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Pulsatilla patens (Ranunculaceae), a perennial herb, is ectomycorrhizal in northeastern Poland and likely shares ectomycorrhizal fungi with *Pinus sylvestris*

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Abstract

The sharing of species of ectomycorrhizal fungi (EMF) among different co-occurring host plant species could allow the formation of common mycorrhizal networks, which can alter plant–plant interactions and succession. Such sharing of EMF among woody species is thought to be common in many forests, but very few herbaceous plants form EMF, so they are assumed to be excluded from EMF networks in forests. We studied the EMF on roots of a common coniferous tree, *Pinus sylvestris*, and a co-occurring rare herbaceous perennial plant, *Pulsatilla patens* (Ranunculaceae), in northeastern Poland. We examined roots from co-occuring *P. sylvestris* and *P. patens*, visually classified EMF into morphotypes, studied tissue sections of mycorrhizal structures using compound microscopy, and used DNA sequencing to identify the fungi. On both host plant species, we observed EMF colonization, with colonized root tips exhibiting a swollen appearance, as well as a variety of colors and textures of fungal mycelium covering and emanating from those swollen tips. Sectioning and microscopic examination of an EMF morphotype common on *P. patens* confirmed the presence of a mantle and Hartig net, indicating the likely presence of functional ectomycorrhizal structures. The two most frequent EMF were *Cenococcum geophilum* and *Piloderma olivaceum*, and the latter was found to associate with both host plant species. Several EMF found here only on *P. patens*, including *C. geophilum* and two *Russula* species, are known from previous studies to also associate with *P. sylvestris* and other tree species. The observation of shared EMF between a coniferous tree and an understory herb indicates the potential for common mycorrhizal networks to alter interactions between these two species and may also indicate a unique way in which the distribution and abundance of a rare herbaceous plant may be influenced by shared mutualisms with a common co-occurring woody plant.

Keywords

ectomycorrhizal fungi; mycorrhizal networks; *Pulsatilla patens*; *Pinus sylvestris*

Introduction

Host-specificity in mutualisms can have significant consequences for the outcomes of species interactions and for coexistence in communities. For example, in mycorrhizal mutualisms between plants and root-symbiotic fungi belowground, host-specific mycorrhizal fungi could promote niche-partitioning among plant species and alter plant–soil

feedbacks [1,2], changing the likelihood of species coexistence. Generalist mycorrhizal fungi can be shared among multiple plant species in a common mycorrhizal network (CMN), potentially altering interactions among plant species and changing the course of succession [3–5]. Here, we report for the first time the occurrence of ectomycorrhizal fungi (EMF) on the perennial herb *Pulsatilla patens* (L.) Mill. in northeastern Poland, and the likely sharing of EMF between *P. patens* and co-occurring trees including *Pinus sylvestris* L.

Ectomycorrhizal symbiosis typically develops between woody plants and Basidiomycota or Ascomycota soil fungi, with the fungi obtaining carbohydrates from the plant in exchange for mineral nutrients taken up from the soil. Plants in these interactions often have a wide fungal symbiont range, being colonized by 10's to 100's of species of EMF from diverse lineages. The fungi, in contrast, exhibit more variation in specificity, with many broad generalists but some taxa colonizing only specific lineages of plants [5,6]. In most mixed ectomycorrhizal plant communities that have been examined, multihost fungi are common (e.g., [7]), suggesting that CMNs could be ubiquitous in these communities. If true, this observation is significant, as CMNs have the potential to significantly alter the mechanisms and outcomes of interactions among plant species through resource transfers among individual plants and asymmetric relationships of different plant and fungal species with the CMN [3,4,8–10].

CMNs have particular potential to alter outcomes of interactions among dominant canopy trees and understory plants, as resource distribution through the CMN could reduce asymmetry in resource availability between canopy and understory plants. Understory plants in several families, such as Orchidaceae and Ericaceae, have been shown to share EMF with overstory trees (e.g., [11], reviewed by [12]). Indeed, in those examples, it has been demonstrated that the understory plants are myco- or mixoheterotrophic, obtaining all or some of their carbon from the fungi in the CMN, presumably resulting in indirect parasitism of overstory trees [13]. As such, the CMN allows these plants to overcome their disadvantage in photosynthesis from being located in the understory. It is possible that additional herbaceous understory plants could use such a strategy, sharing EMF with overstory trees and overcoming a competitive disadvantage through acquisition of one or more resources from the CMN.

In July 2015, we made preliminary observations in northeastern Poland on the root system of a single individual of *Pulsatilla patens* (L.) Mill. (Ranunculaceae), a small herbaceous perennial. These observations suggested that it was colonized by EMF, inspiring the current study. As EMF were detected for the first time on *P. patens*, our aim was to confirm this EM association, unusual for a herbaceous Ranunculaceae. As this observation was made on *P. patens* individuals co-occurring with Scots pine (*Pinus sylvestris*), we tested if these EMF could be generalists, shared with Scots pine, and contribute to form a CMN between *P. patens* and the overstory trees.

Material and methods

Study species

Pulsatilla patens is a lowland species of Boreo-meridional-continental and circumpolar distribution [14] in the area between 44°30′ and 63°30′ north latitude and 12° and 70° east longitude [15] (Fig. 1A). Its European distribution includes central Poland [16,17] (Fig. 1B), where its frequency decreases towards the west and south [18]. *Pulsatilla patens* is a threatened plant species in Europe, listed in the Annex II of European Union Habitats Directive (92/43/ETY) [19] and in Appendix I of the Bern Convention [20]. This species is considered critically threatened in the flora of Czech Republic [21]; it is included in the red list and/or red data books of Germany (endangered) [22,23], Sweden (vulnerable) [24,25], Lithuania (relatively restored), Leningrad region of Russia (decreasing number), Slovakia [26], and Kaliningrad District of Russian Federation [27]. In Poland, *P. patens* has been a strictly protected species since 1958 and requires active protection [28]. This species is now protected by Natura 2000 program [29]. The "Red list of vascular plants in Poland" classifies *P. patens* as critically endangered (E category) [30], while *The red data book of the Polish Carpathians* (CR category) [31] and the *Red*
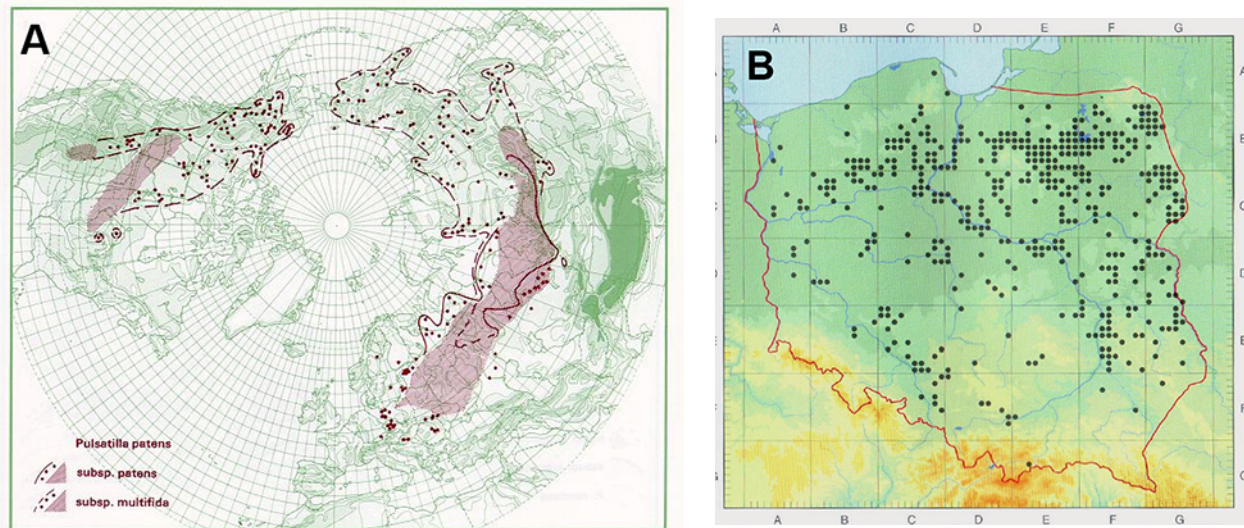
Fig. 1 Current distribution of *Pulsatilla patens* (L.) Mill. (**A**) in Europe, Asia, and North America (source: Den virtuella floran – Naturhistoriska riksmuseet, http://linnaeus.nrm.se/flora/di), and (**B**) in Poland [79].

data book of Poland consider *P. patens* as a low risk (LR) species [32]. Therefore, *P. patens* is classified as an endangered species in Poland, as a vulnerable species (V) in the West Pomerania and Greater Poland regions [33], as critically endangered (CR) in Lesser Poland Upland [34], as a threatened species with extinction (E) in Silesia [35], and as a species extinct in the region (RE) in Lower Silesia and Opole provinces [36–38].

Pinus sylvestris (Pinaceae) is a conifer that is widespread across Eurasia from northern Spain and Scotland in the west to Russia in the east, and from Lapland in the north to Turkey in the south. It also occurs in the United States, Canada, and New Zealand, where it has been introduced. It is an economically important species in many areas, where its abundance has been augmented by planting for forestry. It commonly forms open pine forests and woodlands, sometimes associated with other conifers such as *Picea*, and sometimes with broad-leaved trees such as *Betula* spp. and *Populus tremula*. Although they can occasionally host AM fungi (e.g., [39]), pines are predominantly ectomycorrhizal. Studies are accumulating on the ectomycorrhizal fungi associated with *P. sylvestris* throughout its range, demonstrating that it associates with a broad diversity of EMF species (e.g., [40–42]), and promotion of abundance of *P. sylvestris* may also augment abundance of EMF. Throughout much of the range of Scots pine, *P. patens* can co-occur with it, especially in northeastern Poland and at forest edges and in disturbed areas, leading us to hypothesize that Scots pine and *P. patens* share EMF species in common.

Study area

The study was conducted in northeastern Poland, in the Supraśl Forest Division in the Knyszyńska Forest, in three localities (Kopna Góra, Lipowy Most, Woronicza) (Fig. 2, Tab. 1), which belong to the Natura 2000 network [43,44]. The Knyszyńska Forest includes an area of approximately 17,439.94 ha located in the central part of the Forest (Forestry Commission Forest Management Plan Supraśl, 2006–2015). The climate of the region is continental, with mean annual temperature of 7°C and mean annual rainfall of 610 mm. Snow cover is on average 10 cm thick for 85–90 days a year. The growing season (at 5°C threshold) lasts for about 200 days [45].

Phytosociological characterization of study sites

Knyszyńska Forest vegetation is distinguished by the occurrence of boreal and sub-boreal communities and the communities with the optimum of occurrence in Central and Southeastern Europe. The vegetation of Knyszyńska Forest represents 23 forest
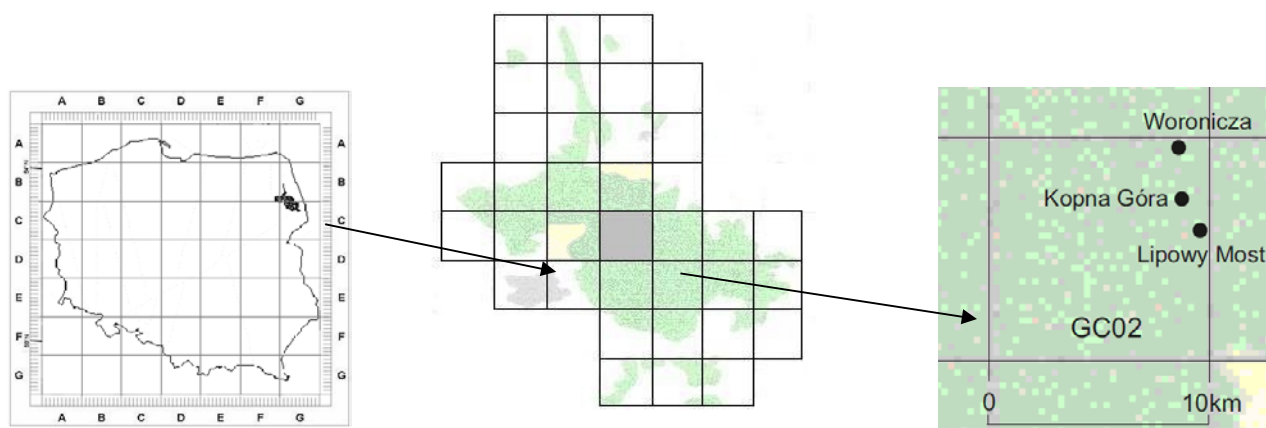
Fig. 2 Distribution of the investigated localities within the Polish geobotanical grid (ATPOL – GC02) area.

Tab. 1 Characterization of the study localities in the Knyszyńska Forest.

Location of study sites	Geographic coordinates	Elevation above sea level (m)	Type of community	Dominant undergrowth species
Kopna Góra	N 53 07.123 E 023 12.575	150	*Serratulo-Pinetum typicum* (W. Mat. 1981)*	*Vaccinium myrtillus, Fragaria vesca, Convallaria majalis, Poa trivialis, Agrostis capillaris, Pleurozium schreberi*
Lipowy Most	N 53 13.273 E 023 30.164	160	*Serratulo-Pinetum typicum* (W. Mat. 1981)**	*Vaccinium myrtillus, Fragaria vesca, Calluna vulgaris*
Woronicza	N 53 15.031 E 023 29.271	140	*Serratulo-Pinetum typicum* (W. Mat. 1981)*	*Vaccinium vitis-idaea, Vaccinium myrtillus, Calamagrostis arundinacea, Festuca ovina, Agrostis capillaris, Pleurozium schreberi*

* *Carici digitatae-Piceetum* [80]. ** *Serratulo-Piceetum* [81].

and scrub associations and 835 species of vascular plants [43,46]. The characteristic feature of the vegetation in this forest is a considerable contribution of *Picea abies* and *Pinus sylvestris* in tree stands and the lack of *Fagus sylvatica*, *Acer pseudoplatanus*, and *Abies alba* (Tab. 1). At each of the nine *P. patens* collection sites, we conducted phytosociological analyses of plant communities with the use of GPS technique and identification of habitats of the plant patches studied, in an area of 400 m² (20 × 20 m), using Braun-Blanquet releves. The assignment of species to particular syntaxonomic units was assumed after Matuszkiewicz [47], vascular plants nomenclature was assumed after Mirek et al. [48], and nomenclature of bryophytes after Ochyra et al. [49]. At every *P. patens* location, the vegetation and their biotic (cover of dominant undergrowth species, competition herbal layer) and abiotic factors (types of soil, temperature air, temperature soils, chemical soil analysis) were earlier characterized [43,46,50,51].

Sample collection and ectomycorrhizal morphotyping

Root samples and rhizosphere soil samples of *P. patens* and *P. sylvestris*, three samples per site per species, were collected, resulting in a total of 18 samples (3 sites × 2 species × 3 samples). At each site, soil was carefully excavated around the roots of three distinct individuals of *P. patens* and small subsamples of roots were collected for mycorrhizal

examination, leaving the plants alive with most of their root system intact. Due to the endangered status of *P. patens*, great care was taken to disturb the plant as little as possible. Moreover, the rarity of *P. patens* did not allow more extensive sampling at those sites. Small root samples of *P. sylvestris* were taken from pines within 5 m of each *P. patens* plant sampled. *Pinus sylvestris* roots were excavated from the base of each tree to ensure correct species collection. Each pair of samples collected (*P. patens* and adjacent *P. sylvestris*) was separated by at least 5 meters and most by more than 10 meters.

Root samples were returned to the laboratory on the same day stored at 4°C. Within 3 days, each sample was rinsed and soaked in petri dishes for 30 minutes before examination. Each sample was evaluated under a dissecting microscope and distinct variations, or morphotypes, of colonized root tips were counted. Each morphotype was categorized based on appearance (shape, color, surface texture of mantle, and emanating hyphae or rhizomorphs) [52]. A representative sample (1–4 root tips) of all the root tips observed for each morphotype in each sample was isolated for molecular identification. Several samples of a common EMF morphotype on *P. patens* were saved in CTAB buffer or in 50% ethanol for sectioning and compound microscopy to confirm anatomical structures of EMF including mantle and Hartig net. Those EMF tips were cut with a razor blade, and fine transverse sections were observed with a compound light microscope (Olympus BX 51).

Molecular identification

Identities of all ectomycorrhizal morphotypes were confirmed through Sanger DNA sequencing and comparison of sequences with public databases. Sequences were then classified into operational taxonomic units (OTUs) for comparison of EMF between *P. patens* and *P. sylvestris*. Genomic DNA was extracted from sampled fungal root tips using a modified protocol of Extract-N-Amp Tissue Kit Extraction and Neutralization Buffers (Sigma-Aldrich, USA). A collection of fungal tissue was taken from sampled mycorrhizal root tips using forceps and was submerged in 10 µL of Sigma extraction buffer and incubated at 65°C for 10 min, 95°C for 10 min. Thirty µL of Sigma neutralization buffer was added and DNA was diluted to 20% with 160 µL of PCR-grade water. DNA was amplified in 8 µL PCR reactions containing 4 µL of 2.0X Apex RedTaq PCR Master Mix (Genesee Scientific Corporation, USA), 0.4 µL of each 10 µM fungal-specific primer: ITS-1F and ITS-4, 2.2 µL of PCR-grade water, and 1 µL of DNA extract. Incubations were thermocycled at an initial denaturation of 94°C for 3 min, then 30 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 45 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min, then run on a 1% agarose gel. PCR products were cleaned enzymatically using 10 µL incubations containing 0.25 µL ExoSAP-IT enzyme mix (ThermoFisher Scientific, USA), 4.75 µL of PCR-grade water, and 5 µL of PCR product. Incubations were thermocycled at 37°C for 30 min, 80°C for 20 min, 4°C for 5 min. PCR products were sequenced using 10 µL reactions containing 0.4 µL of BigDye Terminator v3.1 Ready Reaction Premix, 1.8 µL of 5× BigDye Terminator v3.1 Sequencing Buffer (Applied Biosystems), 0.5 µL of 10 µM fungal-specific primer ITS-5, 6.3 µL of PCR-grade water, and 1 µL of PCR product. Sequencing reactions were thermocycled at an initial denaturation of 96°C for 1 min, followed by 45 cycles of denaturation at 95°C for 20 sec, annealing at 52°C for 20 sec, and extension at 60°C for 4 min. Products were dried using a Vacufuge (Eppendorf, Germany) at 45°C for 30 min and then shipped to the Arizona State University DNA Lab for cleaning and reading. Sequences were compared with the UNITE database of fungal sequences [53], which contains curated sequences for most major taxa of ectomycorrhizal fungi, to confirm suspected identities, and were grouped into OTUs of 97% similarity using Geneious software. Fungal sequences recovered from *P. patens* roots were also queried against the UNITE and INSD databases, including environmental sequences, to investigate if other host plants have been associated with the same fungi. All sequences more than 97% similar to the query sequence were checked for host plant associations.

Results

We found an average of 3.0 and 3.3 different EMF morphotypes per root sample of *P. patens* and *P. sylvestris* roots, respectively. In all, 958 root tips were observed (522 on *P. patens*, 436 on *P. sylvestris*, an average of 16.8 tips per morphotype per sample). All observed root tips appeared to be ectomycorrhizal, based on a lack of root hairs, swollen appearance, emanating fungal structures, and often differences in color from originating roots (Fig. 3). Eight fine roots with several EMF root tips of a smooth orange morphotype from *P. patens* were subsampled for microscopic observations. All such root tips proved to be colonized by an external mycelium (Fig. 4A,B) and all fine sections were covered by a mycelial mantle and presented a clear Hartig net (Fig. 4B,C). Root tips were brown, concolorous with the root, sometime branching (Fig. 4B) regularly, and were relatively long (3 mm; Fig. 4A) and covered by a short distance mycelium. The mantle was transparent, with a few emanating hyphae, and not hydrophobic. The mantle was complete, without cystidia nor granules. The Hartig net organized on two rows, not reaching the endodermis, with round cells. The Hartig net was particularly ornamented, of the Palmetti type (Fig. 4C,D).
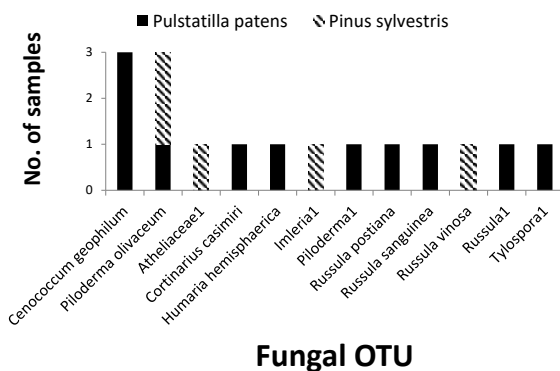
Nine morphotypes of EMF were observed on *P. patens* roots (see Fig. 3 for examples), and 15 morphotypes on *P. sylvestris* roots. All nine *P. patens* individuals (three from each site) hosted EMF, and EM morphotypes were observed on all of these individuals. From samples of those morphotypes, DNA was extracted from 76 and 91 root tips of *P. patens* and *P. sylvestris*, respectively, and PCR amplification success was 60% and 79%, respectively. Many sequences had signals indicating likely presence of more than one fungal species, and those sequences could not be used; 39 usable fungal sequences were obtained, 12 from *P. sylvestris* and 27 from *P. patens*. Those sequences revealed 15 different operational taxonomic units (OTUs) of fungi on roots of *P. patens*, and four different OTUs of fungi on roots of *P. sylvestris* (Fig. 5). *Cenococcum geophilum* was found on roots of *P. patens* in three samples from two different sites. Six of the 15 fungal OTUs isolated from *P. patens* roots were known or suspected to be nonmycorrhizal, including Lecanorales1 (lichenaceous; one sample), Coleosporium1 (plant pathogen; one sample), Malassezia1 (saprobe; one sample), Luellia1 (saprobe; one sample), Mycena1 (saprobe; four root tips from two different sites), and *Hygrocybe acutoconica* (saprobe; two root tips from the same sample). *Mycena* and *Hygrocybe* species have been found previously as endophytic fungi or otherwise associated with plant roots [54,55].
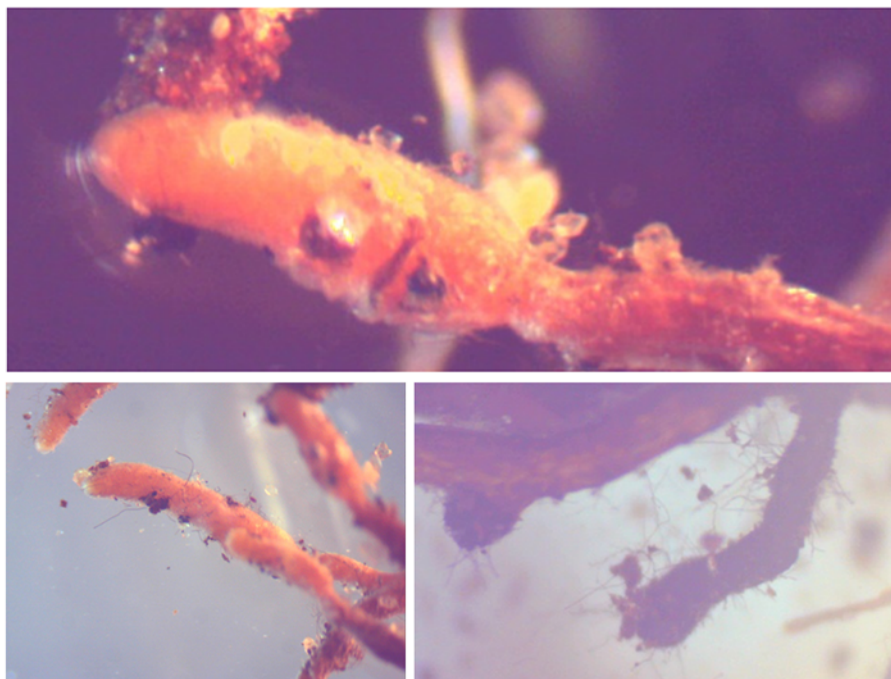

Fig. 3 Examples of apparent ectomycorrhizal morphotypes observed on roots of *Pulsatilla patens* in northeastern Poland.
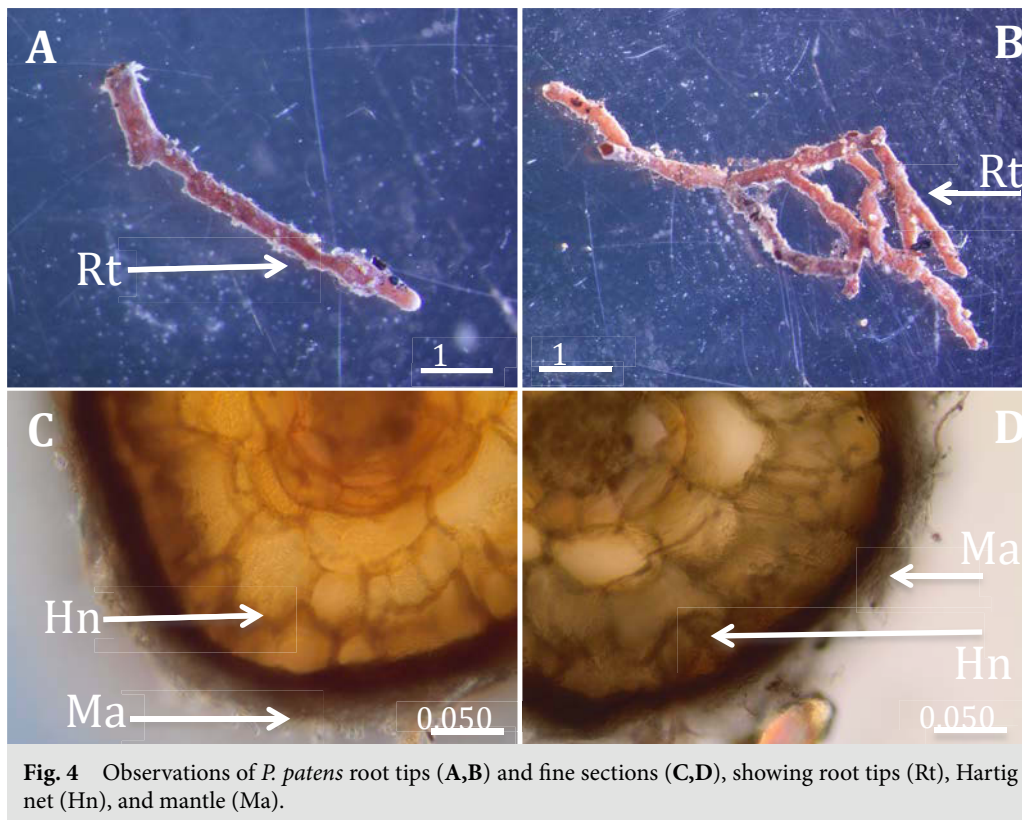
Fig. 4 Observations of *P. patens* root tips (**A,B**) and fine sections (**C,D**), showing root tips (Rt), Hartig net (Hn), and mantle (Ma).

Fig. 5 Putative ectomycorrhizal (EM) fungal operational taxonomic units (OTUs) recovered from Sanger sequencing of EM morphotypes observed on *P. patens* and *P. sylvestris*.
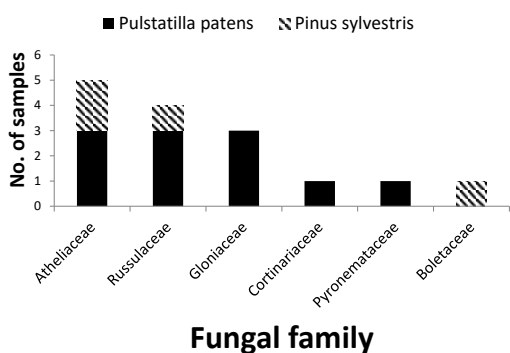
Fig. 6 Family composition of putative ectomycorrhizal (EM) fungal operational taxonomic units (OTUs) recovered from Sanger sequencing of EM morphotypes observed on *P. patens* and *P. sylvestris*.

Putative ectomycorrhizal fungal OTUs isolated from the two host plants included one that was found on samples from both species – *Piloderma olivaceum* (Atheliaceae); indeed, this OTU was found on root tips of both host plant species in the same sample from one site, plus a sample of *P. sylvestris* from a different site. The other 11 OTUs were found only on either *P. patens* or *P. sylvestris* (Fig. 5). Like *P. sylvestris*, *P. patens* associated mainly with Atheliaceae and Russulaceae (Fig. 6).

Among the 27 fungal sequences from *P. patens* roots, nine were not detected on other hosts in searches of environmental sequences on the UNITE and INSD databases (Tab. S1). Ten were detected on a deciduous tree, 12 were detected on conifers, and one on an herb only. Among the sequences reported on conifers, three were shared with an herbaceous plant. Six sequences (including four EM OTUs and one nonmycorrhizal sequence) were previously detected on *P. sylvestris* in other studies.

The EM fungal OTUs belonged to six families, with Atheliaceae found in the most samples (Fig. 6).

Discussion

Pulsatilla patens is ectomycorrhizal in northeastern Poland

Our observations of classic ectomycorrhizal morphotypes on tips of fine roots of *P. patens*, one of which was confirmed by microscopic examination of cross sections to have a well-developed mantle and Hartig net, support our hypothesis that this rare herbaceous perennial plant engages in ectomycorrhizal symbiosis, at least in this small part of its large geographic range. Moreover, this conclusion is supported by PCR amplification

and sequencing from those *P. patens* EM morphotypes of nine fungal OTUs belonging to taxa known to have an ectomycorrhizal ecology from previous studies on other host plants, including *Cenococcum*, *Cortinarius*, and three different species of *Russula*. Despite its wide distribution, its mycorrhizal ecology has been studied very little. Moora et al. [56] compared the symbionts and germination of *P. patens* with a more common species of *Pulsatilla*, *P. pratensis*. According to their study, *P. patens* associates with site-specific arbuscular mycorrhizal fungi (AMF) [57], which increase the germination rate of this species. However, those site-specific AM fungi are not rare [57], and are rather different between conifer forests and grasslands. Therefore, these fungi may not limit the distribution of *P. patens*. Given those previous reports, we suspect that *P. patens* engages in AM symbiosis throughout its range (regardless of habitat), but we did not look for AMF, and it would be interesting to know whether abundance of EMF is related to abundance of AMF in this plant.

As far as we are aware, *P. patens* is fully autotrophic, not receiving significant carbon from symbiotic fungi. Thus, our observations likely add to the small number of examples of fully autotrophic herbaceous (nonwoody) plants that engage in EM symbiosis (e.g., [58]). In some examples of herbaceous EM plants, engagement in the symbiosis may be facultative, occurring only under some conditions. For example, *Polygonum viviparum* has been shown to be EM in young soils near retreating glaciers in the presence of other EM plants [59], but has been reported to lack EMF under other circumstances (e.g., [60]). Massicotte et al. [61] hypothesized that the ability to form EM associations may vary with plant ontology, and only older individuals might develop EM roots. Such observations raise the question of whether *P. patens* is also facultatively ectomycorrhizal, engaging in EM symbiosis only at some sites under specific conditions, e.g., only where it co-occurs with EM trees, or whether its EM symbiosis is widespread and ubiquitous. Analysis of what abiotic or biotic contextual factors drive any variation in engagement in the AM versus EM symbiosis could lend significant insight into the proximate mechanisms underlying costs and benefits of these widespread mutualisms. A geographic study of the occurrence of AM and EM symbiosis on *P. patens*, and environmental correlates of such occurrence, is thus recommended as a potentially fruitful next step.

Pulsatilla patens likely shares EM fungal species with co-occurring dominant trees

Not only did we find one EM fungal OTU – *Piloderma olivaceum* – in common between *P. patens* and nearby *P. sylvestris* (Fig. 5), despite very shallow sampling of the EM fungal community, but several of the fungal OTUs found here only on *P. patens* are known from previous studies as EM associates of *P. sylvestris* or other trees, including broadleaf trees (Tab. S1). For example, *Cenococcum* spp. are well-known generalist EMF found across diverse hosts around the world [62]. *Russula sanguinea* is a wide-ranging EMF species that associates with multiple pines, including *Pinus sylvestris* (e.g., [63]). *Russula postiana* has been reported to associate with Scots pine several times previously from Slovakia to Spain [64,65] and with *Pyrola rotundifolia* in Estonia [66]. *Cortinarius casimiri* and *Humaria hemisphaerica* have both been found as EMF on broad-leaved trees (e.g., [67]), raising the possibility that they could facilitate CMNs between *P. patens* and such trees where they co-occur. Indeed, database queries of our fungal sequences recovered from *P. patens* roots showed that most of the fungi found here have been previously found to associate with other plants, including a diverse array of hosts ranging from conifers, to woody deciduous trees, to herbaceous plants (Tab. S1).

The likelihood of shared EM fungal species between *P. patens* and co-occurring trees such as *P. sylvestris* means that common mycorrhizal networks (CMNs) could form between *P. patens* and those tree species. As a consequence, competitive effects of trees on *P. patens*, which may be substantial for soil resources and even asymmetric for light, could be somewhat ameliorated by CMNs. Limiting resources, especially significant amounts of N and water, can move through CMNs from plant to plant, altering the balance of competition for those resources [4,9,10]. Although direct root–root interactions between such plants may still be strongly competitive, CMN connections through shared EMF have the potential to offset these negative effects of trees on subordinate plants (e.g., [68]). Of course, we lack data to demonstrate the functioning of such a CMN, but surrounding trees could still contribute to *P. patens* germination and growth

by providing a diverse and abundant EM inoculum. This "nursing" effect of a distinct host species has been applied to herbaceous plants, such as *Helianthemum bicknellii*, which favors *Quercus* spp. germination by providing shared EMF [58].

Some of the best examples of CMNs between trees and understory plants come from studies of myco- and mixotrophic plants, i.e., plants that obtain at least some of their carbon from a mycorrhizal fungal partner. In some of these cases, the fungi involved have been shown to be EMF likely shared with overstory trees, meaning that CMNs are mediating transfer of carbon from those trees to the understory plants. The detection of EMF fungi on green orchid roots has even been a clue to detect mixotrophy, and the functioning of these CMNs has been confirmed for many forest-dwelling Orchidaceae and Ericaceae from the temperate region (reviewed by [12]). Could it be that an understory herb such as *P. patens*, although assumed to be completely autotrophic, could obtain some carbon from overstory trees when sharing EMF with those trees? Further studies are required to test this hypothesis, perhaps beginning with comparison of stable isotope signatures between *P. patens* and co-occurring plants, which have been used as an indicator of mixotrophy in other herbaceous plants [13].

Conservation status of *P. patens* and potential management implications of its ectomycorrhizal status in northeastern Poland

To realize any management implications for *P. patens* populations resulting from knowledge of its status as an ectomycorrhizal plant, we will require data demonstrating under what conditions and contexts *P. patens* engages with EMF, AMF, or both. Coupled with information on the habitat requirements of *P. patens*, and factors already implicated in its decline where it is threatened, we could potentially understand how the ectomycorrhizal symbiosis could be involved in the population dynamics of this plant. Within Poland, *P. patens* prefers well-sunlit locations with southwestern and southern exposure, typically on fringes of boreal forests of the class *Vaccinio-Piceetea* [47] or in slightly shady areas [18]. It may also occur in ploughed sections of forests, forest glades and fire-protection forest belts [46,69] or, sporadically, in xerothermic and psammophilous grasslands [70]. Thus, *P. patens* is found under a wide variety of abiotic conditions in Poland. Even if we assume that *P. patens* is ectomycorrhizal wherever it co-occurs with ectomycorrhizal trees, the benefits and functions of that symbiosis may be highly variable, depending, for example, on variation in availability of light and various soil nutrients.

The reasons for the threatened status of *P. patens* in Poland include a constant decrease in the forest area, and severity of different forms of anthropogenic pressure in agricultural, industrial, or urban areas [35,37,38,69,71,72]. Some of these same threats are reducing populations of *P. patens* outside of Poland as well, including Finland, Germany, and Estonia [23,73–78]. Our results raise the possibility that *P. sylvestris* trees may support populations of EMF that are beneficial for *P. patens*, potentially helping to explain why deforestation is correlated with the decline of *P. patens*. However, testing this hypothesis and using this knowledge to ameliorate population threats will require broader studies on the mycorrhizal status of this plant.

Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3572/0:

Tab. S1   Fungal sequences associated with roots of *P. patens* in our study in northeastern Poland.

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