nine distinct morphotypes among which *T. terrestris*, *Hebeloma* sp. and *Suillus luteus* were the most abundant and colonised 793 (39.7%), 355 (17.8%) and 122 (6.1%) of all fine roots, respectively (Table 1). The remaining six morphotypes were rare and all together colonised 51 (2.6%) of roots. Three of those morphotypes could not be matched to published descriptions and remained unidentified (Table 1).

All plants sampled in this study were ECM but the level of colonisation varied considerably between individual plants, ranging between 20% and 100% (Fig. 1). *T. terrestris* occurred in 92.0% of plants, *Hebeloma* sp. in 54.0%, *S. luteus* in 23.0%, and the remaining morphotypes in 21.0% of plants (Table 1). Non-mycorrhizal roots were found in 98.0% of plants (Fig. 1). Different ECMs showed distinct patterns of spatial distribution at the study site. Consequently, Clark–Evans nearest neighbour method revealed an even distribution of dominant ECM morphotypes of *T. terrestris*, *Hebeloma* sp. and *S. luteus* ($R=1.21$–2.10, $p<0.03$), but random distribution of rare morphotypes of *Suillus* sp., *Wilcoxina* sp. and *Piceirhiza bicolorata* ($R=1.05$-1.19, $p>0.05$). Unidentified no.1 and no.2 morphotypes were found in a single plant each, and unidentified no.3 was found in two plants (Fig. 1), thus estimation of their distribution in the study plot was not possible. In some cases, individual plants that were colonised by dominant ECMs of *T. terrestris* and/or *Hebeloma* sp. to a lower degree, showed relatively higher diversity of ECM taxa (Fig. 1).

Community of Root Inhabiting Fungi

Of 2,000 fine roots used for fungal isolation into pure culture, 606 (30.3%) gave fungal growth, and the rest 1,394 (69.7%) either remained sterile or were colonised by bacteria and/or overgrown by rapidly growing fungi from root samples placed in the vicinity within the same dish. Even if occasionally more than one mycelial morphotype was growing out from an individual root sample, only one of those was overgrowing the others, becoming dominant. Consequently, the transfer to a new medium and subculturing was done for a single mycelial morphotype per root sample. In total, this yielded 606 pure cultures which following morphological and molecular identification were found to represent 71 distinct taxa (Fig. 2). Of those, 50 (70.4%) were identified at least to genus level. For unidentified taxa, only four (5.6%) could be matched to ITS rDNA sequences available in the databases, and 18 (25.3%) showed unique sequences. The most commonly isolated fungi were the ascomycetes *Neonectria macrodidyma* (20.3%), *Phialocephala fortinii* (13.5%), *Neonectria radicicola* (6.3%) and the basidiomycete *H. cavipes* (4.5%; Table 2).

Of the total fungal community, 89.9% were asco- and deuteromycetes and 10.1% were basidiomycetes. Results showed that many taxa of asco- and deuteromycetes started

Table 2 Frequency of fungi isolated from root tips of *Pinus sylvestris* seedlings bare-root cultivated in forest nursery

Fungal species	GenBank accession no.	Colonisation (%)	
		Plants	Root tips[a]
Ascomycetes and Deuteromycetes			
Auxarthron conjugatum	HM036583	8.0	1.3
Cadophora finlandica	HM036584	15.0	2.6
Chaetomium funicola	HM036585	22.0	4.5
Chaetomium globosum	HM036586	2.0	0.3
Chaetomium hispanicum	HM036587	6.0	1.0
Chalara microchona	HM036588	1.0	0.2
Coniothyrium sp. aurim1156	HM036589	4.0	0.8
Cylindrocarpon pauciseptatum	HM036590	9.0	1.5
Eupenicillium idahoense	HM036591	1.0	0.2
Eurotium repens	HM036592	2.0	0.3
Fimetariella rabenhorstii	HM036593	1.0	0.2
Fusarium equiseti	HM036594	1.0	0.3
Fusarium oxysporum	HM036595	21.0	3.8
Gibberella avenacea	HM036596	1.0	0.2
Gliomastix murorum	HM036597	2.0	0.5
Hypoxylon serpens	HM036598	1.0	0.2
Lecythophora mutabilis	HM036599	17.0	4.3
Leptodontidium orchidicola	HM036600	1.0	0.2
Neonectria lucida	HM036601	2.0	0.3
Neonectria macrodidyma	HM036602	58.0	20.3
Neonectria radicicola	HM036603	30.0	6.3
Penicillium canescens	HM036604	2.0	0.7
Penicillium chrysogenum	HM036605	5.0	1.2
Penicillium janthinellum	HM036606	2.0	0.3
Penicillium pinophilum	HM036607	11.0	2.3
Penicillium sp. KRP51	HM036608	4.0	0.7
Penicillium sp. KRP56	HM036609	1.0	0.2
Phialocephala fortinii	HM036610	57.0	13.5
Phoma macrostoma	HM036611	3.0	0.5
Pseudeurotium bakeri	HM036612	1.0	0.2
Pseudodictyosporium wauense	HM036613	1.0	0.2
Pseudogymnoascus roseus	HM036614	1.0	0.2
Tetracladium maxilliforme	HM036615	3.0	0.5
Trichoderma gamsii	HM036616	1.0	0.2
Truncatella angustata	HM036617	3.0	0.5
Tuber sp. aurim734	HM036618	2.0	0.3
Ulocladium consortiale	HM036619	1.0	0.2
Unidentified sp. aurim724	HM036620	16.0	3.6
Unidentified sp. KRP100	HM036621	1.0	0.2
Unidentified sp. KRP11	HM036622	7.0	1.2
Unidentified sp. KRP15	HM036623	1.0	0.2
Unidentified sp. KRP24	HM036624	1.0	0.2
Unidentified sp. KRP4	HM036625	1.0	0.2
Unidentified sp. KRP53	HM036626	2.0	0.3
Unidentified sp. KRP54	HM036627	2.0	0.5
Unidentified sp. KRP62	HM036628	1.0	0.2

Table 2 (continued)

Fungal species	GenBank accession no.	Colonisation (%)	
		Plants	Root tips[a]
Unidentified sp. KRP73	HM036629	17.0	3.0
Unidentified sp. KRP76	HM036630	1.0	0.2
Unidentified sp. KRP80	HM036631	8.0	1.5
Unidentified sp. KRP88	HM036632	3.0	0.5
Unidentified sp. KRP90	HM036633	1.0	0.2
Unidentified sp. KRP92	HM036634	1.0	0.2
Unidentified sp. KRP93	HM036635	1.0	0.2
Unidentified sp. KRP94	HM036636	1.0	0.2
Unidentified sp. NS176A	HM036637	5.0	1.2
Unidentified sp. olrim349	HM036638	2.0	0.3
Unidentified sp. PM29C	HM036639	24.0	4.6
Wilcoxina mikolae	HM036640	3.0	0.5
Wilcoxina sp. aurim720	HM036641	2.0	0.3
Zalerion varium	HM036642	1.0	0.2
All Ascomycetes and Deuteromycetes		97.0	89.9
Basidiomycetes			
Armillaria ostoyae	HM036643	1.0	0.2
Asterophora sp. aurim714	HM036644	14.0	2.3
Flagelloscypha minutissima	HM036645	1.0	0.2
Hebeloma cavipes	HM036646	20.0	4.5
Hebeloma helodes	HM036647	1.0	0.2
Psilocybe sp. KRP22	HM036648	1.0	0.2
Rhizopogon roseolus	HM036649	1.0	0.2
Suillus granulatus	HM036650	1.0	0.2
Suillus luteus	HM036651	6.0	1.7
Unidentified sp. KRP27	HM036652	2.0	0.3
Unidentified sp. KRP96	HM036653	2.0	0.3
All Basidiomycetes		43.0	10.1

[a] A single isolate was obtained from each individual root tip with fungal growth

to grow out from the roots significantly earlier as compared to basidiomycetes (Mann-Whitney test, $p<0.0003$; Fig. 3a). An average time required for each taxon isolated in this study to grow out from the surface sterilised fine roots of pine seedlings is presented in Fig. 3b. In many cases, early outgrowing fungi were the asco- and deuteromycetes *Trichoderma gamsii*, *Chaetomium* spp., *Neonectria* spp., *Penicillium* spp. and late outgrowing fungi were the ECM asco- and basidiomycetes *Wilcoxina* spp., *Hebeloma* spp., *Suillus* spp. and *Rhizopogon roseolus*. Unidentified taxa were present among both early and late outgrowing fungi (Fig. 3b).

Although the ECM morphotype of *T. terrestris* was the most abundant in seedling roots (Fig. 1, Table 1), it was never isolated into pure culture. The isolation of the *Hebeloma* sp. morphotype yielded numerous cultures of *H. cavipes* and one of *Hebeloma helodes* while *S. luteus* and *Suillus* sp. yielded cultures of *S. luteus*, *S. granulatus* and *R. roseolus*. Among other root-inhabiting fungi, *Armillaria ostoyae*, a common pathogen in forest stands and decomposer of coarse woody debris [47], was also isolated from a single mycorrhizal root tip.

Intraspecific Diversity of *H. cavipes* Isolates

In total, 27 strains of *H. cavipes* were isolated and analysed in this study. One isolate of *H. helodes* was included for comparison. First, intraspecific diversity of *Hebeloma* isolates was studied by using PCR-RFLP of IGS2 rDNA region, and amplification of this region with primers 5SRNAP and invSR1R resulted in single banded PCR products ca. 2.5 Kbp in size. Double-restriction digestion of those products showed polymorphic fingerprint patterns within area 500–700 bp in size (Fig. 4a). The cluster

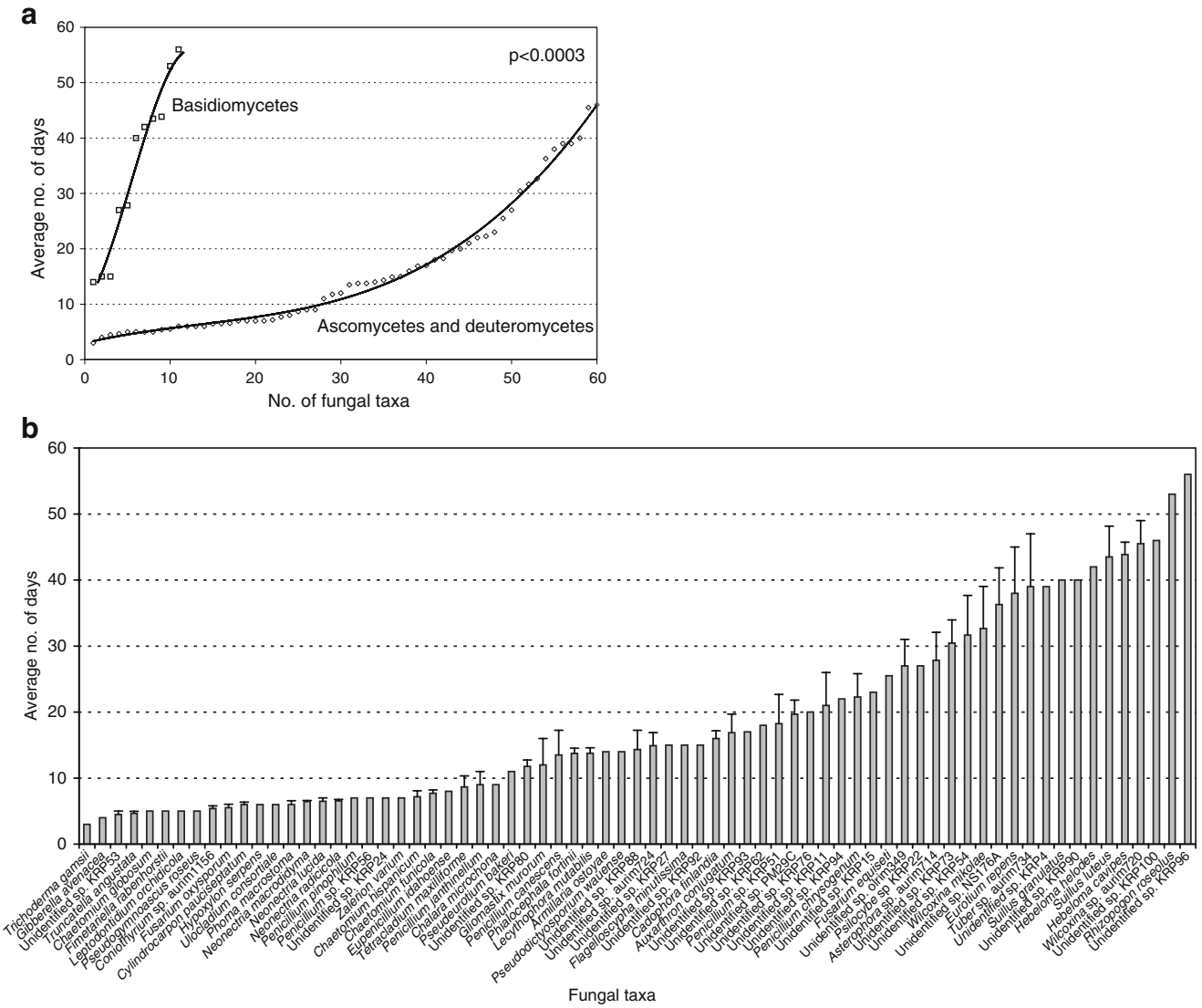


Figure 3 Average time required for fungal mycelia to grow out from surface-sterilised fine roots: **a** as compared ascomycetes/deuteromycetes vs. basidiomycetes, solid lines represent trendlines of actual data; **b** as compared between different fungal taxa isolated in this study. *Error bars* represent standard error of the mean

analysis based on the UPGAMA method detected intraspecific diversity among 27 isolates of *H. cavipes* subdividing those into five clades distinct IGS2 rDNA alleles (Fig. 4b). The largest clade was composed of 18 isolates. Dice's similarity coefficient between different IGS2 rDNA alleles of *H. cavipes* was of at least 72%.

Second, intraspecific diversity of *H. cavipes* isolates was studied using genealogical concordance of five genetic markers. The complete alignment of combined GDHA, EF1a, ITS rDNA, nLSU and mtSSU datasets was 2,638 bp long with 42 polymorphic characters, and of those, 29 were non-informative. Partition homogeneity test showed no significant conflict between the genetic markers used (p= 0.16) allowing to perform analysis on the concatenated sequence data. UPGMA analysis of sequence information of 27 *H. cavipes* isolates identified eight clades denoted A-H in Fig. 4c. Four of those (A, B, F, H) were identical to the ones identified by PCR-RFLP, including the largest clade (A) composed of 18 isolates placement of which was significantly supported by bootstrap analysis (Fig. 4b, c). F clade included three isolates and the remaining six clades were each represented by a single isolate. Mapped positions for each *H. cavipes* isolate and genotype in the nursery plot is shown in Fig. 5. Isolates belonging to the A genotype were positioned in the larger part of the nursery plot and the distance between its most distal isolates was ca. 34.8 m. In several cases, multiple strains (2-5) of *H. cavipes* were isolated from the same root system, but within respective root systems all isolates were of the same genotype.

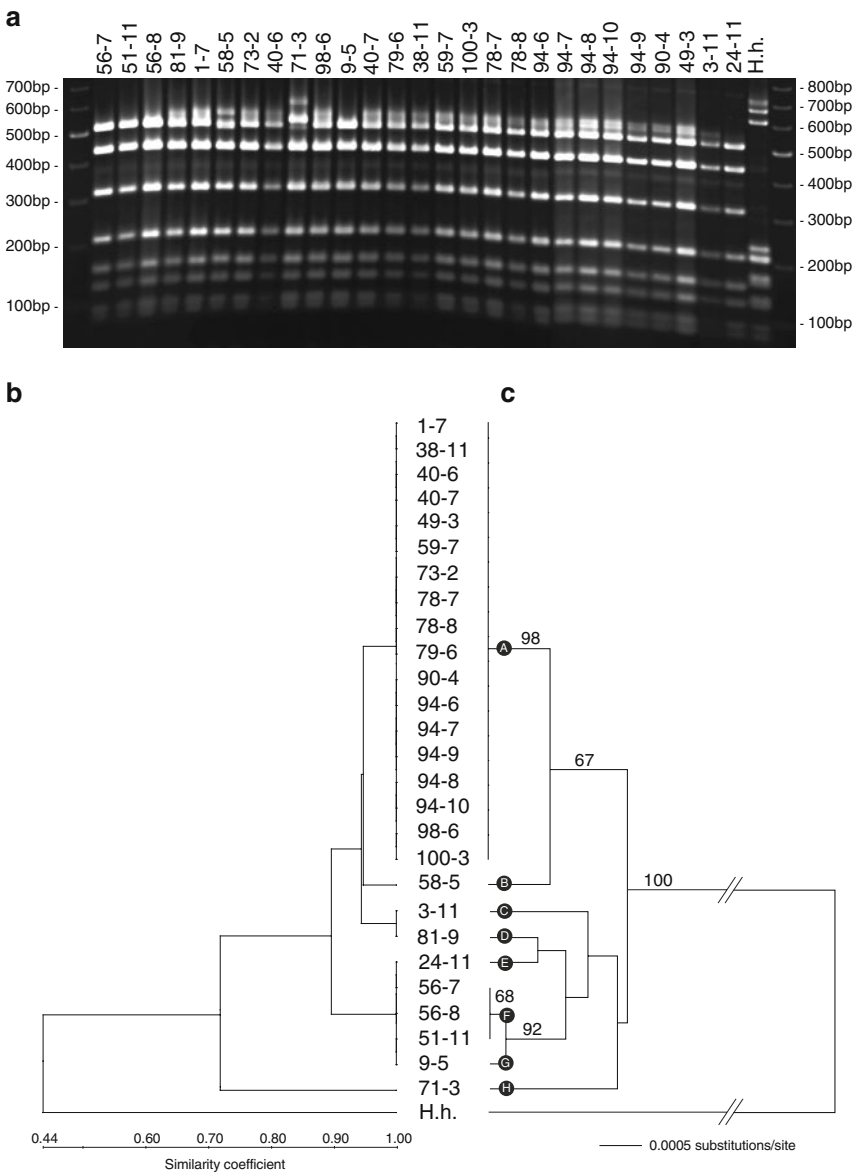


Figure 4 Intraspecific diversity of *Hebeloma cavipes* strains isolated from fine roots of *Pinus sylvestris* seedlings in forest nursery plot: **a** electrophoresis gel with PCR-RFLP patters of IGS2 rDNA region and strain names on the top; **b** UPGAMA tree produced from PCR-RFLP patterns; **c** UPGMA PAUP generated tree based on sequence information of combined five genetic marker dataset. Bootstrap branch support values of 1,000 replicates are indicated near the branches. The tree was rooted using *Hebeloma helodes* (H.h.) as outgroup. Each distinct genotype is labelled by the *capital letters A-H*. Strain names are indicated between the trees

Discussion

The results of the present study on ECM morphotyping demonstrated similar degree of root mycorrhization but lower diversity of ECM morphotypes when compared with related studies conducted in other forest nurseries [22, 25, 36, 46, 54]. A lower degree of morphotype diversity can probably be explained by the predominant colonisation of roots and plants by *T. terrestris*, which capability to outcompete some ECMs was previously reported from dual culture inoculation experiments in forest nursery [44]. Other morphotypes found in this study, especially of *Hebeloma* sp. and *S. luteus*, occurred in a number of roots and plants but all of those taken together were less abundant than that of *T. terrestris*. The observed patterns in spatial distribution of different ECM morphotypes in the studied forest nursery plot were determined by their relative abundance. Consequently, even distribution was found of commonly encountered mycorrhizas (*T. terrestris*, *Hebeloma* sp. and *S. luteus*), and random in rare ones. The majority of ECM morphotypes found in the present study were also reported from both planted and naturally regenerated forest plantations of *P. sylvestris* [21, 23, 37].

The majority of ECM morphotypes were represented by basidiomycetes, but fungal isolation into the pure culture frequently yielded asco- and deuteromycetes. Such bias towards predominant isolation of asco- and deuteromycetes can be explained by their higher in vitro growing rates. Significantly shorter periods of time required for asco- and deuteromycetes to emerge from roots lead to overgrowth of basidiomycetes and, consequently, misrepresentation of the latter in the isolated fungal community. A careful selection

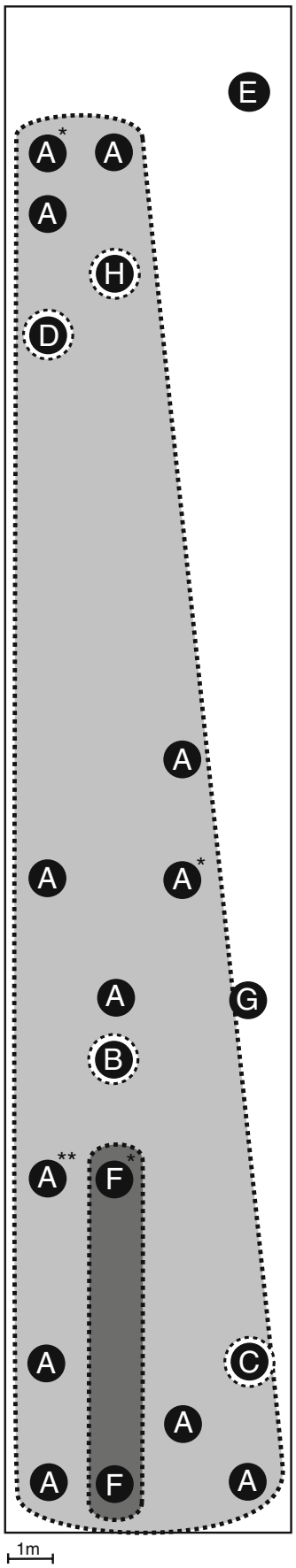


Figure 5 Occurrence and spatial distribution of different genotypes of ECM basidiomycete *Hebeloma cavipes* in confined forest nursery plot. *Capital letters A-H* represents distinct genotypes as in Fig. 4c. Differently shaded area represents the smallest possible size of each genet with multiple isolates. The seedlings from which multiple cultures (two or five) of *H. cavipes* were isolated are marked with one or two stars, respectively

of root surface sterilisation method as was shown in isolation trials of *T. terrestris* [33] and supplementation of nutrients media with fungicides selectively inhibitory to asco- and deuteromycetes (e.g. benomyl) [53] is likely to increase recovery of basidiomycetous taxa. This has to be taken into account while studying fungal communities in roots by mycelial isolations. Obtained dataset indicated that there was an average probability of finding previously undetected taxon in every 33.6 root tip, suggesting that a more diverse fungal community could be revealed with increased sampling effort. Nevertheless, many isolated taxa remained unidentified as their ITS rDNA sequences could not be matched (even to the genus level) to any of reference sequences available in the databases. However, phylogenetic analysis carried out revealed their proximity to the identified taxa and possible affiliation with the certain fungal genera.

In this work, many taxa of saprotrophs and necrotrophs were commonly isolated from the healthy-looking root tips of pine seedlings. *N. macrodidyma* was the dominant fungal isolate. This fungus was recently described as a new species [17], commonly associated with black foot disease of wine grapes in southern Europe and around the southern hemisphere [2, 5]. In our previous studies in Lithuania, this species was also commonly isolated from both healthy-looking and diseased roots of *P. sylvestris* and *Picea abies* seedlings planted in forest nurseries, clearcuts and farmland [36, 38]. This suggests that similarly as many other *Neonectria* spp., *N. macrodidyma* is a plant pathogen well adapted to a wide range of hosts and habitats. However, often isolation of this species from the asymptomatic fine roots may suggest that in those *N. macrodidyma* is present as dormant propagules, or that it is an opportunistic pathogen switching from endophytic to pathogenic behaviour under certain environmental conditions.

Only a small fraction of fungi isolated into pure culture were ECM among which *H. cavipes* was the most abundant. In our previous study conducted in forest nurseries in Lithuania [36], ECM roots of greenhouse cultivated *P. sylvestris* seedlings were commonly colonised by *Hebeloma* sp. DQ068955 for which taxonomic name at that time could not be established in the absence of reference sequence at any of available databases. Comparison of 614 bp long ITS rDNA sequences of all *H. cavipes* strains isolated in this study to the latter sequence showed 99.5-100% homology, indicating that all of those represent

the same fungal species. This suggests that *H. cavipes* is a common ECM symbiont of *P. sylvestris* seedlings in forest nurseries in Lithuania.

In the present study, the two different molecular fingerprinting methods used were largely consistent in revealing genetic diversity within *H. cavipes* isolates. In particular, this was notable for most common genotypes. Consequently, a simple and relatively fast PCR-RFLP approach using species specific primers, as shown before [15], should allow both to process a large number of samples (also environmental) and to assess diversity in local ECM populations. The results of this study suggest that *H. cavipes* is largely disseminated by vegetative means of local genotypes and that nursery cultivation practices are likely to contribute to the dissemination of this species in the forest nursery soils. However, taken into the account that the nursery site was derived in the past from the forest land, the possibility should not be excluded that observed large genet (A) preceded the establishment of the nursery.

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