Fungi associated with disease symptoms on *Pinus mugo* needles in the Polish Tatra Mountains

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**Abstract.** Dwarf mountain pine, *Pinus mugo*, is an important component of the subalpine and alpine zone in Europe. For several years, in one of the natural areas of the occurrence of this species in the Polish Tatras, an intensified decline of the assimilation apparatus has been observed. The studies conducted in 2016–2020 were aimed at determining the types of disease symptoms occurring on *P. mugo* needles and at identifying the species composition of fungi within symptomless and diseased needles. We isolated 57 taxa from 6 types of disease symptoms and from needles without disease symptoms, identified on the basis of morphological features and molecular analyses. Absolute parasites were represented by only one species of *Coleosporium senecionis*, which was identified by the aecium stage. The genus *Lophodermium* was represented by three species: *L. conigenum*, *L. corconticum* and *L. pini-mugonis*. So far, *L. corconticum* was known only from the Karkonosze Mountains in Poland, and *L. pini-mugonis* from the German Alps. In addition, the more frequently isolated species included: *Sydowia polyspora*, *Hendersonia sp.*, *Nemania serpens*, *Lepptomelanconium allescheri*, *Cladosporium spp.*, *Biscogniauxia nummularia* and *Alternaria spp.* Some taxa were associated with only one type of disease symptom, while some species of fungi were found to be associated with different symptoms. Twelve species of fungi were isolated from living symptomless needles, some of which were subsequently found in association with necrotic areas on needles, e.g., *Lophodermium corconticum*, *L. conigenum* and *Lepptomelanconium allescheri*. *L. pinastris* has not been found in the present studies. In the discussion, an attempt was made to assess the role of some of the identified species of fungi in causing the disease process of *P. mugo* needles.

**Key words:** dwarf mountain pine, endophytes, needle diseases, *Lophodermium corconticum*, *L. pini-mugonis*, *L. conigenum*

**Introduction**

Dwarf mountain pine, *Pinus mugo*, is a key element in the sub-alpine flora of many mountain ranges of Europe (Boratyńska 2002; Boratyńska et al. 2005). In the Tatra Mountains, this shrub grows mainly above the mountain zone at an altitude of 1550–1850 m a.s.l. (Boratyńska 2002). *P. mugo* plays an important role in preventing avalanches and soil erosion in high mountains (Ballian et al. 2016). It also helps to maintain favorable thermal conditions of the soil and reduces the effects caused by frost weathering. As a pioneering species, *P. mugo* inhabits areas damaged by landslides, avalanches and human activity (Mróz & Perzanowska 2004). For these reasons, this species is currently under legal protection in Poland.

In various areas of *P. mugo* occurrence, research is carried out on the participation of fungi in reducing the health condition of this species, growing in relatively difficult environmental conditions (Schnell 1987). Known diseases of *P. mugo* needles include, in particular, red band disease caused by *Dothistroma septosporum* and brown spot needle blight caused by *Mycosphaerella dearnessii* (anam. *Lecanosticta acicola*) (Pehl & Butin 1992; Maschning & Pehl 1994; Holdenrieder & Sieber 1995; Pehl 1995; La Porta & Capretti 2000; Bednárová et al. 2006; Boroń et al. 2016). *P. mugo* has also been shown as a host to the recently described new species *Dothistroma pini* (Barnes et al. 2011). *P. mugo* needles are also often infested by fungi of the genus *Lophodermium* (Andjelić 2000; Hirose & Osono 2006; Hou et al. 2009). This phenomenon is characteristic also for other tree species of the genus *Pinus* (Minter & Millar 1980; Ortiz-Garcia et al. 2003; Salas-Lizana & Oono 2018). In some studies, considerable attention was paid to *P. mugo* needle endophytic fungi. The following fungi are mainly shown as *P. mugo* needle endophytes: *Cenangium ferruginosum*, *Cyclaneusma minus*, *C. niveum*, *Lophodermium*
pinastri and Sydowia polyspora (= Hormonema dematioides) (Schnell 1987; Hata & Futai 1995; Sieber et al. 1999). Some of them, under stressful conditions, cause premature dying off and dropping of needles (Sieber 2007; Giordano & Gonthier 2011).

In Poland, research on P. mugo needle diseases is relatively sparse. One of the first observations was made in the Tatra National Park by Lutyk (1978), who found the presence of several species of fungi on the needles of this shrub, of which he considered L. pinastri to be the most dangerous. Results of later studies conducted by Pusz et al. (2015) also come from this region. The authors found frequent presence of yellow spots on the needles. In the needles from all stands, they isolated large numbers of L. conigenum and to a lesser extent L. pinastri. In similar studies in the Karkonosze National Park (Pusz et al. 2013), the presence of L. pinastri and Lophodermium sp., which was later described as a new species L. corconticum (Koukol et al. 2015), was observed. Research by Kowalski et al. (2018) led to the first identification of the presence of the pathogenic fungus Leptomelancognium allescheri on the needles of P. mugo in the Tatra National Park and the description of the accompanying disease symptoms. Results of previous studies show that the emerging health problems of mountain pine needles growing at higher altitudes may be the result of activities of the various pathogenic fungi. The relatively poor identification of the species composition of fungi present in the P. mugo needle in Poland and observation in recent years of increased occurrence of diseases prompted the undertaking of further research in this field.

The aim of this study was: i) to determine the species composition of fungi inhabiting P. mugo needles with symptoms of necrosis and discoloration, and for comparison – fungi in living symptomless needles; ii) determination of the species of fungi for the distinguished types of discoloration; iii) examining selected species of fungi using molecular techniques and phylogenetic analysis.

Material and methods

The research was carried out on seven selected plots located in the Western Tatras within the Chocholowska Valley (Table 1; Fig. 1). The plant material was collected during the growing season (May, July, September and November) in 2016, 2017 and 2020. Samples were collected at different weather conditions. In total, 35 randomly selected shoots, each 50 cm long, from 35 P. mugo bushes were collected. Shoots were packed separately in polyethylene bags and transported directly to the laboratory, where they were stored at 5°C. 23,500 double needles from 3 years (30% of all needles on the shoots) were analyzed for necrosis and discoloration and the percentage of needles with symptoms was calculated. A detailed analysis of the symptoms of diseases on the needles resulted in the identification of 7 types of needles: needles without symptoms; needles with numerous yellow and yellow-brown spots; needles with a brown spot or...
streaked necrosis, then associated with a dead top; dead needles with only a viable epiphyseal part remaining; needles completely dead, attached to the shoots; needles alive, with necrosis progressing from the base; needles with numerous irregular whitish-yellow spots. In order to identify the fungi accompanying the symptoms of the disease, both etiological signs on necrotic tissues were examined, and needle isolation was also performed. Isolation of fungi was carried out with 520 needles (2060 examined, and needle isolation was also performed. Isolation of fungi was carried out with 520 needles (2060 needle fragments) considering each type described above. Observations of conidomata structure and measurements of morphological structures were made using the Zeiss Discovery V12 stereomicroscope and the Zeiss Axiophot light microscope with differential interference contrast (DIC) illumination. Appropriate literature and mycological keys were used for the analyses (Sutton & Chao 1970; Minter & Millar 1980; Sutton 1980; Minter 1981; Vujanovic & St-Arnaud 2001; Hou et al. 2009). For groups of identical isolates, one or more representative cultures were selected for molecular analysis.

On the basis of 23,500 needles analyzed, the percentage of needles with discoloration and necrosis was recalculated for every single plot (Table 1). In order to determine the incidence of vesicular rust in pine needles, 3691 needles were analyzed on five randomly selected shoots, and then the percentage of needles with aeciospores and symptoms remaining after aeciospores was determined. Needles with necrotic discoloration and without discoloration were surface sterilized by soaking first for 1 min in 96% ethanol, then 30 s in a solution of sodium hypochlorite (approx. 4% available chlorine) and finally for 30 s in 96% ethanol and 3 min in sterile water followed by drying in layers of blotting paper and removal of superficial tissue. Next six small fragments were cut out from one sample and placed in the Petri dishes (9 cm) on malt extract agar supplemented with tetracycline (MEA+T medium: 20 g/L malt extract, 15 g/L agar, Difco, Sparks, MD, USA; 200 mg/L tetracycline, TZF Polfa, Poland). The MEA+T cultures were incubated in the dark for 10 weeks at 20°C and controlled every 5–8 days for the occurrence of sporulation.

### Table 1. Characteristic of study plots.

| Plot number | Location                        | Altitude (m a.s.l.) | Clump surface (are) (a) | Mean percentage of needles with discoloration and necrosis (%) |
|-------------|---------------------------------|---------------------|-------------------------|----------------------------------------------------------------|
| 1           | 49°12′57″N; 19°46′11″E          | 1556                | 5.5                     | 44                                                              |
| 2           | 49°12′59″N; 19°46′08″E          | 1582                | 5.5                     | 50                                                              |
| 3           | 49°13′03″N; 19°46′05″E          | 1624                | 4.3                     | 45                                                              |
| 4           | 49°13′05″N; 19°46′00″E          | 1667                | 3.0                     | 50                                                              |
| 5           | 49°13′08″N; 19°45′55″E          | 1737                | 4.6                     | 65                                                              |
| 6           | 49°13′09″N; 19°45′53″E          | 1746                | 5.1                     | 58                                                              |
| 7           | 49°13′50″N; 19°46′14″E          | 1592                | 1.5                     | 51                                                              |

### Molecular analyses

Samples for genetic analysis were prepared for the ITS region of fungal material according to the procedure by Kowalski et al. (2018). Total genomic DNA was extracted using Genomic Mini AX Plant (A&A Biotechnology, Gdynia, Poland) according to the manufacturer’s protocol, and diluted DNA extracts were used as templates in PCR. The molecular identification of fungi was performed by comparing the sequences of the ITS regions of rDNA.

In our study the ITS rDNA region (ITS1-5.8S-ITS2) was amplified with the primers ITS4 and ITS5 (White et al. 1990). DNA fragments were amplified in a total volume of 25 μl containing: 0.25 μl of Phusion Green High-Fidelity DNA Polymerase Thermo Scientific (Fermantas, Finnzymes, Pierce, Abgene), 5 μl Phusion HF buffer (5×), 0.5 μl of dNTPs (10 mM), 0.75 μl DMSO (100%) and 0.5 μl of each primer (25 μM). Amplifications were run in a Thermocycler Biometra T-Personal 48 (Biometra GmbH, Goettingen, Germany) with 35 cycles comprising 30 s denaturation at 98°C, 10 s annealing at 52–56°C (depending on the primer melting temperature and fungal species) and 30 s at 72°C, and a final elongation step at 72°C for 8 min. PCR effectiveness was verified by 2% agarose gel electrophoresis stained with Midori Green Advance DNA Stain (Nippon Genetic Europe), and positive amplification products were purified with a Clean-Up DNA purification kit (A&A Biotechnology, Gdynia, Poland). Bidirectional sequencing of purified products was carried out with the use of PCR primers. Amplified products were sequenced with the BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the products were resolved with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), at the DNA Research Centre (Poznań, Poland) using the same primers that were used for the PCR. The obtained barcode sequences were processed with Chromas Pro 1.6 software (Technelysium, Australia) and queried against the NCBI GenBank database with the BLAST search tool to retrieve the most similar sequences.

### Phylogenetic analyses

Phylogenetic analyses were conducted only for Lophodermium spp. BLAST searches using the BLASTn algorithm were performed to retrieve similar ITS sequences from GenBank (http://www.ncbi.nlm.nih.gov) and accession numbers for these sequences are presented in the phylogenetic tree (Fig. 2). Datasets were curated with the MEGA X program (Kumar et al. 2018). The ITS dataset included all available sequences for reference species previously used by (Koukol et al. 2015) that could be retrieved from GenBank (Fig. 2) to show the placement of our isolates within this genus. The outgroup taxon for the ITS dataset analysis was Colpoma quercinum.

ITS dataset were aligned online using MAFFT v 7 (Katoh & Standley 2013) with the E-INS-i option, a gap-opening penalty of 1.53 and an offset value of 0.00. The alignments were checked manually with BioEdit.
v.2.7.5 (Hall 1999) to ensure the correct alignment of intron, ITS1 and ITS2 regions. Introns were excluded from further phylogenetic analyses. Then, the dataset were tested using the corrected Akaike information criterion (AICc) for the best fitted substitution model in jModelTest 2.1.10 (Guindon & Gascuel 2003; Darriba et al. 2012) and analysed in terms of maximum likelihood (ML) and Bayesian inference (BI).

Phylogenetic trees were inferred for each of the datasets using three different methods: Maximum likelihood

Figure 2. Etiological signs and symptoms of Pinus mugo needles: A – streaked necrosis (symptom type C); B – ascomata Lophodermium corconticum; C – ascomata Lophodermium conigenum; D – conidiomata Leptomelanconium allescheri; E – conidiomata and immature hysterothecia Lophodermium pini-mugonis; F – conidiomata Hendersonia sp.; G – needles with numerous irregular whitish yellow spots (type G symptoms); H – aecium Coleosporium senecionis (Photo A–G – T. Kowalski, Photo H – C. Bartnik). Scales: A–G = 2 mm; H = 5 mm.
Maximum likelihood (ML) analysis was run in PhyML 3.0 (Guindon et al. 2010) via the Montpellier online server (http://www.atgc-montpellier.fr/phyml/) with 1000 bootstrap replicates. The best evolutionary substitution model for the ITS region was HKY+G. The BI analysis was carried out in MrBayes v 3.1.2 (Ronquist & Huelsenbeck 2003). The MCMC chains were run for 10 million generations using the best-fitted model. Trees were sampled every 100 generations, resulting in 100,000 trees from both runs. The burn-in value for each dataset was determined in Tracer v 1.4.1 (Rambaut & Drummond 2007).

MP analyses were performed using PAUP* 4.0b10 (Swofford 2003). Gaps were treated as fifth state. Bootstrap analysis (1000 bootstrap replicates) was conducted to determine the levels of confidence for the nodes within the inferred tree topologies. Tree bisection and reconnection (TBR) was selected as the branch swapping option. The tree length (TL), Consistency Index (CI), Retention Index (RI), Homoplasy Index (HI) and Rescaled Consistency Index (RC) were recorded for each analysed dataset after the trees were generated.

Estimates of average evolutionary divergence over sequence pairs within and between groups L. corconticum and L. conigenum were conducted using the Maximum Composite Likelihood model (Tamura et al. 2004). Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

**Results**

Fifty seven taxa were found in the needles of P. mugo, of which 33 were identified to species, 13 to genus, 2 to family, 3 to class and 6 were distinguished as unidentified (Table 3; Figs 2–3). Out of the seven types of disease symptoms identified on needles, the greatest species diversity of fungi was found in dead needles (29 taxa), and the smallest (5 taxa) in needles with symptoms of type C and F (Table 2). Fungi were most often isolated from needle fragments completely dead and living needles with necrosis progressing from the root (only 22.5% and 21.1% of the fragments were sterile, respectively), and least frequently from needle fragments with G symptom (90.3% sterile) and needles without disease symptoms (Table 2).

Much attention was paid to determining the species diversity of fungi from the genus *Lophodermium*. Comparison of the ITS sequences obtained from all representative isolates, initially assigned by their morphological characteristics to the genus *Lophodermium*, with sequences in GenBank database confirmed their genetic affinities among the different species of this genus. The isolates were distributed into three species. One isolate belonged to *L. pini-mugonis*, ten isolates belonged to *L. conigenum* and 33 isolates represented *L. corconticum*.

Based on the BLAST results, the currently obtained ITS sequences showed similarities ranging from 98.82% to 99.73% for *L. conigenum*, 98.20% to 100% for *L. corconticum*, and 100% for *L. pini-mugonis* when related to references sequences. The 44 complete ITS region sequences ranged in length from 538 to 716 bp. The shortest sequence belonged to *L. pini-mugonis*. The length of the *L. conigenum* sequence ranged from 690 to 705 bp. The longest sequences belonged to the *L. corconticum* sequence from (700–716 bp). The ITS sequences obtained from all isolates contain intron at the 3’ end of 18S subunit, except *L. pini-mugonis*. The intron length for *L. conigenum* ranged from 200 to 203 bp, and for *L. corconticum* from 2012 to 2014 bp. Alignments for the ITS dataset used in phylogenetic analyses contained 549 characters; including gaps.

The affiliation of the studied isolates to three known taxa, i.e., *L. pini-mugonis*, *L. conigenum* and *L. corconticum*, was also confirmed on the basis of ITS phylogenetic analyses (Fig. 4). The ITS sequences of these species form clades clearly distinguished from other species belonging to the genus *Lophodermium*. These clades are strongly supported by the results of analyses carried out with the use of ML and BI methods (Fig. 4). Evolutionary analyses involved 65 nucleotide sequences of *L. corconticum* and *L. conigenum*. There were a total of 549 positions from needle fragments completely dead and living needles with necrosis progressing from the root (only 22.5% and 21.1% of the fragments were sterile, respectively), and least frequently from needle fragments with G symptom (90.3% sterile) and needles without disease symptoms (Table 2).

Table 2. Types of disease symptoms on needles and data on the isolation of fungi from needles.

| Type of disease symptoms | Characteristics of the symptom | Number of needles tested | Number of fragments | Sterile fragments (%) | Number of isolated fungal taxa |
|-------------------------|--------------------------------|-------------------------|---------------------|----------------------|--------------------------------|
| A                       | Needles without symptoms      | 50                      | 240                 | 77.5                 | 12                             |
| B                       | Needles with numerous yellow and yellow-brown spots | 125                     | 500                 | 45.8                 | 26                             |
| C                       | Needles with a brown spot or streaked necrosis, then associated with a dead top | 55                      | 140                 | 72.0                 | 5                              |
| D                       | Dead needles, only the living epiphyseal part (0.7–1.5 cm long) remained | 75                      | 190                 | 41.2                 | 24                             |
| E                       | Needles completely dead       | 150                     | 600                 | 22.5                 | 29                             |
| F                       | Live needles, with necrosis progressing from the base | 30                      | 180                 | 21.1                 | 5                              |
| G                       | Needles with numerous irregular whitish yellow spots | 35                      | 210                 | 90.3                 | 6                              |
| **Total**               |                                | **520**                 | **2060**            |                      |                                |
Table 3. The species composition and the share of fungi inhabiting the needles of mountain pine with various symptoms. A–G – as in Table 2, OE – fungi were identified on the basis of their etiological signs; ‘+’ – needles with etiological signs.

| The name of the fungus                                      | Most similar sequence in GenBank | Similarity (%) | The symptom type and % of the inhabited needles with this symptom | OE |
|-------------------------------------------------------------|----------------------------------|----------------|-------------------------------------------------------------------|----|
| Alternaria alternata (Fr.) Keissl                         | MH384939.1                       | 100.0          | A–G                                                               |    |
| Alternaria caesiptosa (de Hoog & C. Rubio) Woudenberg & Crous | MH861255.1                       | 99.5           |                                                                   |    |
| Alternaria chartarum Preuss                                 | MH430101.1                       | 99.8           |                                                                   |    |
| Alternaria infectiona E.G. Simons                          | MK460954.1                       | 99.5           |                                                                   |    |
| Alternaria sp.                                              | MG020276.1                       | 99.4           |                                                                   |    |
| Alternaria tenella (Kunze) Wiltshire                        | MT573466.1                       | 99.8           |                                                                   |    |
| Aureobasidium pullulans (de Bary & Löwenhah) G. Arnaud      | JX462671.1                       | 99.0           |                                                                   |    |
| Basidiomycetes                                              | GU566227.1                       | 98.0           | A–G                                                               |    |
| Biscogniauxia nummularia (Bull.) Kunzite                    | MH860015.1                       | 99.3           |                                                                   |    |
| Botrytis cinerea Pers.                                      | KP050616.1                       | 100.0          |                                                                   |    |
| Cadophora sp.                                               | JF327416.1                       | 99.0           |                                                                   |    |
| Cenangium acuem Cooke & Peck                                | LT158445.1                       | 98.2           |                                                                   |    |
| Cenangium ferruginosum Fr.                                  | LT158467.1                       | 100.0          |                                                                   |    |
| Cladosporium allicinum (Fr.) Bensech, U. Braun & Crous      | KP701895.1                       | 100.0          |                                                                   |    |
| Cladosporium cladosporoides (Fresen.) G.A. de Vries         | KU314943.1                       | 99.0           |                                                                   |    |
| Daldysa species                                              | JN225913.1                       | 99.2           |                                                                   |    |
| Davidiella sp.                                              | KPT14607.1                       | 99.0           |                                                                   |    |
| Desmazierella acicola Lib.                                  | MT790315.1                       | 99.3           |                                                                   |    |
| Dimerosporium sasae A. Hashim., Sat. Hatak. & Kaz. Tanaka   | NR_155038.1                      | 99.3           |                                                                   |    |
| Dothideomycetes                                             | KY436101.1                       | 99.8           |                                                                   |    |
| Epicoccum nigrum Link                                       | MH861752.1                       | 99.6           |                                                                   |    |
| Fimiaria rubroeburistis (Niessl) N. Lundq.                  | JX421715.1                       | 100.0          |                                                                   |    |
| Hendersons sp.                                              | KT000192.1                       | 99.8           |                                                                   |    |
| Herpophotiapia sp.                                          | KTS581851.1                      | 98.0           |                                                                   |    |
| Hormonema sp.                                               | KPT14567.1                       | 98.0           |                                                                   |    |
| Lachnellula calyciformis (Batsch) Dharne                    | MH858771.1                       | 99.8           |                                                                   |    |
| Lachnellula subtillissima (Cooke) Dennis                    | MH752069.1                       | 98.6           |                                                                   |    |
| Lesistomyces sp.                                            | KP900974.1                       | 99.8           |                                                                   |    |
| Leptosphaerium allesiacheri (Schnabl) Petr.                 | MF573935.1                       | 98.0           |                                                                   |    |
| Lopadostoma turidium (Pers.) Traverso                       | KC774617.1                       | 100.0          |                                                                   |    |
| Lophodermium conigenum (Brunaud) Hilüter                   | KY742578.1                       | 99.6           |                                                                   |    |
| Lophodermium coroticum Koukol, Pusz & Minter                | HG939564.1                       | 99.4           |                                                                   |    |
| Lophodermium pini-mugonis C.L. Hou & M. Piepenbr.           | JF332165.1                       | 100.0          |                                                                   |    |
| Massarinaceae                                               | KJ486534.1                       | 99.4           |                                                                   |    |
| Mollisia sp.                                                | MH86008.1                        | 95.2           |                                                                   |    |
| Mycosphaera tassiana (De Not.) Johanson                     | HG935310.1                       | 99.0           |                                                                   |    |
| Nemania serpens (Pers.) Gray                                | KU141386.1                       | 99.0           |                                                                   |    |
| Parastagonospora aveanae (A.B. Frank) Quadell., Verkle & Crous | LT603040.1                      | 99.6           |                                                                   |    |
| Periconia macrospinoa Lefebvre & Aar.G. Johnson             | MN547378.1                       | 99.4           |                                                                   |    |

*OE* – fungi identified on the basis of etiological signs; ‘+’ – needles with etiological signs.
in the final dataset (Table 4). The *L. corconticum* groups, which consisted of 33 sequences, indicated some variation. Subpopulations of *L. corconticum* represented by groups 1 and 1R indicated low within-group (respectively 0.0052, 0.0054) and between-group (0.0052) divergences. It can therefore be concluded that the sequences of *L. corconticum* obtained in the present study do not differ significantly from reference sequences deposited in GenBank. The differences between the groups representing *L. corconticum* and *L. conigenum* ranged from 0.0646 to 0.0798. The *L. conigenum* groups, which consisted of 10 sequences, indicated substantial variation. Subpopulations of *L. conigenum* represented by groups 2, 2R-a and 2R-b indicated low divergences within-group (from 0.0014, to 0.0050). Divergences between-group for subpopulations represented by groups 2 and 2R-a were low (0.0031).

Table 4. Estimates of average evolutionary divergence over sequence pairs within (d) and between groups (D).

| The name of the fungus | Most similar sequence in GenBank | Similarity (%) | The symptom type and % of the inhabited needles with this symptom | OE |
|------------------------|----------------------------------|----------------|---------------------------------------------------------------|-----|
|                        |                                  |                | A   | B   | C   | D   | E   | F   | G   |
| *Pezizomycetes*        | GQ153137.1                       | 99.0           | –    | 1.6 | –   | –   | –   | –   | –   |
| *Phaeosphaeria* sp.    | EF432300.1                       | 99.6           | –    | 3.2 | –   | –   | –   | –   | –   |
| *Phialocephala* sp.   | FR774054.1                       | 99.8           | –    | 1.6 | –   | 1.4 | –   | –   | –   |
| *Rhytismataceae*      | KF889704.1                       | 96.0           | –    | –   | –   | 1.4 | 0.7 | –   | +   |
| *Sydowia polyspora*    | JN944640.1                       | 100.0          | –    | 1.4 | 7.3 | 7.1 | 0.7 | 56.7 | 2.9 |
| *Trichoderma laxis*   | KU394235.1                       | 99.0           | –    | –   | –   | –   | 2.0 | –   | –   |
| *Xylaria* sp. Pm 61   | KP174691.1                       | 98.0           | –    | 0.8 | –   | –   | 2.0 | –   | –   |
| Unidentified Pm 32    |                                  | –              | –    | –   | –   | –   | 0.8 | –   | –   |
| Unidentified Pm 49    |                                  | –              | –    | –   | –   | 0.8 | –   | –   | –   |
| Unidentified Pm 50    |                                  | –              | –    | –   | 2.4 | –   | –   | –   | –   |
| Unidentified Pm 52    |                                  | –              | –    | –   | 0.8 | –   | –   | –   | –   |
| Unidentified Pm 62    |                                  | –              | –    | –   | 0.8 | –   | –   | –   | –   |
| Unidentified Pm 67    |                                  | –              | –    | –   | 0.8 | –   | –   | –   | –   |

Therefore, it can be concluded that this is the level of nucleotide differences corresponding to intraspecific variations. Divergences between-group for subpopulations represented by groups 2, and 2R-a vs 2R-b were relatively high (respectively 0.0386 and 0.0399). This result indicates a high probability that the sequence group 2R-b represents a species other than *L. conigenum* (Table 4). It was also confirmed on the basis of ITS phylogenetic analyses which revealed that sequences of *L. conigenum* obtained from GenBank formed strongly supported sister lineage with some of the sequences obtained in this study on the phylogram (Fig. 4).

Twelve taxa were isolated from needles without disease symptoms, of which 10 were also found in needles with various disease symptoms. These included primarily: *L. conigenum*, *L. corconticum*, Desmazerella acicola and Leptomelanconium allescheri. The frequency of isolation of these fungi from needles without symptoms ranged from 6.0% for *Lep. allescheri* up to 12.0% for *L. conigenum*.

Among the identified fungi, the needles were colonized by four species with a high frequency: Lophodermium corconticum, L. conigenum, Sydowia polyspora and Hendersonia sp. The *L. corconticum* was isolated from the 6 types of needles tested (except type F). The most numerous were on type B (32.0%) and type C (21.8%) (Fig. 2A). On dead needles, this fungus produced the characteristic ascomata, conidiomata and black lines (Fig. 2B). In vitro, developed a white colony with black sclerotic mycelium in the form of patches or lines and colorless, bacilliform conidia (Fig. 3A–B). *L. conigenum* inhabited all types of needles, and was most often isolated from needles type B (17.6%) and type E (17.3%). On the dead needles, this fungus produced ascocarps, conidiomata and sometimes brown fuzzy oblique stripes (Fig. 2C). On the other hand, on the MEA medium, it produced colonies with sparse air mycelium, white-cream to light brown in color, and rod-shaped conidia (Fig. 3G–H).

Sydowia polyspora was isolated from most types of symptoms (except type A), but most often this fungus
inhabited live needles, with necrosis progressing from the root (56.7%).

*Hendersonia* sp. was isolated from 5 types of needles, most often from dead needles (46.5%) and needles with yellow and yellow-brown spots, and from needles with a viable epiphyseal part only (9.6% and 7.1%, respectively). This fungus produced black pycnidial conidiomata on dead needles (Fig. 2F).

The group of fungi with a lower frequency of occurrence and usually isolated from 2 or 3 (occasionally 4–5)

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**Figure 3.** Cultures of fungi isolated from the *Pinus mugo* needles: A – culture *Lophodermium corconticum* (no. 381KS); B – conidia *Lophodermium corconticum;* C – culture *Lophodermium pini-mugonis;* D – conidia *Lophodermium pini-mugonis* (no. 445KS); E – culture *Leptomelanconium allescheri* (no. Pm68KS); F – macroconidia *Leptomelanconium allescheri;* G – culture *Lophodermium conigenum* (no. 386KS); H – conidia *Lophodermium conigenum* (Photo T. Kowalski). Scales: B, F, H = 10 µm; D = 5 µm.
Figure 4. Phylogram obtained from Maximum Likelihood (ML) analyses of the ITS data for the isolated fungal taxa belonged to *Lophodermium* genus. Sequences obtained during this study are presented in bold type. The Bootstrap values ≥ 75% for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses.* Bootstrap values < 75%. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Colpoma quercinum* represent the outgroup.
types of symptoms included 18 taxa: Alternaria infectoria, Biscogniauxia nummularia, Cladosporium spp., Desmazerella acicola, Leptomelanconium allescheri, Cenangium ferruginosum, Nemania serpens, Alternaria spp., Cenangium acuum and one taxon from the Rhytismataceae family. The frequency of their occurrence depending on the type of disease symptom is given in Table 3. Some of them produced etiological signs on the needles in situ. Lep. allescheri produced black acervular conidiomata (Fig. 2D) on dead and dying needles, and on MEA medium it developed colonies yellowish-olive to yellowish brown, with weak aerial mycelium, sometimes with visible black tar drops (macroconidia) (Fig. 3E–F). An unidentified species of the fungus from the Rhytismataceae family also produced hysterothecia with club-shaped, mucus-surrounded ascospores on dead parts of needles. Very limited growth on MEA medium was observed for this species, both from ascospores and from fragments of infested needles, which made identification difficult. In the case of Lophodermium pini-mugonis, immature ascomata and conidiomata were found on a dead attached needle in autumn (Fig. 2E), containing hyaline, rod shaped, 2–3.5 × 0.8–1.2 μm conidia (Fig. 3D). After four weeks on the MEA medium, the colony reached a diameter of 60–80 mm at 20°C. It is compact, adherent to the nutrient solution, with a modest air suede mycelium, cream-orange to orange-brown, sometimes with darker zones (Fig. 3C). The frequency of the obligate parasite, Coleosporium senecionis, was determined by the ecium stage on the needles (Fig. 2H). The presence of this species was found on 3.3% of the needles (Table 3). This fungus is responsible for the pine blister rust.

Most taxa (61.4%) were isolated from only one type of symptom (B, D or E), and their frequency of occurrence was relatively low and ranged from 0.7 to 4.3% (average 2.1%) of the inhabited needles. There are two known pathogenic species in the group of these fungi: Botrytis cinerea and Herpotrichia sp. (Table 3).

Needles with numerous irregular whitish yellow spots (Fig. 2G) were only sporadically populated by fungi. Only six species of fungi were isolated from 9.7% of needle fragments. The frequency of their occurrence did not exceed 3%, with the exception of L. conigenum (5.7%).

Discussion

The obtained results indicate a large diversity of disease symptoms on the needles of P. mugo and a significant diversity of species of fungi present in such needles. One of the symptoms on P. mugo needles was whitish-yellow spots (Type G). From such needles, it was possible to isolate fungi only sporadically, which suggests that their cause should be seen among the abiotic factors. The occurrence of similar spots on P. mugo and with a particularly high frequency on Picea abies is reported by Schütt et al. (1985). In the case of P. abies, such discoloration intensified with the increase of altitude above sea level in mountainous areas. There were numerous indications that they could be caused by photo-oxidants (Lang & Holdenrieder 1985; Schütt et al. 1985). Evidence for potential impacts of ozone was also observed at some mountain sites in Europe on Pinus cembra (Wieser et al. 2005) and on other conifers in mountain forest stands in California (Bytnerowicz et al. 2008).

When considering the role of the identified species of fungi, it should be borne in mind that the assimilation apparatus of forest trees is commonly inhabited by endophytic fungi that live inside plant cells for a significant period of their life cycle without causing visible disease symptoms (Sieber 2007). The colonization frequency and species diversity of endophytic fungal communities are affected by host species, tissue age and geographic location (Saikkonen et al. 1998; Sieber 2007). The endophyte communities in angiosperms are frequently dominated by species of Diaporthales and those in gymnosperms by species of Helotiales (Sieber 2007). In Poland, over 80 taxa of fungi have been found in the symptomless needles of P. sylvestris and 40 taxa in the needles of P. nigra (Kowalski 1993; Kowalski & Zych 2002). In P. mugo needles, the endophytic fungal community seems to be significantly poorer. Sieber et al. (1999) in symptomless needles of P. mugo ssp. uncinata in Switzerland found 11 species. In the current research in the Tatra Mountains, 12 species have been isolated from such needles. It is important to know the species composition of endophytes because some of them are, however, opportunistic and can cause disease after the host has been weakened by some other factor (Sieber 2007). This is one of the important, difficult to resolve the problems, which endophytes contribute to the disease of plant organs, and which, taking advantage of their death for other reasons, produce their fruiting bodies on them. This problem was highlighted in Germany in relation to the common premature browning disease and spruce needle fall (Butin 1986). This aspect should also be considered when analyzing the role of fungi as the cause of P. mugo needle disease in the Tatra Mountains. Among the endophytes we found, there are those found also on P. mugo and other pine species in other regions of the world. A typical representative of this group is Cenangium ferruginosum (Hata & Futai 1995; Sieber et al. 1999). In turn, Desmazerella acicola is a common endophyte in the needles of P. sylvestris and P. nigra in Poland (Kowalski 1993; Kowalski & Zych 2002), which then occurs on fallen needles in the litter as a saprotroph (Kowalski 1988). In contrast, representatives of the genera Alternaria, Cladosporium and Epicoccum are isolated as endophytes from a wide range of conifers and deciduous plants.

P. mugo needles in the Tatra Mountains showed very diverse types of discoloration during the study period, sometimes preceded by distinct tissue necrosis. The discoloration of more than 40 taxa of fungi in needles with various types of discoloration indicates a large species diversity of fungi able to colonize the tissues of P. mugo needles. The only representative of the obligate parasites was Coleosporium senecionis, which produced the aecium stage on living needles. The remaining species, in terms of trophic conditions, should be classified as relative parasites or saprotrophs, secondarily inhabiting dying tissues. Some species found in symptomatic needles may have existed previously in needles as endophytes, confirming
the views expressed by other authors (Saikkonen et al. 1998; Sieber et al. 1999; Sieber 2007).

The fungi known for frequent colonization of the needles of *Pinus* spp. include numerous species of the genus *Lophodermium*, with different ability to cause disease symptoms (Minter & Millar 1980; Kowalski 1982; Ortiz-Garcia et al. 2003; Salas-Lizana & Oono 2018). On *P. sylvestris* in Europe, *L. piniastri* and *L. seditiosum* and locally *L. conigenum* are most often recorded (Minter & Millar 1980; Reignoux et al. 2014), and also *L. pini-excelsae*, which occurs mainly in 5-needle pines (Minter et al. 1978).

Among these species, only *L. seditiosum* shows clear pathogenic character on *P. sylvestris*, and *L. conigenum* inhabits mainly trash needles. *Lophodermium piniastri* fructifies mainly on fallen needles in the litter (Minter & Millar 1980), but it is also a common endophyte. Reignoux et al. (2014) drew attention to the significant differentiation of *L. piniastri* endophytically occurring in living needles, distinguishing three cryptic taxa within it. In Poland, only *L. piniastri* and *L. seditiosum* were found in *P. sylvestris* nurseries and stands, and their occurrence depended on the age of the stand and the age of the needles (Kowalski 1982, 1988, 1993). According to Butin (1984), in Germany, there are mainly three *Lophodermium* species on *P. nigra*, with *L. conigenum*, *L. piniastri* inhabiting mainly dead or dying needles, while sometimes only *L. seditiosum* has phytopathogenic significance.

In the current research, three species of *Lophodermium* were found on the needles of *P. mugo*: *L. conigenum*, *L. corconticum* and *L. pini-mugonis*. Of these, only *L. conigenum* is more widely known from various pine species in many regions of Europe (Minter et al. 1978; Minter & Millar 1980; Butin 1984; Koukol et al. 2015). While on *P. sylvestris* and *P. nigra* it is treated more like a saprotroph, whereas, on *P. mugo* it was often found as an endophyte. It was also isolated from symptomatic needles and then its fructification was observed on dead needles. *L. corconticum* was described on the needles of *P. mugo* from the Karkonosze Mountains in Poland (Koukol et al. 2015) and so far it has not been recorded outside of this region. The Polish Tatras are therefore the second recognized site for this species. Its finding on needles showing various symptoms of necrosis and discoloration may suggest that it exhibits some pathogenic properties, while its presence in living symptomless needles may indicate that it is capable of living as an endophyte. However, it should be noted that after a successful infection, pathogenic fungi begin the incubation period, after which disease symptoms appear. To obtain a credible answer in this regard, it will be necessary to carry out pathogenicity tests. As Koukol et al. (2015) already indicated, *L. corconticum* produces hysterotheca, conidiomata and black lines on the needles, very similar to those produced by *L. piniastri*. The in vitro colonies are also similar (Kowalski 1982). Therefore, it cannot be ruled out that *L. corconticum* also occurs in other regions of Europe on *P. mugo*, but can be treated as *L. piniastri* sensu lata. In the present research, *L. piniastri* has not been found.

Recently, a new species of *L. pini-mugonis* was described on the needles of *P. mugo* in the German Alps (Hou et al. 2009). Until now, this species is only known from the type collection. During the current research in the Polish Tatras, its presence was confirmed on one needle. Despite good genetic compatibility with the holotype, there are some differences in the microscopic features of conidia. The conidia we observed in conidiomata in situ were slightly longer and rather rod shaped or cylindrical, quite typical in shape to many other *Lophodermium* spp. (Minter 1981). They were significantly shorter than those of *L. conigenum* or many other *Lophodermium* spp. (Minter 1981). Hou et al. (2009) specify that the conidia were ellipsoidal in shape. In the present work, a colony of this species in vitro is also presented for the first time. To determine the degree of variability within fruiting body and in vitro colonies, it would be necessary to find a larger number of needles colonized by this species.

Summing up the aspect of the presence of *Lophodermium* spp. on *P. mugo* in the Polish Tatras, it should be stated that it is significantly different from the species composition of *Lophodermium* fungi occurring in Poland on *P. sylvestris* (Kowalski 1982, 1988, 1993).

Currently conducted research has provided numerous data on *Lophodermium* at the molecular level. Koukol et al. (2015) describing *L. corconticum* demonstrated the ability to identify isolates of the species based on the analysis of the ITS, which was also confirmed in the present work. Unfortunately, these authors have deposited the sequences with GenBank without describing the ITS regions. This is of particular importance for the appropriate preparation of data for phylogenetic analysis and for ensuring the comparability of the results with other studies. In this work, as in the publication by Ortiz-Garcia et al. (2003), fragments of the ITS sequence containing the intron were excluded from the phylogenetic analysis. All *L. corconticum* sequences obtained in this study contained an intron within the 18S fragment. Comparing them with the reference sequences obtained by Koukol et al. (2015), it can be concluded that the intron was present in all sequences except one, the length of which did not allow the assessment of the presence of the intron. Common, but not mandatory presence of an intron sequence in the region of 18S fungi of the genus *Lophodermium* was pointed by Ortiz-Garcia et al. (2003), and confirmed in this study. There was no intron in the ITS sequence of *Lophodermium pini-mugonis*, while the *L. corconticum* and *L. conigenum* sequences had introns of different lengths. The length of these introns, however, did not exceed that reported by Ortiz-Garcia et al. (2003) of 225 bp. According to Ortiz-Garcia et al. (2003) species of the genus *Lophodermium* differ in the level of intraspecific variation in the ITS sequence. For example, the differences between the sequences obtained from isolates were for: *L. piniastri* 5.6%, *L. agathidis* 2.9% and *L. conigenum* 0%. Koukol et al. (2015) reported that the nucleotide variation in ITS sequences for *L. corconticum* does not exceed 0.5%, which was also confirmed by the results of this study. The subpopulation of *L. corconticum* from the Tatra Mountains does not differ in terms of ITS sequence from the subpopulation from the Sudetes. This is also confirmed by the lack of lineage in the phylogram.
(Fig. 2) representing the geographical variation of ITS sequences for this species.

The result of the phylogenetic analysis shows that the ITS sequences obtained from the isolates of fungi designated as *L. conigenum* do not form one clade. There are two sister clades in Fig. 2. A similar system of relations between *L. conigenum* isolates was also found by Koukol et al. (2015). *L. conigenum* sequences obtained in the present study from isolates derived from *P. mugo* needles form one clade with sequences of fungi isolated from *P. sylvestris* in Scotland, most of which come from studies by Reignoux et al. (2014). The second clade includes sequences of fungi designated as *L. conigenum* and *L. austrole*. Among this group of sequences, a separate lineage is formed by sequences obtained from *L. conigenum*, for which the host plants were *Pinus radiata* from New Zealand and *P. thunbergii* from China. Large differences between *L. conigenum* sequences were also presented in the phylogenogram by Wang et al. (2010). Such a system of relations between two sister clades in Fig. 2. A similar system of relations between *L. conigenum* and *P. mugo* do not form one clade. There are large differences in the ITS sequences obtained in the isolates of fungi designated as *L. conigenum* and *L. austrole.*

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