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Diversity and ecology of *Armillaria* species in virgin forests in the Ukrainian Carpathians

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Abstract In this study, we investigated the diversity and ecology of *Armillaria* species in virgin pure beech and mixed conifer forests (15,000 ha) of the Carpathian Biosphere Reserve in Ukraine. *Armillaria* rhizomorphs were systematically sampled, both from the soil and from the root collar of trees (epiphytic), on 79 plots (25×20 m) of a 1.5×1.5 km grid. In both forest massifs, rhizomorphs were present in the majority of the soil samples, with an estimated dry weight of 512 kg/ha in the pure beech forests and 223 kg/ha in the mixed conifer forests. Similarly, in both forest massifs, most of the trees inspected had rhizomorphs at the root collar. Species identification based on DNA analyses showed that all five annulated European *Armillaria* species occur in these virgin forests, as previously observed in managed forests in central Europe. However, differences in the frequencies of the single species were observed. The predominance of the preferentially saprotrophic *A. cepistipes* and *A. gallica* (84 and 15% of the specimens, respectively) and the absence of significant pathogenic activity suggest that in these virgin forests *Armillaria* species are most likely to behave as saprotrophs. Forest management may increase the frequency of the pathogenic species *A. ostoyae*, which is rare in virgin forests.

Keywords Rhizomorphs · Wood-decaying fungi · Natural forests · The Carpathian Biosphere Reserve · Forest management

Introduction

The basidiomycete genus *Armillaria* (Fr.: Fr.) Staude is an important natural component of the mycoflora in forest ecosystems worldwide (Shaw and Kile 1991). All *Armillaria* species are able to survive saprotrophically by degrading woody substrates, and typically produce, in the soil or under the tree bark, highly differentiated filamentous aggregations named rhizomorphs (Garraway et al. 1991). Several species can also act as primary or secondary pathogens causing root rot on a wide range of trees and shrubs (Kile et al. 1991). At present, about 40 *Armillaria* species are known (Watling et al. 1991; Pegler 2000), 7 of which also occur in European temperate and boreal forests (Guillaumin et al. 1989; Zolciak et al. 1997). The individual species differ in ecological behavior, geographical distribution, and host preference (Shaw and Kile 1991).

The occurrence and ecology of *Armillaria* species have been well investigated in managed forest stands of western, southern, and south-eastern Europe (Guillaumin et al. 1993; Rigling et al. 1998; Tsopelas 1999; Prospero et al. 2003a; Keča et al. 2009; Oliva et al. 2009; Lushaj et al. 2010). In contrast, little detailed information is available for eastern European regions (Lochman et al. 2004; Łakomy 2006; Kaliszewski et al. 2007), and most studies only refer to *Armillaria mellea* sensu latu (e.g., Dudka et al. 1997).

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Moreover, since most European forest stands have been managed for centuries (Bengtsson et al. 2000), very little is known about the diversity, ecology, and distribution of *Armillaria* species in natural, undisturbed forest ecosystems in temperate Europe. The few remnants of natural forests that could be potentially investigated are not larger than 50–100 ha, while continuous forest areas of more than 1,000 ha are very rare.

In the Transcarpathian region of Ukraine (south-west), the Carpathian Biosphere Reserve (CBR) offers a unique opportunity for studying the biodiversity and natural processes in virgin or primeval forest ecosystems, i.e. forests that have never been significantly modified by human activity. The region covers an area of about 53,650 ha and became part of the World Network of Biospheres Reserves of UNESCO in 1992. It consists of six detached massifs and two botanical reserves (Brändli and Dowhanytsch 2003; Brändli et al. 2008; Hamor et al. 2008). About 80% of the CBR's territory is covered with mainly virgin (i.e., without any anthropogenic influences from the past; Parviainen 2005) or natural (i.e., naturally developing but with possible anthropogenic influences from the past; Parviainen 2005) forests that have never been clear-cut or artificially replanted (Brändli and Dowhanytsch 2003). Thus, the forest associations that occur there are probably representative of those in forests in central Europe prior to human influence. Oak (*Quercus robur* and *Q. petraea*) stands are predominant in the foothills, whereas at the mountainous level beech (*Fagus sylvatica*), mixed, and spruce (*Picea abies*, *Abies alba*) forests are present. Subalpine and alpine vegetation include pine (*Pinus mugo*) and green alder (*Alnus viridis*) forests and meadows. Regarding age structure, almost 50% of the CBR's stands can be considered mature and old overgrowth, with trees aged up to 250 years for beech and 400 years for silver fir (Hamor et al. 2008; Brändli and Dowhanytsch 2003). The forest area is characterized by a high abundance of dead wood in various stages of decay, which harbors a rich variety of fungal species involved in wood decomposition (Brändli et al. 2008).

Virgin forests are of interest for researchers because they have preserved their original structure and dynamics. Findings from studying these forests can benefit the close-to-nature management of exploited forests (Brändli et al. 2008). In natural forest ecosystems, native pathogenic organisms are thought to be important in regulating plant species diversity and distribution (Castello et al. 1995). Soil-borne fungal pathogens, such as the *Armillaria* species or *Phellinus weirii*, selectively remove the less vigorous trees, thereby producing canopy gaps and woody substrates which help forest regeneration (Holah et al. 1997; Bendel et al. 2006a). Artificial changes due to management practices (e.g., logging, modification of tree

species composition) may greatly alter the ecological balance by increasing the impact of pathogens on forest structure and composition (Castello et al. 1995; Jactel et al. 2009). For example, when native forests are converted to exotic plantations, indigenous *Armillaria* species can cause considerable tree mortality (e.g., *Armillaria* root rot in *Pinus radiata* stands in New Zealand; Van der Pas 1981). The creation of fresh stumps through sylvicultural operations provides new food-bases for *Armillaria* species and stimulates the production of rhizomorphs in the soil (Stanosz and Patton 1991). Forest management may also modify the natural balance between different *Armillaria* species, sometimes resulting in pathogenic behavior by hitherto preferentially saptrotrophic species (Legrand et al. 1996).

The main aim of this study was to characterize the large-scale occurrence of *Armillaria* species in two virgin forest massifs (pure beech and conifer/mixed forests) of the CBR. Specifically, we: (1) quantified the presence of *Armillaria* rhizomorphs in the soil and on the root surface of trees; (2) assessed the impact of vegetation type, soil pH, and altitude on the occurrence of *Armillaria* rhizomorphs; and (3) determined the composition of the *Armillaria* species community. These findings provided a basis for making inferences about the ecological role of the genus *Armillaria* in the virgin forests of the CBR.

Materials and methods

Study area

Our study was conducted in the core and buffer zones of two protected massifs (Uholsko-Shyrokoluzhanskyi and Chornohirskyi) of the CBR (Fig. 1). Here, forests, because of the absence of human activity (e.g., clear and selective cuttings, artificial plantations of non-indigenous trees, or livestock grazing), have preserved their natural structure and dynamic and can, therefore, be considered virgin (Shelyag-Sosonko 1997; Commarmot et al. 2005; Parviainen 2005; Brändli et al. 2008; Rizun and Chumak 2008).

In the Uholsko-Shyrokoluzhanskyi massif (10,383 ha), pure beech virgin forests occupy 88% of the total area and are included in UNESCO's World Heritage list. Typical characteristics of these forests are a high frequency of trees in the upper diameter classes (30–80 cm), a volume of standing and lying deadwood considerably higher than in comparable managed beech stands, and a small-scale gap dynamic (Commarmot et al. 2005). The massif, which consists of two contiguous areas (Uholka and Shyrokyi Lug forestry), is located between 400 and 1,350 m a.s.l., with the timberline at about 1,150 m a.s.l. (Brändli and Dowhanytsch 2003). The region is characterized by acidic
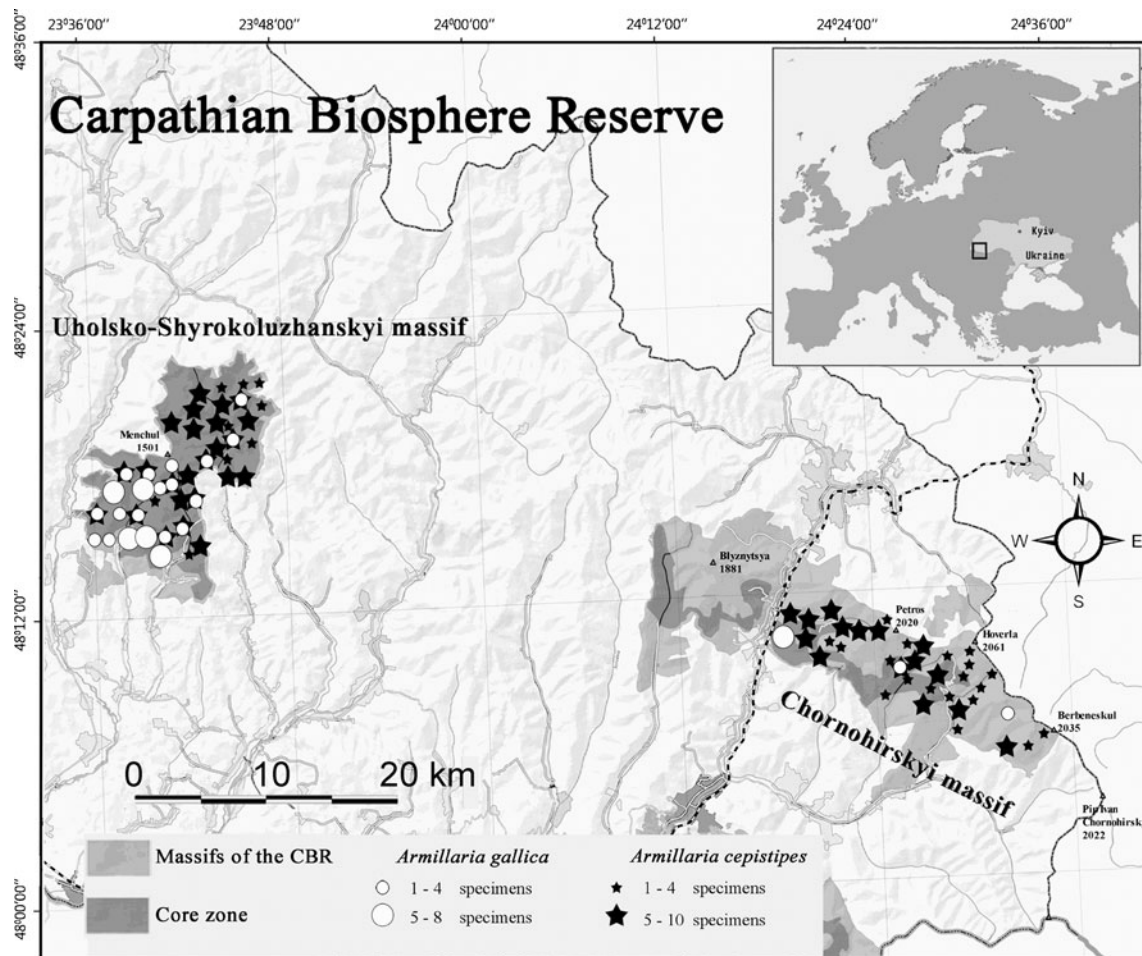
Fig. 1 Location of the two investigated protected forest massifs (Uholsko-Shyrokoluzhanskyi and Chornohirskyi) of the Carpathian Biosphere Reserve in south-western Ukraine and spatial distribution and frequency of *Armillaria gallica* and *A. cepistipes* rhizomorphs in the soil

brown soils (dystric cambisols) with high content of rough humus (12–15%) in the upper horizons and a litter layer of about 5 cm (Voitkiv 2008; Hamor et al. 2008).

The climate is characterized by an annual average temperature of +7°C and an annual average precipitation of 948 mm (Brändli and Dowhanytsch 2003). In the Uholka area, the annual temperatures are slightly higher and the vegetation period longer than in the Shyrokyi Lug area (Tasenkevich et al. 1982).

In the Chornohirskyi massif (4,127 ha), 67% of the forests are considered to be natural or virgin (Brändli and Dowhanytsch 2003). Most of the massif (79%) is covered by coniferous mountain forests (fir–spruce forests) and mixed beech–fir–spruce forests (Hamor et al. 2008). The region lies between 700 and 2,061 m a.s.l., with the timberline at 1,500–1,550 m a.s.l. (Shelyag-Sosonko et al. 1997). Soils (dystric cambisols) are generally acidic but present a minor content of rough humus (10–12%) in the upper horizons and a thinner litter layer (3 cm) than in the Uholsko-Shyrokoluzhanskyi massif (Voitkiv 2008; Hamor et al. 2008). The massif belongs to three different

climate zones with annual average temperatures ranging from 0 to +7°C and annual average precipitation varying between 1,000 and 1,500 mm (Brändli and Dowhanytsch 2003).

Sampling

In both forest massifs, *Armillaria* was systematically sampled on the plots of the 1.5×1.5 km square grid of the large-scale inventory. Seventeen out of 96 plots were not included in the sampling because they were located outside the core or buffer zones of the protected massif, i.e., in areas where influences of past human activity could not be completely excluded. In each plot, a 20×25 m (500 m^2) rectangle was established and samples of soil (15×15× 15 cm) were taken at all four corners of the rectangle. The soil samples were sieved through a 9-mm square mesh to separate the roots and rhizomorphs from the soil (Prospero et al. 2003b). All rhizomorphs found were collected and taken to the laboratory where, for each soil sample, the number of individual rhizomorphs, their diameter, fresh

weight, and dry weight (after lyophilisation for 12 h) were determined. For pH measurement, 100 g of the sieved soil were taken at each sampling point. In addition, the nearest tree to each soil sampling point (i.e., four trees per plot) was checked for epiphytic rhizomorphs (i.e., rhizomorphs attached to the surface) on the root collar. Samples of these epiphytic rhizomorphs were then detached and the species identified in the laboratory.

In the lowland of the Uholsko-Shyrokoluzhanskyi massif, 20 Armillaria fruiting bodies were collected along a trail. Species identification showed that all belonged to A. gallica, apart from one which was of A. mellea. Giving that fruiting body sampling was not systematically conducted, these data were only used to confirm the presence of A. mellea in the investigated area.

Armillaria isolation

Armillaria was isolated from the rhizomorphs as described by Prospero et al. (2003a). Three segments 1 cm in length of one rhizomorph (randomly selected) from each soil sample and one epiphytic rhizomorph (randomly selected) from each tree were dipped in 50% ethanol for 15–20 s. Subsequently, they were surface-sterilized in 30% hydrogen peroxide (H_2O_2) for 25–40 s and placed on a semi-selective agar medium (12 g 1^{-1} malt extract, 15 g 1^{-1} Bacto Agar amended with 2 mg 1^{-1} benomyl and 100 mg 1^{-1} strepto-mycin; Maloy 1974). The isolation plates were incubated in the dark at 20–25°C. After 1–3 weeks, pure cultures were transferred to Diamalt agar (15 g 1^{-1} Bacto Agar, 20 g 1^{-1} Diamalt; Hindelbank, Switzerland). All remaining rhizomorphs were frozen at −20°C for genetic analyses.

Soil pH

The pH of each soil sample was determined as described by Voznyuk and Kuzmich (1984). Briefly, 8 g of air-dry soil were dissolved in 20 ml of distilled water and the actual acidity was determined at room temperature using a Knick pH-Meter 761 Calimatic.

Species identification

The first attempt to identify the Armillaria species involved conducting classic interfertility tests (Korhonen 1978) and PCR-RFLP analysis of a portion of the intergenic spacer (IGS-1) region of the ribosomal DNA (Harrington and Wingfield 1995). Many of the results were ambiguous. Thus, additional genetic analyses, i.e., sequencing of the IGS-1 and ITS regions of the ribosomal DNA, and PCR-RFLP analysis and sequencing of the translational elongation factor 1-α region of the nuclear DNA, had to be conducted to identify the species clearly.

Interfertility tests Twenty-two unknown isolates were paired with three different haploid tester strains (Korhonen 1978) of the five annulated European Armillaria species (A. borealis Marxmuller and Korhonen, A. cepistipes (Velenovsky), A. ostoyae (Romagnesi) Herink, A. mellea (Vahl:Fr.) Kummer, A. gallica Marxmüller and Romagnesi) as described in Harrington et al. (1992). The tester strains originated from the mycological culture collection of WSL (Birmensdorf, Switzerland) and were initially provided by J.-J. Guillaumin (INRA, Clermont-Ferrand, France).

DNA extraction DNA was extracted from 50 mg of lyophilized (12 h) rhizomorphs (one per soil sample and one per tree) and from 30 mg of lyophilized (12 h) mycelium, which was obtained from 3-week-old Armillaria pure cultures on Diamalt agar. The extraction was performed using the CTAB method described in Gardes and Bruns (1993). The DNA was re-suspended in 50 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4°C until use.

PCR amplification The IGS-1 and the ITS (ITS-1, 5.8S, and ITS-2) regions of the ribosomal DNA and the translational elongation factor 1-α (EF1-α) region of the nuclear DNA were amplified by PCR. The PCR reactions were performed in 50-μL volumes with the following final concentrations: 1× reaction buffer (Sigma), 4 mM $MgCl_2$, 100 μM dNTPs (Promega), 20 pmol of each primer (10 pmol for the ITS region), 2.5 U Taq DNA polymerase (Sigma), and about 50 ng of DNA template. For each region, specific forward and reverse primers were used (IGS-1: LR12R and O-1, Veldman et al. 1981; Duchesne and Anderson 1990; ITS: ITS1 and ITS4, White et al. 1990; EF-1α: EF595F and EF1160R, Maphosa et al. 2006). The IGS-1 and ITS regions were amplified using a PCR program with an initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 2 min, and 1 cycle of 72°C for 30 min. For amplification of the EF1-α region, the following PCR program was used: 1 cycle of 94°C for 2 min, followed by 33 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, and 1 cycle of 72°C for 30 min. The PCR products were checked for successful amplification and their length was determined in 1.5% agarose gels.

RFLP analysis For species identification, the PCR products of the IGS-1 region were digested with the four restriction enzymes Alu I, Hinc II (Hind II), Mva1269 I (Bsm I), and Nde I (Fermentas) (Harrington and Wingfield 1995). The digest was performed according to the manufacturer's protocol using 10 μL of the PCR product. The sizes of the restriction fragments were determined on 3% agarose gels after running at 99 V/cm for 50 min. Species

identification was considered successful if the resulting pattern corresponded to one of the previously described patterns for the five annulated European *Armillaria* species (Harrington and Wingfield 1995; Kim et al. 2000; Keča et al. 2006; Pérez-Sierra et al. 1999). Samples with a mixed *A. cepistipes* and *A. gallica* RFLP pattern in the IGS-1 region were further analyzed in the EF1-α region by digesting the EF1-α amplicon (approx. 600 bp) with the restriction enzyme *Alu* I. The three haploid tester strains of *A. cepistipes* and *A. gallica* previously used for interfertility tests served as references for the EF1-α RFLP analysis. Samples that showed the same pattern as the three *A. cepistipes* testers (i.e. two bands of approx. 444 and 156 bp) were considered to belong to *A. cepistipes*, and samples with the same pattern as the three *A. gallica* testers (i.e. two bands of approx. 560 and 40 bp) were considered to be *A. gallica*.

Sequencing of the IGS-1, ITS, and EF-1α regions The PCR products were purified using PCR purification columns (MinElute PCR Purification Kit; Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. Sequencing reactions were carried out with the BigDye Terminator Cycle Sequencing Reaction v3.1 Kit (Applied Biosystems, Carlsbad, CA, USA) following the protocol provided by the manufacturer. Post-reaction clean-up was performed using DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD, USA) according to the manufacturer's protocol. Each region was sequenced separately in both directions using the above-mentioned specific primers. The sequences were generated using an ABI 3130 sequencer (Applied Biosystems). All sequences were edited manually using the software GeneStudio (TM) Professional Edition Version 2.1.2.3, and then analyzed with CLC Sequence Viewer Version: 4.6.1 (CLC bio).

Statistical analysis

The data were analysed statistically with the software JMP 8 (SAS Institute). For an effect to be considered statistically significant, we used a level of significance of 5% ($p<0.05$). The relationship between dry weight, diameter, and the number of rhizomorphs in the soil (dependent variables) and the altitude and soil pH of the plots (independent variables) was analysed using a multiple regression analysis (B = regression coefficient, R = coefficient of multiple correlation, r^2 = coefficient of multiple determination, and p = probability of error). The relationship between the presence of epiphytic rhizomorphs on the roots (yes/no) and the amount of rhizomorphs in the corresponding soil samples (dry weight) was investigated with Pearson's coefficient of correlation r. Finally, the relationship

between the presence of epiphytic rhizomorphs (dependent variable) and the altitude and soil pH of the plots (independent variables) was investigated with logistic regression. The frequencies of *A. cepistipes* and *A. gallica* rhizomorphs in the soil and on the root surface of trees were compared using chi-square.

Results

Incidence of *Armillaria* rhizomorphs

Armillaria rhizomorphs were found in all 79 sample plots in the two forest massifs, in a total of 216 soil samples (68%) and on the roots of 240 trees (76%). In the massif with pure beech forests, rhizomorphs were present in 85% of the soil samples and on the root collar of 81% of the inspected trees (Table 1). In the conifer and mixed forest massif, rhizomorphs showed a lower frequency and were more frequent on the root collars (65% of the observed trees) than in the soil (53% of total samples). Consequently, the estimated total rhizomorph biomass in the soil was considerably higher in the pure beech forests (512 kg/ha) than in the conifer and mixed forests (223 kg/ha). Within the same soil pH class and altitudinal range, the rhizomorph biomass in the soil was about three times higher in the pure beech forests than in the conifer and mixed forests.

Multiple regression analyses showed that, in both massifs, the altitude of the plots significantly affected the amount of rhizomorphs (dry weight and number) in the soil (Table 1). The frequency of rhizomorphs in the soil and on the surface of the root collar significantly decreased with increasing altitude (Fig. 2a). In the pure beech forest massif, 80% of all soil rhizomorph dry mass was found at altitudes between 500 and 910 m a.s.l., but in proximity to the timberline (1,000±150 m a.s.l.) rhizomorphs were less frequent. The same trend was observed in the conifer and mixed forest massif with an altitude correction due to the different altitude of the timberline (80% of the rhizomorph's mass found between 590 and 1,350 m a.s.l.; rhizomorphs were rare above 1,400 m a.s.l.). Similarly, epiphytic rhizomorphs on the root collars were significantly less frequent at high altitudes (Fig. 2a). In the massif with pure beech forests, up to 1,000 m a.s.l. 92% of the inspected beech trees had *Armillaria* rhizomorphs on their root collars. Near the timberline (1,000±150 m a.s.l.) only 75% of the trees were positive for rhizomorphs. In the conifer and mixed forest massif, below 1,400 m a.s.l. epiphytic rhizomorphs were present on the root collars of 77% of the trees, whereas near the timberline (approx. 1,400 m a.s.l.) they were only on 39% of the trees (Table 1).

Table 1 Characteristics of the *Armillaria* rhizomorphs found in the soil and epiphytically on the roots of trees in the Uholsko-Shyroko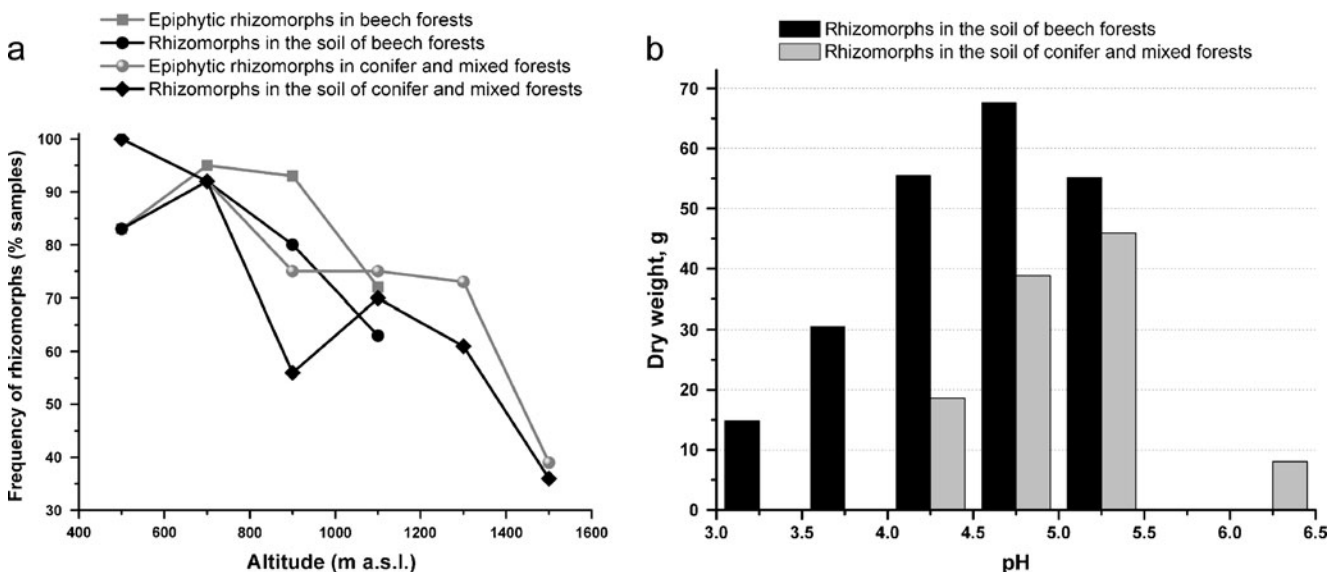luzhanskyi (pure beech forests) and in the Chornohirskyi (conifer and mixed forests) massifs in the Carpathian Biosphere Reserve in Ukraine and influence of altitude of the plots, soil pH, and altitude × soil pH on their abundance

Rhizomorphs	Beech forests	Conifer/mixed forests
In the soil		
Incidence of positive sample points	140/172 (81%)	76/144 (53%)
Mean weight per sample, g	1.18±0.08	0.51±0.06
Biomass (dry weight), kg/ha	520	226
Mean rhizomorph diameter per sample, mm	1.17±0.05	0.82±0.05
Altitudinal range with the majority of rhizomorphs, m a.s.l. (80% of total dry weight)	500-910	590–1,350
pH range with the majority of rhizomorphs (80% of total dry weight)	3.5-5.0	4.0–5.0
Influence of altitude of the plots[a]	$r=-0.42$, $r^2=0.18$, $p<0.0001$	
Influence of soil pH[a]	$r=0.28$, $r^2=0.08$, $p=0.012$	
Influence of altitude of the plots×soil pH[b]	$R=0.54$, $r^2=0.26$, $p<0.0001$	
	BpH=0.33, Balt=−0.46	
Epiphytic on the roots		
Incidence of positive trees	146/172 (85%)	94/144 (65%)
Altitudinal range with 80% of all positive trees (m a.s.l.)	500–970	590–1,350
Frequency of positive trees (% of inspected trees)	97	78
Frequency of positive trees above the altitudinal range (% of inspected trees)	75	43
Influence of altitude of the plots[a]	$r=-0.36$, $r^2=0.13$, $p<0.0001$	
Influence of soil pH[a]	$r=0.06$, $r^2=0.004$, $p=0.26$	
Influence of altitude of the plots×soil pH[b]	$R=0.37$, $r^2=0.136$, $p<0.0001$	
	BpH=0.093, Balt=−0.37	

[a] *r* Pearson's coefficient of correlation; r^2 coefficient of determination; *p* probability of error

[b] *R* coefficient of multiple correlation; r^2 coefficient of multiple determination; *B* regression coefficient; *p* probability of error

In both massifs, the amount of rhizomorphs (dry weight) in the soil was also affected by the soil pH (Table 1). Most rhizomorphs (74% of total dry weight) were found in plots with soil pH between 4.0 and 5.0, and less than 5% below pH 3.5 and above pH 5.5 (Fig. 2b). In contrast, the soil pH had no significant influence on the frequency of epiphytic rhizomorphs on the root collars (Table 1).

Fig. 2 Relationship between the presence of *Armillaria* rhizomorphs and **a** plot altitude (soil and epiphytic rhizomorphs) and **b** soil pH (soil rhizomorphs) in beech forests and conifer/mixed forests in the Uholsko-Shyrokoluzhanskyi and Chornohirskyi massifs

The simultaneous presence of rhizomorphs in a soil sample and on the root collar of the nearby standing tree was observed at 85% of the sample points in the beech forests and for 74% in the conifer and mixed forests (Fig. 3). Pearson's correlation tests showed that the presence of epiphytic rhizomorphs on the root collars was significantly correlated to the presence of rhizomorphs in the soil ($r=0.46$, $p<0.0001$).

Armillaria species identification

In total, 424 rhizomorphs could be assigned to an *Armillaria* species. *Armillaria cepistipes* was the dominant species (84% of the specimens), followed by *A. gallica* (15% of the specimens). The remaining 1% of the specimens were classified as *A. ostoyae* (five specimens) or *A. borealis* (one specimen). The two rare species were easily discriminated using interfertility tests and a PCR-RFLP analysis of the IGS-1 region. In fact, the same restriction fragment patterns were observed as previously reported for European *Armillaria* species (Harrington and Wingfield 1995; Kim et al. 2000; Keča et al. 2006; Pérez-Sierra et al. 1999). In contrast, the identification of the two genetically, morphologically and ecologically close *A. cepistipes* and *A. gallica* was much more problematic. Interfertility tests for some isolates gave ambiguous results and sequencing the IGS-1 and ITS amplicons revealed heterogeneous intra-specific and interspecific variations in 35% of the specimens. These problematic specimens could only be assigned to either *A. cepistipes* or *A. gallica* by performing sequence and PCR-RFLP analyses of the EF1-α region. The EF1-α region of the three tester strains of both *A. cepistipes* and *A. gallica* were sequenced

beforehand. A restriction enzyme was then identified (*Alu* I) that produced different fragment patterns for the two species (*A. cepistipes*: approx. 560 and 44 bp; *A. gallica*: approx. 444 and 156 bp). All PCR products of the EF1-α region of the problematic specimens were digested with *Alu* I, and only one of the two expected patterns (*A. cepistipes* or *A. gallica*) were observed from each specimen.

Armillaria species ecology

The five *A. ostoyae* specimens (rhizomorphs) were collected at four different locations in the pure beech forest massif. Three rhizomorphs were found in two plots on the root collar surface of three beech trees at an altitude ranging from 600 to 700 m a.s.l, whereas two rhizomorphs were found in the soil in two plots at 1,000–1,100 m a.s.l. and with a soil pH 4.01–5.0. The single *A. borealis* rhizomorph found originated from a soil sample (pH 4.74) taken in a plot (1,210 m a.s.l.) dominated by silver fir (*Abies alba*) in the conifer forest massif.

The two dominant species, *A. cepistipes* and *A. gallica*, were found both in the soil and epiphytically on the root collars of trees (Table 2). In 67% of the plots only *A. cepistipes* occurred, in 4% only *A. gallica* and in 27% both species. When considering only the plots with either *A. cepistipes* or *A. gallica*, the estimated rhizomorph biomass (dry weight) in the soil was similar for both species, i.e., 397 kg/ha for *A. cepistipes* and 384 kg/ha for *A. gallica*. In plots with both species co-occurring, the same species was observed in the soil and on the root surface in 70% of the sample points. Based on a chi-square test, the frequencies of both *Armillaria* species in the soil and on the root collars were not significantly different ($\chi^2=0.09$, $p=0.76$).

Armillaria cepistipes was the dominant species at all altitudes in all the forest associations investigated. Compared to *A. gallica*, *A. cepistipes* was more frequent at higher altitudes (up to 1,500 m a.s.l.) and in soils with a pH ranging from 3.5 to 5.5 (Table 3). *Armillaria gallica* was preferentially found (76% of the specimens) in the pure beech forest of the Uholka area in the Uholsko-Shirokoluzhanskyi massif (Fig. 1). Most *A. gallica* rhizomorphs (96%) were collected in plots at low altitudes (509–1,060 m a.s.l.), with only one specimen collected in a *Piceetum-abietis* stand at 1,480 m a.s.l. Almost 67% of the *A. gallica* soil rhizomorphs originated from acid soils (pH 3.0–4.51), and no rhizomorphs of this species were found in soils with a pH>5.4 (Table 3).

Discussion

In this study, we investigated the occurrence and distribution of *Armillaria* species in two virgin forest massifs in the Ukrainian Carpathians by systematically
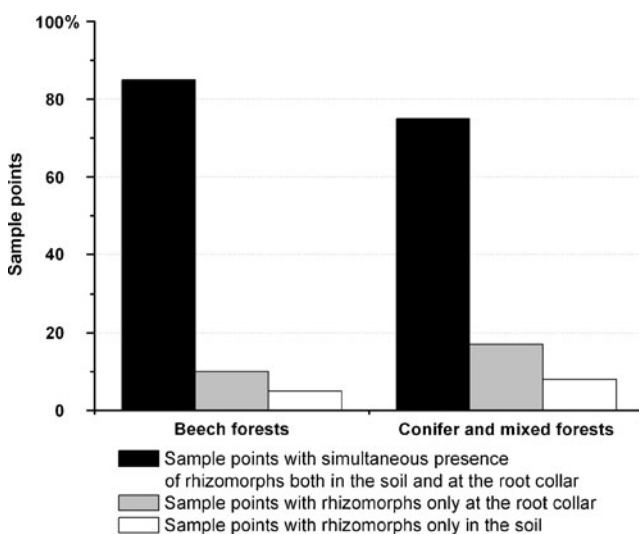

Fig. 3 Incidence of *Armillaria* rhizomorphs in a soil sample and on the root collar of the nearby tree in beech forests and in mixed conifer forests in the Uholsko-Shyrokoluzhanskyi and Chornohirskyi massifs

Table 2 Incidence of *Armillaria cepistipes* and *A. gallica* rhizomorphs in the soil and on the root collar of trees (epiphytic rhizomorphs) in the different forest associations present in the Uholsko-Shyrokoluzhanskyi and Chornohirskyi forest massifs

Protected massif	Forest association[a]	Plots (*n*)	Inspected trees (*n*)	Dry weight of soil rhizomorphs[b] (g/plot)	Frequency of trees with epiphytic rhizomorphs	*A. cepistipes*, rhizomorphs (epiphytic/soil)[c]	*A. gallica*, rhizomorphs (epiphytic/soil)[c]
Uholsko-Shyrokoluzhanskyi	Fagetum (silvaticae)	40	160	4.79±1.54	0.88	100/89	31/20
Chornohirskyi	Other broadleaved associations	3	12	2.19±0.44	1.00	10/7	2/4
Chornohirskyi	Piceetum (abietis)	19	76	1.92±0.85	0.62	44/36	1/0
Uholsko-Shyrokoluzhanskyi and Chornohirskyi	Other mixed conifer and broadleaved associations	12	48	3.08±1.62	0.81	29/23	0/5
Chornohirskyi	Other conifer associations	5	20	0.89±0.11	0.50	9/8	0/0
	Total	79	316	-	-	192/163	34/29

[a] According to Shelyag-Sosonko et al. (1997)

[b] Mean values and standard errors are given

[c] Number of epiphytic rhizomorphs/Number of soil rhizomorphs

sampling rhizomorphs in the soil and on the root collars of trees (epiphytic rhizomorphs).

The high abundance of rhizomorphs suggests that the genus *Armillaria* is an important component of the mycoflora in the virgin forests investigated, where it most likely plays a significant role as a wood decomposer. This hypothesis is supported by (1) the total absence of remarkable tree mortality caused by pathogenic fungi, and (2) the predominance of the preferentially saprotrophic *Armillaria* species. In the region studied, *A. cepistipes* is the most frequent species, followed by *A. gallica*. *Armillaria ostoyae*, *A. borealis*, and *A. mellea* are also present, but their frequency is very low. Thus, our study confirms the presence of all five annulated European *Armillaria* species in south-western Ukraine, as previously reported for the neighboring countries (Łakomy 2006; Kaliszewski et al. 2007; Antonin et al. 2009).

The two predominant species, *A. cepistipes* and *A. gallica*, are considered to be preferential saprotrophs, forming dense networks of rhizomorphs in the soil (Guillaumin et al. 1993; Rigling et al. 1998; Prospero et al. 2003b). Through soil rhizomorphs they may reach new, still unexploited resources (i.e., food bases), such as living trees. Our study indicates that, in natural, unmanaged beech and conifer/mixed forests, whenever *A. cepistipes* and *A. gallica* rhizomorphs are present in the soil, they are also most likely to be found epiphytically on the root collars of living trees. Abundant epiphytic rhizomorphs of *A. gallica* and *A. cepistipes* have been previously reported from the root collars of various tree species, including pedunculate oak (*A. gallica*; Marçais and Caël 2006), sugar maple (*A. gallica*; Marçais and Wargo 2000), and Norway spruce (*A. cepistipes*; Prospero et al. 2003a). Given that healthy trees

can usually prevent colonization of their roots by *A. cepistipes* and *A. gallica*, epiphytic rhizomorphs of these two species probably wait until conditions are favorable to penetrate the bark. In managed forests, bark penetration usually happens after trees are felled and fresh stumps are created (Prospero et al. 2006). In unmanaged natural forests, roots may be penetrated by *Armillaria* when trees are broken or uprooted by strong winds or weakened by drought or defoliating insects (Marçais and Bréda 2006). Why soil rhizomorphs are more abundant in pure beech forests than in conifer and mixed forests probably has not solely to do with differences in altitude and soil pH. In fact, even within the same altitude and soil pH class, soil rhizomorphs are generally more abundant in pure beech forests than in conifer and mixed forests. Thus, it seems likely that vegetation type plays an important role. Previous studies have shown that the density of rhizomorphs is increased by the presence of woody substrates, such as small woody debris, understory vegetation, and root fragments (Lamour et al. 2007). Supporting these observations, in the pure beech forests we investigated, woody substrates were more abundant than in the conifer and mixed forests (Hamor et al. 2008). Comparing our data on abundance of soil and epiphytic rhizomorphs with those from managed forests is difficult because of the absence of similar studies. In managed Norway spruce stands in the Swiss Alps, Prospero et al. (2003a) found that the rhizomorph network formed by *A. cepistipes* can be very dense, reaching up to 25 m/m^2. Similarly, Stanosz and Patton (1991) evidenced an abundant presence of soil rhizomorphs around stumps in managed aspen (*Populus* spp.) stands in Wisconsin. Both in natural and managed forests, the abundance of rhizomorphs in the soil and on the roots of living trees may give

Table 3 Incidence of *Armillaria cepistipes* and *A. gallica* rhizomorphs in the soil of the Uholsko-Shyrokoluzhanskyi and Chornohirskyi forest massifs in relation to the altitude of the plots and soil pH

| | Altitude (m a.s.l.) | | | | | | | Soil pH | | | | | | |
	401-600	601-800	801-1000	1001-1200	1201-1400	1401-1600	Total	3.0-3.5	3.51-4.0	4.01-4.5	4.51-5.0	5.01-5.5	> 5.51	Total
Samples, N	56	176	112	104	112	72	632	40	88	192	208	64	40	632
With *A. cepistipes*, %	9	31	18	16	19	7	100	3	13	34	41	7	2	100
With *A. gallica*, %	11	56	22	10	0	2	100	19	24	24	19	14	0	100

Armillaria an advantage over other wood-decaying fungi in acquiring new resources immediately after these are produced by anthropogenic or natural forest disturbances.

Although *A. cepistipes* and *A. gallica* are ecologically very similar, their occurrence in the two forest massifs differed. While *A. cepistipes* was the dominant species in all the forest types investigated, *A. gallica* was mainly restricted to the pure beech forests at low altitudes. Within the beech forest massif, *A. gallica* rhizomorphs were more frequent in the Uholka area where annual temperatures are slightly higher and the vegetation period longer than in the Shyrokyi Lug area. This distribution pattern of the two species supports previous findings indicating that *A. gallica* is generally confined to hardwood forests at low altitudes, while *A. cepistipes* can occur in both hardwood and coniferous forests (Guillaumin et al. 1993; Tsopelas 1999; Prospero et al. 2003b; Keča et al. 2009). Our study suggests that soil pH may also be a selective factor for the occurrence of either *A. cepistipes* or *A. gallica*. The relatively greater sensitivity of *A. cepistipes* to soil acidity compared to *A. gallica* may give the latter an advantage in occupying acidic soils in forests where, according to the vegetation type and altitude, both species could theoretically occur. However, additional data would be necessary to better understand the factors affecting the distribution of these two species. In about 27% of the plots, *A. cepistipes* and *A. gallica* co-occurred. The sympatrical co-existence of these two species was also observed in pure beech forests in Central France (Legrand et al. 1996), but it seems to be relatively rare. More frequently reported is the co-existence in the same stand of species characterized by a different ecological behavior, such as *A. cepistipes* and *A. ostoyae* (Prospero et al. 2003b; Bendel et al. 2006b) or *A. gallica* and *A. mellea* (Baumgartner and Rizzo 2001).

The ecological similarity between *A. cepistipes* and *A. gallica* might account for the high genetic and morphologic (i.e., fruiting bodies) similarities. Our study confirms that the two species can hardly be discriminated using traditional methods (interfertility tests, analysis of IGS and ITS). Based on our results and those of previous studies (Maphosa et al. 2006; Antonin et al. 2009), *A. cepistipes* and *A. gallica* can be distinguished by PCR-RFLP analysis of the EF-1α region. This method was the only one that gave unambiguous results with our isolates.

Armillaria borealis and *A. ostoyae* were also identified among the rhizomorphs sampled. Since a few fruiting bodies of *A. mellea* were also found in the Uholsko-Shyrokoluzhanskyi massif, our study indicates that all five annulated European *Armillaria* species are present in the Ukrainian Carpathians. Previously, only *A. mellea* s.l. was described for this region (Dudka et al. 1997). As the frequency of *Armillaria* species was estimated based on the abundance of rhizomorphs in the soil and on the root collar

of trees, an objection could be that the incidence of the pathogenic, less rhizomorph-producing species *A. ostoyae* and *A. mellea* may have been underestimated. However, sampling rhizomorphs has been shown to be appropriate for detecting *A. ostoyae* in mixed conifer forests in the Alps (Prospero et al. 2003b) or in pure and mixed beech forests in Central France (Legrand et al. 1996). In addition, on all the plots of the two massifs, no tree mortality caused by *Armillaria* infections was observed. Generally, forests in these protected regions can be described as stable and self-regenerating ecosystems in the climax stage of succession (Rizun and Chumak 2008). The only ecological niche for *Armillaria* not considered in the sampling design we used was heart rot. As previous studies have reported the presence of both pathogenic (*A. ostoyae*) and preferentially saprotrophic (*A. cepistipes*) species in decayed heartwood (Piri et al. 1990; Prospero et al. 2003a), this omission should not greatly modify our results.

The mainly saprotrophic *Armillaria borealis* was found only once and it probably plays a marginal ecological role in these particular forest ecosystems. Previous investigations indicated that *A. ostoyae* is mainly associated with conifers and can behave as an aggressive pathogen in managed stands (Mallett and Maynard 1998; Lung-Escarmant and Guyon 2004; Lushaj et al. 2010). In undisturbed stands and in beech or beech-fir stands, however, *A. ostoyae* may in contrast occur as a saprotroph or a secondary parasite (Guillaumin et al. 1989; Legrand and Guillaumin 1993; Tsopelas 1999). Surprisingly, we only found *A. ostoyae* rhizomorphs in pure beech forests, both in the soil (two plots) and epiphytically on the root collar (two plots), and their presence was never related to visible tree mortality. In the CBR's forests, *A. ostoyae*, like *A. cepistipes* and *A. gallica*, seems to preferentially behave as a saprotroph and rarely as a secondary/opportunistic parasite. Because of its frequent pathogenicity toward conifers, *A. ostoyae* might be an important regulator of stand composition, leading forest succession in the direction of stable pure beech stands. However, as *A. ostoyae* shows significant intraspecific differences in virulence (Morrison and Pellow 2002; Prospero et al. 2004), to support such a hypothesis the virulence of the local genotypes toward conifers should be determined by performing inoculation tests.

In conclusion, our study indicates that, in virgin, unmanaged beech and mixed conifer forests in continental Europe, *Armillaria* is a very frequent saprotroph. By forming a dense network of rhizomorphs in the soil, *Armillaria* reaches the roots of living trees and occupies their surface with rhizomorphs. This strategy may allow *Armillaria* to rapidly colonize the roots and the root collars as soon as the trees are weakened by other factors. Given that wood-decaying fungi are essential for the functioning of forest ecosystems (Lonsdale et al. 2008), *Armillaria* may

play an important ecological role in virgin forests. Although in the two protected forest massifs the same *Armillaria* species were observed as have been found in managed forests in central Europe, the frequencies of the single species differ. In particular, this applies to *A. ostoyae*, which in Europe has a very broad distribution range, from sea level in south-western France up to the timberline in the Alps (Guillaumin et al. 1993; Bendel et al. 2006a). Its higher frequency in managed forests compared to virgin forests may be a consequence of intensive forest management. Starting from the end of the eighteenth century, the frequent replacement of broadleaved tree species (especially beech) with conifers (especially Norway spruce and *Pinus* species) and the production of fresh food bases (stumps) could have created more favorable conditions for *A. ostoyae*. As modern and sustainable forest management aims to re-convert these coniferous monocultures into natural broadleaved stands (Farrell et al. 2000), detailed knowledge about the diversity and ecology of *Armillaria* species in virgin forests will help us to understand changes in the *Armillaria* community after forest re-conversion.

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