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New regional records of Dothistroma needle blight pathogens from Slovakia: distribution, hosts and pathogens characterization

Katarína Adamčíková[1]✉, Katarína Pastirčáková[1], Zuzana Jánošíková[1], Radovan Ostrovský[1], Martin Pastirčák[2], Jozef Pažitný[1], Marek Kobza[1], Slavomír Adamčík[3,4], Miriam Kádasi-Horáková[1], Emília Ondrušková[1]

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Abstract Dothistroma needle blight is one of the most severe needle diseases of pines caused by two closely related species, *Dothistroma septosporum* and *D. pini*. The further spread and distribution of this disease were investigated in Slovakia, and new hosts and stand types were identified at the regional level. *Dothistroma septosporum* was recorded in a natural stand at higher altitude on *Pinus cembra* in the High Tatras and the *P. uncinata* records are new host reports for Slovakia for this pathogen. Moreover, for *D. pini*, *P. cembra* as a new host at the country level was recorded and *P. armandii* was identified as new host species worldwide. Mating types for all collected samples and ITS haplotypes for *D. pini* isolates were determined. For *D. pini*, five localities with the presence of both mating types and three ITS haplotypes (Dp_HAP.1, Dp_HAP.2 and Dp_HAP.4) were reported. Samples where both mating types of the pathogens were identified, were selected for the microscopic examination of fruiting bodies aimed to detect sexual reproductive organs. In all inspected needle samples of *D. pini*, only conidiomata with typical hyaline cylindrical conidia were identified. The sexual state of *D. septosporum* was recorded in one sample of *P. nigra* needles.

Keywords: *Dothistroma septosporum*, *Dothistroma pini*, Slovakia, mating types, ITS haplotypes, sexual state.

Addresses: [1]Institute of Forest Ecology of Slovak Academy of Sciences, Department of Plant Pathology and Mycology, Nitra, Slovakia.| [2]National Agricultural and Food Centre, Research Institute of Plant Production, Piešťany, Slovakia.| [3]Plant Science and Biodiversity Center of Slovak Academy of Sciences, Institute of Botany, Laboratory of Molecular Ecology and Mycology, Bratislava, Slovakia.| [4]Comenius University in Bratislava, Faculty of Natural Sciences, Department of Botany, Bratislava, Slovakia.

✉ **Corresponding Author:** Katarína Adamčíková (katarina.adamcikova@ife.sk).

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Introduction

Pines, which cover 7% of the forest land in Slovakia, thus belong to the most important forest trees with the two most commonly planted pine species, Scots pine (*Pinus sylvestris* L.) and Austrian pine (*Pinus nigra* J.F. Arnold), suffering from numerous fungal diseases (Anonymous 2011, Kunca & Leontovyč 2013). These mostly affect needles causing needle blights. One of the most serious is Dothistroma needle blight (DNB), caused by two species of *Dothistroma*, *D. septosporum* (Dorogin) M. Morelet and *D. pini* Hulbary (Ascomycota, Mycosphaerellales, Mycosphaerellaceae) (Barnes et al. 2004). Over the last few decades, DNB has increased in both incidence and severity in many European countries (Barnes et al. 2022). The host range list of DNB pathogens registered 113 Pinaceae taxa, among them 99 belong to the *Pinus* genus (Barnes et al. 2022).

DNB was for the first time recorded in Slovakia in 1996 in the locality of Modrý Kameň, located in south central Slovakia, close to the Hungarian border. However, only morphological methods were used to observe the symptoms of *P. nigra* (Kunca & Foffová 2000). *Dothistroma septosporum* and *D. pini* were first confirmed using molecular methods several years later by Ondrušková et al. (2017).

Lately, Jánošíková-Hečková et al. (2018) reported the host and geographic range of DNB in Slovakia when both native and planted stands were investigated. The disease was widespread and confirmed in 73 localities in Slovakia, while negative results were recorded in natural and naturally regenerated *P. mugo* Turra and *P. cembra* L. stands (Ondrušková et al. 2017, Jánošíková-Hečková et al. 2018). DNB was identified to affect 11 pine species (*P. mugo*, *P. nigra*, *P. sylvestris*, *P. cembra*, *P. ponderosa* Douglas ex C. Lawson, *P. aristata* Engelm., *P. jeffreyi* Balf., *P. coulteri* D. Don, *P. densiflora* Siebold & Zucc., *P. flexilis* E. James, *P.* × *schwerinii* Fitschen) and two spruce species (*Picea abies* (L.) H. Karst. and *P. pungens* Engelm.) (Jánošíková-Hečková et al. 2018). However, in Slovakia DNB poses a risk primarily to plantations of non-native *P. nigra*, followed by *P. mugo* trees in urban greenery as well as some introduced pine species in parks or arboreta. The most common hosts were *P. mugo*, *P. nigra* and *P. sylvestris* (Jánošíková-Hečková et al. 2018).

DNB pathogens are heterothallic ascomycete fungi for which both mating types (*MAT1-1-1* and *MAT1-2* idiomorphs) have been identified (Groenewald et al. 2007). For these fungi the presence of corresponding mating types is required for sexual reproduction, thus mating type surveys are a simple way to detect their potential for sexual reproduction (Milgroom 1996, Turgeon & Yoder 2000). A better knowledge of the reproductive biology of a pathogen can be crucial for disease management (Milgroom 1996). Both mating types for both *Dothistroma* species were found in Slovakia (Adamčíková et al. 2021, Jánošíková et al. 2021). Although both mating types of *D. septosporum* were found to be in an almost perfect 1:1 ratio, random mating was rejected in the entire dataset, but supported at a smaller scale (*P. mugo* group planted in urban areas; two localities: Kálnica, Litava). These findings suggest a mixed mode of reproduction with an important component of sexual reproduction, although the sexual state (teleomorph) of the fungus (known as *Mycosphaerella pini* Rostr.) has not been physically observed in Slovakia yet (Jánošíková et al. 2021). The sexual state was described in 1957 by Munk from material collected by E. Rostrup in Denmark in 1880, but with no link made to any asexual state or disease (Munk 1957). The connection of the sexual state of the fungus with DNB was first described by Funk and Parker from material collected in British Columbia in 1966, lately being recorded in 11 European countries (Drenkhan et al. 2016).

In the case of *D. pini*, only a single mating type was identified per location while *MAT1-2* idiomorph (92%) was the most dominant and *MAT1-1-1* idiomorph was only present in two localities suggesting that sexual reproduction is not occurring in the studied locations in Slovakia (Adamčíková et al. 2021). The sexual state of *D. pini* has never been described, despite the presence of both mating types reported in the USA and Europe from France, Slovenia, Switzerland and Ukraine and in the same sampling site (Drenkhan et al. 2016).

The recorded climatic variables and deterioration of the disease intensity at localities suggest that climatic conditions are suitable for the spread, growth and life cycle of DNB pathogens in Slovakia (Ondrušková et al. 2020, Woods et al. 2016), expecting further geographical and host expansion. Therefore, the objective of the present study was to determine DNB pathogens through molecular identification in new regions, stand types and hosts. Further, we aimed to characterize *Dothistroma* pathogens by the mating system and/or ITS haplotypes. Additionally, in locations/samples with both mating types, we looked for the sexual reproductive organs of DNB pathogens.

Materials and Methods

Sampling

The sampling of pine needles with typical symptoms was conducted from 2018 to 2020. The presence of the disease was investigated based on characteristic symptoms indicated in EPPO descriptions (EPPO 2015), particularly dead needle tips, central zones with spots in green tissue and small black fruiting bodies (acervuli) in affected parts of the needles. The needles were collected randomly from different parts of the tree crown, in which the aforementioned symptoms were observed, with those showing the presence of conidiomata being selected. Although the number of collected needles per sample was dependent on infection intensity and the size of evaluated trees, each sample consisted of at least 30 needles. Needles from each tree were treated as separate samples and stored in a –20°C freezer until processing. The studied samples were obtained from a range of different stand types, namely arboretum, urban greenery, forest plantation, and natural regeneration. Geographical location data of the sampling sites (latitude/longitude coordinates, altitude) were collected using the MobileMapper 10 (Ashtech LLC, Santa Clara, CA, USA). Georeferenced data were used to create a distribution map using the ArcGIS 9.3 software (ESRI Inc., Redlands, CA, USA).

Herbarium specimens of samples were deposited in the Plant Pathology Herbarium (NR) of the Institute of Forest Ecology of Slovak Academy of Sciences, Nitra, Slovakia.

DNA extraction

DNA was extracted from the needles. Having surface-sterilized needles by wiping with a 96% ethyl alcohol-soaked tissue paper, the symptomatic parts of needle tissue, preferentially those with acervuli, were cut into 0.5-mm pieces, transferred to 2-mL microtubes, and stored at –20°C until used for DNA extraction.

DNA was extracted using an E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek Inc., Norcross, GA, USA), following the manufacturer's instructions.

Species and mating type identification

Identification of species level and mating types was done by conventional PCR using the species and mating type-specific primers of Groenewald et al. (2007). Amplification of DNA was performed in a PCR reaction mix consisting of approximately 2 ng/μl of template DNA, forward and reverse primers (10 pmol/μl), 5× HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia) and molecular grade water added up to 20 μl. After an initial denaturation step for 15 min at 95°C, 40 cycles were performed each comprising a denaturation step at 94°C for 20 s, an annealing step (65°C for *D. pini* and 63°C for *D. septosporum*) for 30 s, and an extension step at 72°C for 40 s followed by a final extension step for 5 min at 72°C. The species-specific primers of Groenewald et al. (2007) amplify regions of approximately 820 bp and 480 bp for individual mating types in *D. pini* and *D. septosporum*.

Molecular-grade water was used as a negative amplification control during the preparation of the reaction mix.

All PCR products were visualized by horizontal electrophoresis on 1% (w/v) TBE agarose gels stained with Simply Safe (EURx Ltd. Gdansk, Poland).

Determination of *Dothistroma pini* ITS haplotypes

From needle samples, that were detected as *D. pini* by species specific primers of Groenewald et al. (2007), the pure fungal isolates were obtained as described by Ondrušková et al. (2017) and from 3 weeks old cultures DNA was extracted as specified above.

Identification of isolates to species level was done by sequencing the ITS region of the ribosomal DNA with primers ITS1F and ITS4 (White et al. 1990, Gardes & Bruns 1993). PCR reactions and conditions were set up according to Ondrušková et al. (2017).

The target fragments were purified using a QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and sequenced in both directions using an ABI3130xl sequencer (Applied Biosystems, Waltham, MA, USA) by the SEQme company (Dobříš, Czech Republic). Sequences were deposited in NCBI GenBank.

Dothistroma pini ITS haplotypes were determined using manually improved alignments generated by the Geneious Alignment function in Geneious version R10.2.6 (Kearse et al. 2012) and comparing sequences to the deposited reference sequences of Barnes et al. (2016) for ITS haplotypes Dp_HAP.1 - Dp_HAP.5, and Mullett et al. (2018) for ITS haplotype Dp_HAP.6.

Microscopic examination of fruiting bodies

For microscopic examination, we selected infected pine needles bearing conidiomata from localities/samples collected between 2013-2020, where both mating types of the pathogens were identified. Needles were gently surface-sterilized by wiping with a tissue paper soaked in 96% ethyl alcohol and placed in a damp chamber for 24 h. Ten needles with evident fruiting bodies per sample were selected and maximum 5 fruiting bodies per needle were excised for microscopic examination. The spores were observed using an Olympus BX51 microscope. All microscopic observations were made in distilled water. Lactophenol blue solution (Merck, Darmstadt, Germany) was used to stain hyaline structures. The morphological structures of the fungus were photographed using a Cannon EOS 1300D digital camera and QuickPHOTO MICRO (version 3.1) software. The observed morphological features of the examined fungus were compared to previously published descriptions.

Results

Distribution and hosts

Seventy-four samples from 40 localities in Slovakia were collected and all were identified as DNB-positive. Within assessed localities, 36 new DNB-positive localities were identified. *D. septosporum* was determined in 31 while *D. pini* in 15 localities (Table 1, Figure 1). Four different types of stands were confirmed. The majority of samples were collected in urban greenery (27 localities). The further new localities were forest plantation (7 localities), arboretum (1 locality) and natural stand (1 locality). *Dothistroma septosporum* detection in Popradské pleso locality (in the National Park of High Tatras) on *P. cembra* tree, is the first DNB record in Slovakia at a higher altitude (1494 m a. s. l.) in natural stand.

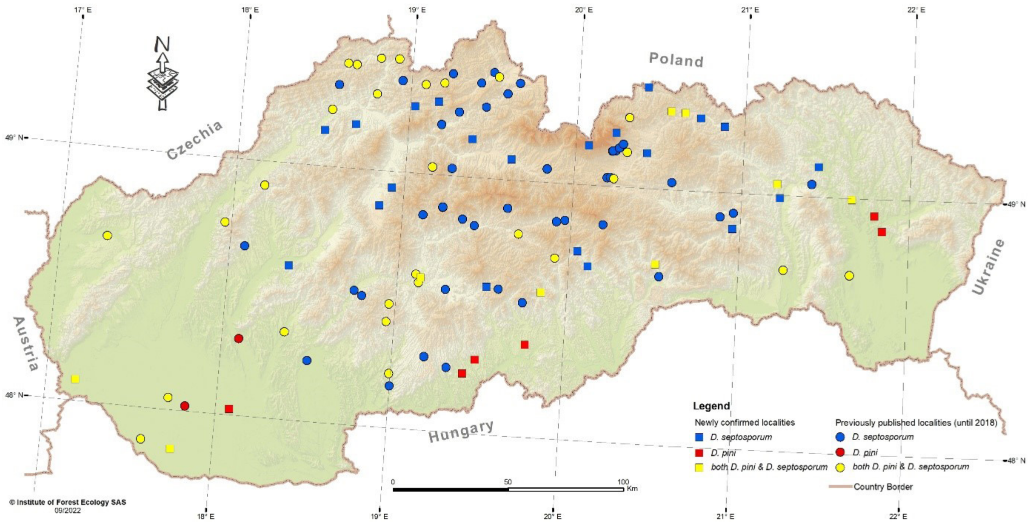

Figure 1 The geographic distribution of Dothistroma needle blight in Slovakia. Square shapes indicate newly confirmed locations in this study, while circles are locations previously published by Ondrušková et al. (2017) and Jánošíková-Hečková et al. (2018). Locations where *D. septosporum*, *D. pini* or both were identified are marked in blue, red and yellow, respectively.

Table 1 List of studied localities and their detailed descriptions

NoL	Locality	No of collected samples	Material analysed	*Pinus* host species	Type of stand	*Dothistroma septosporum*		*Dothistroma pini*	
						NoS	mating type	NoS	mating type
1	Borová hora Arboretum	1	needles	*P. aristata*	A	1	MAT2	0	
		1	needles	***P. armandii*****	A	0		1	MAT2
		2	needles	*P. mugo*	A	2	MAT2	2	MAT2
		1	needles	*P. mugo* 'Hesse'	A	1	MAT1, MAT2	0	
		1	needles	*P. mugo* 'Mops'	A	1	MAT1, MAT2	0	
		1	needles	*P. mugo* 'Slavinii'	A	1	MAT2	1	MAT2
		2	needles	*P. nigra*	A	1	MAT1, MAT2	1	MAT2
		1	needles	*P. nigra* 'Fastigiata'	A	1	MAT2	1	MAT2
		2	needles/ culture	*P. ponderosa*	A	0		2+3	MAT1, MAT2
		1	needles	***P. uncinata*****	A	1	MAT1, MAT2	0	
2	Betliar	1	needles	*P. nigra*	U	1	M AT2	1	MAT2
3	Blatnica	1	needles	*P. mugo*	U	1	MAT2	0	
4	Brodzany	1	needles	*P. mugo*	U	1	MAT1, MAT2	0	
5	Červený Kláštor	2	needles	*P. mugo*	U	2	MAT1, MAT2	0	
	Dolný Kubín	1	needles	***P. uncinata*****	U	1	MAT1, MAT2	0	
6	Gelnica	2	needles	*P. nigra*	U	2	MAT1, MAT2	0	
7	Hniezdne	1	needles	*P. nigra*	U	1	MAT2	1	MAT2
8	Hriňová	1	needles	*P. nigra*	U	1	MAT2	0	
9	Chmeľnica	1	needles	*P. nigra*	U	1	MAT1, MAT2	0	
10	Kežmarok	2	needles	*P. mugo*	U	2	MAT1, MAT2	0	

NoL	Locality	No of collected samples	Material analysed	*Pinus* host species	Type of stand	***Dothistroma septosporum***		***Dothistroma pini***	
						NoS	**mating type**	**NoS**	**mating type**
11	Kokava nad Rimavicou	1	needles	*P. nigra*	U	1	MAT1, MAT2	1	MAT2
		1	needles	*P. ponderosa*	U	0		1	MAT2
	Košice	1	needles/ culture	*P. nigra*	U	0		1+1	MAT1, MAT2
12	Kuková	1	needles	*P. sylvestris*	FP	1	MAT2	0	
13	Kvakovce	1	needles	*P. nigra*	U	1+1	MAT2	1	MAT2
14	Lietava	1	needles	*P. nigra*	FP	1	MAT1, MAT2	0	
15	Liptovský Mikuláš	2	culture	*P. nigra*	U	2	MAT2	0	
16	Ľubotín	1	needles	*P. mugo*	U	1	MAT1	0	
17	Lúčky	1	needles	*P. mugo*	U	1	MAT2	0	
18	Manín	1	needles	*P. nigra*	FP	1	MAT2	0	
	Mlyňany Arboretum	1	culture	***P. cembra*****	A			1	MAT2
19	Muráň	1	needles	*P. mugo*	U	1	MAT1, MAT2	0	
	Nitra	5	needles	*P. nigra*	U	3	MAT1, MAT2	4	MAT2
20	Palárikovo	1	needles	*P. nigra*	FP	0		1	MAT1
21	Podunajské Biskupice	2	needles/ culture	*P. nigra*	FP	0+1	MAT2	2+3	MAT2
22	Pinciná	2	needles	*P. nigra*	U	0		2	MAT1, MAT2
23	Popradské pleso	1	needles	***P. cembra***	N*	1	MAT2	0	
24	Pôtor	1	needles	*P. nigra*	U	0		1	MAT1
25	Prešov	1	needles	*P. mugo*	U	1	MAT2	0	
26	Revúca	1	needles	*P. mugo*	U	1	MAT2	0	
27	Strážske	1	needles	*P. nigra*	U	0		1	MAT1, MAT2
28	Tatranská Kotlina	1	needles	*P. mugo*	U	1	MAT1, MAT2	0	
29	Terchová	1	needles	*P. mugo*	FP	1	MAT1	0	
		1	needles	*P. nigra*	FP	1	MAT1, MAT2	0	
30	Topoľovka	1	needles	*P. nigra*	U	0		1	MAT2
31	Turčianske Teplice	2	needles	*P. mugo*	U	1	MAT1, MAT2	0	
32	Veľký Meder	6	needles	*P. nigra*	FP	1	MAT2	1	MAT2
33	Veľký Šariš	1	needles	*P. nigra*	U	1	MAT2	1	MAT1, MAT2
	Víglaš	1	needles	*P. mugo*	U	1	MAT2	1	MAT2
34	Vyšné Ružbachy	1	needles	***P. cembra*****	U	1	MAT1, MAT2	1	MAT2
		2	needles	*P. nigra*	U	2	MAT1, MAT2	0	
		1	needles	*P. sylvestris*	U	1	MAT1, MAT2	0	
35	Závada	1	needles	*P. nigra*	U	0		1	MAT1
36	Zázrivá	1	needles	*P. mugo*	U	1	MAT2	0	

Note: type of stand: A – arboretum, U – urban greenery, FP – forest plantation, N – natural; NoL – number of newly identified locality; NoS – number of samples detected needles + cultures; in bold letters: *new record, **new host record

With regards to the host spectrum of both DNB pathogens, eight pine species and four cultivars were identified (Table 1), the most frequently infected were *P. nigra* (40 samples) and *P. mugo* (22 samples). Three cultivars were recorded for *P. mugo* (‘Hesse’, ‘Mops’ and ‘Slavinii’), and one cultivar for *P. nigra* (‘Fastigiata’), all collected in Borová hora Arboretum in Zvolen. The other hosts recorded were *P. aristata*, *P. armandii* Franch. (1 sample each), *P. uncinata* Ramond ex DC., *P. sylvestris* (2 samples each), *P. ponderosa* and *P. cembra* (3 positive samples each).

Records of *D. septosporum* on *P. uncinata* (2 needle samples from 2 localities) and *D. pini* on *P. cembra* (one needle sample and one culture from 2 localities) are new host records for Slovakia. In addition, *P. armandii* (in Borová hora Arboretum) was identified as a new host species of *D. pini* worldwide.

Characteristics of *Dothistroma* species

In the case of *D. septosporum*, *MAT1-2* dominated, recorded in 32 localities in total, while only a single mating type *MAT1-2* was present in 17 localities. Only a single mating type, *MAT1-1-1* was detected in one locality. For *D. pini*, the results were similar, mating type *MAT1-2* dominated, recorded in 15 localities from 18 *D. pini* positive. Only a single mating type was detected in 13 localities, while only *MAT1-1-1* in 3 and only *MAT1-2* in 10 localities. Both mating types within the same sample (tree) were present in 5 localities (Borová hora Arboretum, Košice, Pinciná, Strážske, Veľký Šariš; Table 1).

For 11 *D. pini* cultures ITS haplotypes and mating types were determined, results are shown in Table 2. Both mating types within the cultures and three ITS haplotypes: Dp_HAP.1 (4 isolates from 2 localities), Dp_HAP.2 (6 isolates from 4 localities) and Dp_HAP.4 (single isolate) were identified.

Table 2 Details of *Dothistroma pini* isolate´s collection examined in the present study. ID – identification number.

DNA ID	Locality of sample origin	*Pinus* host species	ITS haplotype	Mating type	GenBank accession number
D803	Mlyňany Arboretum	*P. cembra*	Dp_HAP.1	MAT2	OP602351
D824	Podunajské Biskupice	*P. nigra*	Dp_HAP.1	MAT2	OP602352
D825	Podunajské Biskupice	*P. nigra*	Dp_HAP.1	MAT2	OP602353
D826	Podunajské Biskupice	*P. nigra*	Dp_HAP.1	MAT2	OP602354
D931	Zliechov	*P. nigra*	Dp_HAP.2	MAT1	OP602355
D963	Košice	*P. nigra*	Dp_HAP.4	MAT2	OP602356
D970	Pinciná	*P. nigra*	Dp_HAP.2	MAT1	OP602357
D972	Veľký Šariš	*P. nigra*	Dp_HAP.2	MAT2	OP602358
D973	Borová hora Arboretum	*P. ponderosa*	Dp_HAP.2	MAT1	OP602359
D974	Borová hora Arboretum	*P. ponderosa*	Dp_HAP.2	MAT2	OP602360
D975	Borová hora Arboretum	*P. ponderosa*	Dp_HAP.2	MAT2	OP602361

Microscopic examination

For the microscopic examination, 37 samples of *D. septosporum* were selected while 23 samples were collected in previous studies (Ondrušková et al. 2017, Jánošíková-Hečková et al. 2018) and 14 from the current sampling (Table 3). In the case of *D. pini,* five samples were selected, where both mating types were confirmed. One sample (Košice, *P. nigra*) was not inspected due to a low number of needles having fruiting bodies of *Dothistroma*.

In all inspected needle samples of both *Dothistroma* species, conidiomata with typical hyaline cylindrical conidia were identified. The ascomata of *D. septosporum* were found on

Table 3 Details of *Dothistroma septosporum* and *D. pini* samples studied for the presence of sexual reproductive organs.

No	Locality	***Pinus* host species**	**Date of sampling**	**Herbarium specimen**	**Pathogen / Spore type**
1	Banská Belá	*P. sylvestris*	4 Dec 2015	NR6168	DS/C
2	Borová hora Arboretum	*P. mugo*	11 Jun 2020	NR6061	DS/C
3	Borová hora Arboretum	*P. mugo*	11 Jun 2020	NR6062	DS/C
4	Borová hora Arboretum	*P. ponderosa*	3 Sep 2019	NR6056	DP/C
5	Borová hora Arboretum	*P. uncinata*	11 Jun 2020	NR6064	DS/C
6	Červený Kláštor	*P. mugo*	31 Dec 2019	NR5986	DS/C
7	Duchonka	*P. nigra*	14 Nov 2013	NR5815	DS/C
8	Gabčíkovo	*P. nigra*	18 Feb 2015	NR5209	DS/C
9	Gelnica	*P. nigra*	4 Aug 2020	NR5992	DS/C
10	Chmeľnica	*P. nigra*	6 Aug 2020	NR5995	DS/C
11	Kálnica	*P. nigra*	21 Mar 2014	NR5817	DS/C
12	Kežmarok	*P. mugo*	6 Jun 2019	NR6003	DS/C
13	Kluknava	*P. nigra*	27 Jul 2014	NR6005	DS/A,C
14	Krompachy	*P. mugo*	1 Jul 2015	NR5409	DS/C
15	Lietava	*P. nigra*	30 Apr 2017	n.a.	DS/C
16	Lipníky	*P. mugo*	25 Apr 2014	NR5403	DS/C
17	Litava	*P. nigra*	14 Mar 2014	NR5816	DS/C
18	Ľubochňa	*P. sylvestris*	25 Mar 2014	NR5472	DS/C
19	Mlyňany Arboretum	*P. sylvestris*	15 May 2015	NR5836	DS/C
20	Muráň	*P. mugo*	2 Jul 2020	NR6012	DS/C
21	Nemecká	*P. mugo*	24 Apr 2014	NR5262	DS/C
22	Nový Tekov	*P. nigra*	13 Apr 2014	NR5820	DS/C
23	Pinciná	*P. nigra*	3 Jul 2020	NR6024	DP/C
24	Slanica	*P. nigra*	17 May 2016	NR5827	DS/C
25	Spišská Teplica	*P. sylvestris*	20 Jan 2016	NR5837	DS/C
26	Spišská Teplica - Spariská	*P. sylvestris*	29 Jan 2015	NR5834, NR5835	DS/C
27	Stará Lesná	*P. mugo*	25 Feb 2014	NR5253	DS/C
28	Strážske	*P. nigra*	5 Aug 2020	NR6035	DP/C
29	Šajdíkove Humence	*P. aristata*	13 May 2015	NR5795	DS/C
30	Šajdíkove Humence	*P. nigra*	13 May 2015	NR5823	DS/C
31	Šajdíkove Humence	*P. nigra*	13 May 2015	NR6637	DS/C
32	Šajdíkove Humence	*P. mugo*	13 May 2015	NR6636	DS/C
33	Šajdíkove Humence	*P. sylvestris*	13 May 2015	NR5831	DS/C
34	Tatranská Kotlina	*P. mugo*	5 Jun 2019	NR6037	DS/C
35	Tatranská Lomnica	*P. mugo*	24 Feb 2014	NR5228	DS/C
36	Terchová	*P. nigra*	17 Jun 2020	NR6040	DS/C
37	Vaľkovňa	*P. mugo*	3 Mar 2015	NR5802	DS/C
38	Veľký Šariš	*P. nigra*	6 Aug 2020	NR6048	DP/C
39	Vyšné Ružbachy	*P. nigra*	30 Dec 2019	NR6050	DS/C
40	Vyšné Ružbachy	*P. nigra*	30 Dec 2019	NR6051	DS/C
41	Vyšné Ružbachy	*P. sylvestris*	6 Jun 2020	NR6062	DS/C

Note: n.a. – not available; pathogen: DS – *Dothistroma septosporum*, DP – *D. pini*; spore type: A – ascospores, C – conidia

three needles of the *P. nigra* sample from Kluknava (NR6005, Figure 2). No spermogonia or spermatia were observed. A comparison of biometric characteristics of *D. septosporum* on *P. nigra* in Germany (Butin 1985) and Serbia (Karadžić 2004), on *Pinus* spp. in Canada (Funk & Parker 1966), and of the Slovak material is given in Table 4. The finding of the sexual stage of the fungus is reported for the first time in Slovakia.

Discussion

This study has contributed to providing new knowledge about the geographical distribution of DNB pathogens and their characteristics. Not only new regional records for both pathogens but also new stand types and hosts have been reported for Slovakia. As the existence of both mating types of DNB pathogens has been recorded in the country and furthermore results of population study suggested sexual reproduction for *D. septosporum* in Slovakia (Jánošíková et al. 2021), an intensive survey for the presence of ascostromata and ascospores was performed in the present study.

DNB is a highly widespread disease that occurs in both, the Northern and Southern Hemisphere in almost every country where

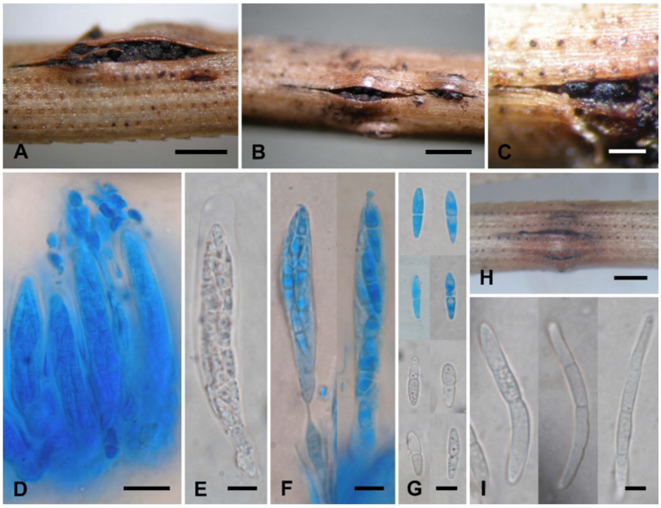

Figure 2 *Dothistroma septosporum* on *Pinus nigra* (Slovakia, Gelnica District, Kluknava, 27 July 2014, NR6005). (A–C) ascostromata in the needle; (D) a cluster of asci; (E) immature ascus; (F) mature asci with ascospores; (G) ascospores; (H) conidiomata in the typical red band breaking through the epidermis; (I) conidia. Scale bars = 500 µm (A, B, H), 100 µm (C), 10 µm (D), 5 µm (E, F, G, I).

Table 4 Biometric characteristics of *Dothistoma septosporum* reported by other authors and of the Slovak material examined.

Morphology	Funk & Parker (1966)	Butin (1985)	Karadžić (2004)	Examined material
Ascostromata (µm diam.)	200-600 × 95-150	500-1000 × 300-400	370-1030 × 198-412	450-1200 × 110-250
Locules (µm diam.)	40-86	70-100	46-144	60-120
Asci (µm)	46-52 × 8-10	35-50	33-48 × 6-9	37-49 × 6-7.5
Ascospores (µm)	13-16 × 3-4	12-15 × 3-3.5	9-17 × 2.4-3.6	10.5-13.5 × 2.7-3
Conidia (µm)	16-64 × 3.5	20-29-36 × 2.5	15-44 × 1.8-3.0	20.5-44 × 2-3.5

susceptible hosts are found (Watt et al. 2009) as well the exact worldwide distribution based on reports validated using molecular methods was also reviewed (Drenkhan et al. 2016). The last study of the geographical distribution of *Dothistroma* species in Slovakia revealed their wide extent in 73 localities mostly situated in the west and central part of the country (Jánošíková-Hečková et al. 2018) with sporadic records from the east. The present study was focused on monitoring in the east part of Slovakia where 16 new DNB localities were reported including the natural stand of *P. cembra* in the National Park of High Tatras. This is as well the single regional record from a natural stand at higher altitude as the Popradské pleso locality is situated at 1494 m a.s.l. The previous DNB reports from Slovakia (Ondrušková et al. 2017, Jánošíková-Hečková et al. 2018) did not confirm the disease presence at higher altitudes. However, there are reports from Europe, where *D. septosporum* was recorded in high mountain pine forests, as in Montenegro from 820-2150 m of altitude (Lazarević et al. 2017) or in Germany at altitudes between 1200 and 1600 m (Maschning & Pehl 1994). Some degree of human-assisted dispersal of *D. septosporum* is suggested in Slovakia (Jánošíková et al. 2021), and as this area (Popradské pleso) with a high degree of protection is a place often visited by tourists, the introduction of DNB there, could have an anthropogenic character.

There are currently 113 known host taxa for *Dothistroma* species, 99 of which are within the genus *Pinus* (Barnes et al. 2022), while the published list of DNB host species from Slovakia comprised 11 pine and 2 spruce species (Jánošíková-Hečková et al. 2018). In this study we enlarged the regional host range of pines by 2 species: *P. armandii* and *P. uncinata*. There are numerous records and new reports of pathogens from arboreta and botanical gardens (Drenkhan & Hanso 2009, Jánošíková-Hečková et al. 2018, Cleary et al. 2019, Oskay et al. 2020, Mesanza et al. 2021) possible also due to higher introduced tree species diversity. In the present study, we surveyed the Borová hora Arboretum, where the disease in 6 different pine species and 4 cultivars and both *Dothistroma* species was recorded. Our discovery of *D. pini* on *P. armandii* is the first record of the fungus detected using species-specific PCR and was collected also in this Arboretum.

Further, we made the monitoring of locations, in the southern part of Slovakia, where most of the previously confirmed *D. pini* samples came from (Adamčíková et al. 2021). Out of 16 newly detected *D. pini* localities 7 are located in the south part of the country. Results still suggest that of the DNB pathogens, *D. septosporum* is the more geographically widespread and also showed that *D. pini* has more limited distribution as so far affirmed (Barnes et al. 2022, Jánošíková-Hečková et al. 2018). On the other hand, *D. pini* is being expanded and spread not only geographically but also in the host range. The results of this study expanded the known host range of *D. pini* from 11 (Jánošíková-Hečková et al. 2018) to 13 (*P. armandii*, *P. cembra*), while for *D. septosporum* by one host species (*P. uncinata*) in Slovakia.

So far, although for *D. septosporum* both mating types were recorded (Jánošíková et al. 2021), for *D. pini* only a single mating type was recorded in the same locality (Adamčíková et al. 2021). The results of *D. septosporum* mating types determination and distribution in the present study were similar to and comparable with the previously detected, existence of both mating types with a dominance of the mating type *MAT1-2*. These results were valid for *D. septosporum* in the *P. mugo* host population, where both mating types were detected in 15 localities, only *MAT1-1-1* in 5 localities and only *MAT1-2* in 13 localities (Ondrušková et al. 2017). Similarly, at the national level, in the *D. septosporum* Slovakian population, *MAT1-2* dominated. However, despite this slight *MAT1-2* dominance the mating type ratio for both scales (*P. mugo* and overall Slovakian population) did not significantly deviate from the 1:1 ratio (Ondrušková et al. 2017).

According to Funk & Parker (1966) who described the sexual state of the *D. septosporum* fungus (as *Scirrhia pini* A. Funk & A.K. Parker) from material in British Columbia the ascostromata were frequently associated with conidial structures and were reported on several native and nonindigenous pine species formed by early June for over a month (Funk & Parker 1966). In Europe, reports of the sexual state are unusual (Butin 1985, Karadžič 1989, Kowalski & Jankowiak 1998), but has been recorded in 11 countries (Drenkhan et al. 2016), whereas it has not been observed in the Slovak Republic yet (Ondrušková et al. 2017). The asci and ascospores have only rarely been observed (EPPO 2015), although extensive observations were performed, for example out of 1800 needles observed they were found on only two four-year-old needles from litter (Kowalski & Jankowiak 1998).

In studies considering the ratio of mating types in Slovakian *D. septosporum* populations (Barnes et al. 2014, Ondrušková et al. 2017) revealed that sexual reproduction was possible in Slovakia. Furthermore, in Jánošíková et al. (2021) using molecular markers (microsatellite loci) the probability of sexual reproduction was investigated. These results showed clear evidence of random mating and indicate that sexual recombination and reproduction have occurred in Slovakia and that sexual reproduction plays, or has played, an important role in the pathogen's life cycle in Slovakia (Jánošíková et al. 2021). Extensive needle observation (3600 needles) of the obtained samples, which also included needles from the two most genetically diverse localities (Kálnica and Litava, according to Jánošíková et al. 2021) and litter, revealed needles with ascostromata in one sample (*P. nigra*, Kluknava, Table 3).

Morphometric data of the Slovak material correspond well with previously published descriptions of European collections (Butin 1985, Karadžić 2004), while sexual state from Canadian collections reported by Funk & Parker (1966) has smaller ascostromata. In Serbia, ascostromata occurred usually only when the needles are completely necrotic, most often on 2-and 3-year-old needles suggesting that teleomorph is a saprophytic stage of the fungus (Karadžić 2004). A more recent study of historical scenarios showed the Central European *D. septosporum* population is derived from the Northeastern European, the ancestral population, by the natural spread of the pathogen relatively recently, ca. 70–190 generations ago, and underwent a weak bottleneck event (Mullett et al. 2021) what could explain the source or origin of high genetic and genotypic diversity in Central Europe including Slovakia. Although the presence of both mating types of the fungus, indicates the potential of sexual reproduction, asexual reproduction remained the primary mode of reproduction (Mullett et al. 2021).

In the present study, five localities with the presence of both mating types for *D. pini* were reported, while until now a single mating type in the individual locality was detected (Adamčíková et al. 2021). The presence of both mating types in a single locality is not a standard record for *D. pini*. So far, it was reported in Ukraine, France (Siziba et al. 2016) and Slovenia (Drenkhan et al. 2016), similarly in the United States (Groenewald et al. 2007, Barnes et al. 2014). In addition, in Switzerland, both mating types have been detected in the same needle (Queloz et al. 2014).

No new *D. pini* ITS haplotypes were revealed in this study, within newly confirmed localities the same three ITS haplotypes were recorded as previously published for Slovakia (Adamčíková et al. 2021) and the same haplotypes as detected in Europe (Barnes et al. 2016, Mullett et al. 2018). The results confirmed the previous geographical spread of ITS haplotypes: Dp_HAP.1 expanded most frequently in the western part of Slovakia, while Dp_HAP.2 in the middle and eastern part of the country with a rare record for Dp_HAP.4 tracking a similar distribution pattern at the European level. Dp_HAP.2 was identified in Russia, Ukraine, and Romania, located eastwards from Slovakia and Dp_HAP.1 was recorded in the Czech Republic, Hungary and Slovenia, located south and westwards from Slovakia. In Western Europe (France, Spain) all three ITS haplotypes were recorded (Barnes et al. 2016, Mullett et al. 2018). Increasing the number of studied samples in the future (for most countries so far single isolate was analysed) could reveal higher ITS haplotype diversity.

For *D. pini* no sexual state has ever been recorded worldwide, however some early reports of *D. septosporum* teleomorph were from the geographic range of *D. pini* as well and due to very similar morphology could have been incorrectly assigned to *Dothistroma* sp. anamorph (Drenkhan et al. 2016).

Conclusions

These new regional reports of DNB pathogens show their progressing spread, confirming the higher risk to the urban plantations of *P. nigra* and *P. mugo* and revealing the colonisation of new stand types and host species. Also, new *D. pini* records are of high value showing expansion to the northern and eastern parts of the country and clarifying that its expanding geographical extent is greater than previously expected. Most probably due to human-mediated dispersal and possibly climate change. Furthermore, although only by a single record, the existence of sexual reproduction in the present *D. septosporum* populations was confirmed. This result supports the previous conclusions of genetic diversity studies by Jánošíková et al. (2021), where the occurrence of sexual recombination and reproduction was indicated for the Slovak population.

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Declaration of the authors

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References

Adamčíková K., Jánošíková Z., van der Nest A., Adamčík S., Ondrušková E., Barnes I., 2021. Population structure and genetic diversity suggest recent introductions of *Dothistroma pini* in Slovakia. Plant Pathology 70(8): 1883-1896. https://doi.org/10.1111/ppa.13428

Anonymous, 2011. Správa o lesnom hospodárstve v Slovenskej republike za rok 2010 – Zelená správa. [Report on the status of forestry in the Slovak Republic of 2010 – Green Report]. Ministry of Agriculture and Rural Development of the Slovak Republic, Bratislava, 84 pp.

Barnes I., Crous P.W., Wingfield B.D., Wingfield M.J., 2004. Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma*, *D. septosporum* and *D. pini*. Studies in Mycology 50: 551-565.

Barnes I., Wingfield M.J., Carbone I., Kirisits T., Wingfield B.D., 2014. Population structure and diversity in an invasive pine needle pathogen reflects anthropogenic activity. Ecology and Evolution 4(18): 3642-3661. https://doi.org/10.1002/ece3.1200.

Barnes I., van der Nest A., Mullett M.S., Crous P.W., Drenkhan R., Musolin D.L., Wingfield M.J., 2016. Neotypification of *Dothistroma septosporum* and epitypification of *D. pini*, causal agents of Dothistroma needle blight of pine. Forest Pathology 46(5): 388-407. https://doi.org/10.1111/efp.12304

Barnes I., van der Nest A., Granados G. M, Wingfield M.J. 2022. Dothistroma needle blight. In Asiegbu F.O., Kovalchuk A. (eds.), Forest Microbiology, Forest tree Health, Vol. 2. Academic Press, London, San Diego, Cambrige, Oxford, pp. 179-199.

Butin H., 1985. Teleomorph- und anamorph-Entwicklung von *Scirrhia pini* Funk & Parker auf Nadeln von *Pinus nigra* Arnold. [Teleomorph and anamorph development of *Scirrhia pini* Funk & Parker of needles from *Pinus nigra* Arnold]. Sydowia, Annales Mycologici Ser. II 38: 20-27.

Cleary M., Laas M., Oskay F., Drenkhan R., 2019. First report of *Lecanosticta acicola* on non-native *Pinus mugo* in southern Sweden. Forest Pathology 49(3): e12507. https://doi.org/10.1111/efp.12507

Drenkhan R., Hanso M., 2009. Recent invasion of foliage fungi of pines (*Pinus* spp.) to the Northern Baltics. Forestry Studies 51: 49-64. https://doi.org/10.2478/v10132-011-0077-7

Drenkhan R., Tomešová-Haataja V., Fraser S., Bradshaw R.E., Vahalík P., Mullett M.S. et al., 2016. Global geographic distribution and host range of *Dothistroma* species: a comprehensive review. Forest Pathology 46(5): 408-442. https://doi.org/10.1111/efp.12290

EPPO, 2015. PM7/46 (3) *Lecanosticta acicola* (formerly *Mycosphaerella dearnessii*), *Dothistroma septosporum* (formerly *Mycosphaerella pini*) and *Dothistroma pini*. Bulletin OEPP/EPPO Bulletin 45(2): 163-182. https://doi.org/10.1111/epp.12217

Funk A., Parker A.K., 1966. *Scirrhia pini* n. sp., the perfect state of *Dothistroma pini* Hulbary. Canadian Journal of Botany 44(9): 1171-1176. https://doi.org/10.1139/b66-128

Gardes M., Bruns T.D., 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology 2(2): 113-118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Groenewald M., Barnes I., Bradshaw R.E., Brown A.V., Dale A., Groenewald J.Z., Lewis K.J., Wingfield B.D., Wingfield M.J., Crous P.W., 2007. Characterization and distribution of mating type genes in the Dothistroma needle blight pathogens. Phytopathology 97(7): 825-834. https://doi.org/10.1094/PHYTO-97-7-0825

Jánošíková Z., Dutech C., Ondrušková E., Adamčíková K., Mullett M., 2021. Population structure and genetic diversity of *Dothistroma septosporum* in Slovakia. European Journal of Plant Pathology 160(4): 771-787. https://doi.org/10.1007/s10658-021-02266-z

Jánošíková-Hečková Z., Ondrušková E., Barta M., Ostrovský R., Kádasi-Horáková M., Pastirčáková K., Kobza M., Adamčíková K., 2018. The hosts and geographic range of Dothistroma needle blight in Slovakia. Forest Pathology 48(3): e12421. https://doi.org/10.1111/efp.12421

Karadžič D., 1989. *Scirrhia pini* Funk et Parker. Life cycle of the fungus in plantations of *Pinus nigra* Arn. in Serbia. European Journal of Forest Pathology 19(4): 231-236. https://doi.org/10.1111/j.1439-0329.1989.tb00257.x

Karadžić D., 2004. The distribution, hosts, epidemiology, impact and control of fungus *Mycosphaerella pini* E. Rostrup apud Munk in Serbia. Bulletin of the Faculty of Forestry, University of Belgrade 90: 7-35. [in Serbian with English summary]. https://doi.org/10.2298/GSF0490007K

Kearse M., Moir R., Wilson A., Stones-Havas S., Cheung M., Sturrock S., Buxton S., Cooper A., Markowitz S., Duran Ch., Thierer T., Asthon B., Meintjes P., Drummond A., 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12): 1647-1649. https://doi.org/10.1093/bioinformatics/bts199

Kowalski T., Jankowiak R., 1998. First record of *Dothistroma septospora* (Dorog.) Morelet in Poland: a contribution to the symptomology and epidemiology. Phytopathologia Polonica 16: 15-29.

Kunca A., Foffová E., 2000. Ohrozenie porastov borovice čiernej fytokaranténnym patogénom *Dothistroma septospora* (Dorog.) Morelet [The threat to Austrian pine plantations from the phytosanitary pathogen *Dothistroma septospora* (Dorog.) Morelet]. In Varínsky J. (ed.), Aktuálne problémy v ochrane lesa 2000, 17-18 April 2000, Banská Štiavnica. Forest Research Institute, Zvolen, pp. 136-139 [in Slovak].

Kunca A., Leontovyč R., 2013. Laboratory experiments with growth potential of *Cenangium ferruginosum* tested on natural nutrition soils. Forestry Journal 59(1): 44-49.

Lazarević J., Davydenko K., Millberg H., 2017. Dothistroma needle blight on high altitude pine forests in Montenegro. Baltic Forestry 23(1): 294-302.

Maschning E., Pehl L. 1994. Bedrohung autochthoner Latschen durch Dothistroma-Nadelbräune [Threat to native dwarf mountain pine by Dothistroma needle blight]. Allgemeine Forstzeitschrift 49(5): 249-252.

Mesanza N., Raposo R., Elvira-Recuenco M., Barnes I., van der Nest A., Hernández M., Pascual M.T., Barrena I., San Martín U., Cantero A., Hernandez-Escribano L., Iturritxa E., 2021. New hosts for *Lecanosticta acicola* and *Dothistroma septosporum* in newly established arboreta in Spain. Forest Pathology 51(1): e12650. https://doi.org/10.1111/efp.12650

Milgroom M.G., 1996. Recombination and the multilocus structure of fungal populations. Annual Review of Phytopathology 34: 457-477. https://doi.org/10.1146/annurev.phyto.34.1.457

Mullett M.S., Adamson K., Bragança H., Bulgakov T.S., Georgieva M., Henriques J., Jürisoo L., Laas M., Drenkhan R., 2018. New country and regional records of the pine needle blight pathogens *Lecanosticta acicola*, *Dothistroma septosporum* and *Dothistroma pini*. Forest Pathology 48(5): e12440. https://doi.org/10.1111/efp.12440

Mullett M.S., Drenkhan R., Adamson K., Boroń P., Lenart-Boroń A., Barnes I., Tomšovský M., Jánošíková Z., Adamčíková K., Ondrušková E., Queloz V., Piškur B., Musolin D.L., Davydenko K., Georgieva M., Schmitz S., Kačergius A., Ghelardini L., Kranjec Orlović J., Müller M., Oskay F., Hauptman T., Halász Á., Markovskaja S., Solheim H., Vuorinen M., Heinzelmann R., Hamelin R.C., Konečný A., 2021. Worldwide Genetic Structure Elucidates the Eurasian Origin and Invasion Pathways of *Dothistroma septosporum*, Causal Agent of Dothistroma Needle Blight. Journal of Fungi 7(2): 111. https://doi.org/10.3390/ jof7020111

Munk A., 1957. Danish Pyrenomycetes. A Preliminary Flora. Dansk Botanisk Arkiv 17(1): 1-491.

Ondrušková E., Hečková Z., Kádasi Horáková M., Koltay A., Ostrovský R., Pažitný J., Adamčíková K., 2017. Distribution and characterization of Dothistroma needle blight pathogens on *Pinus mugo* in Slovakia. European Journal of Plant Pathology 148(2): 283-294. https://doi.org/10.1007/s10658-016-1088-2

Ondrušková E., Ostrovský R., Jánošíková Z., Adamčíková K., Kobza M., 2020. Selected climatic variables in Slovakia are favourable to the development of Dothistroma needle blight. Folia Oecologica 47(2): 144-152. https://doi.org/10.2478/foecol-2020-0017

Oskay F., Laas M., Mullett M., Lehtijärvi A., Doğmuş-Lehtijärvi H.T., Woodward S., Drenkhan R., 2020. First report of *Lecanosticta acicola* on pine and non-pine hosts in Turkey. Forest Pathology 50(6): e12654. https://doi.org/10.1111/efp.12654

Queloz V., Wey T., Holdenrieder O., 2014. First record of *Dothistroma pini* on *Pinus nigra* in Switzerland. Plant Disease 98(12): 1744. https://doi.org/10.1094/PDIS-06-14-0630-PDN

Siziba V.I., Wingfield M.J., Sadiković D., Mullett M.S., Piškur B., Barnes I., 2016. Development of microsatellite markers for the pine blight pathogen, *Dothistroma pini*. Forest Pathology 46(5): 497-506. https://doi.org/10.1111/efp.12282

Turgeon B.G., Yoder O.C., 2000. Proposed nomenclature for mating type genes of filamentous Ascomycetes. Fungal Genetics and Biology 31(1): 1-5. https://doi.org/10.1006/fgbi.2000.1227

Watt S.M., Kriticos, J.D., Alcaraz S., Brown A.V., Leriche A., 2009. The hosts and potential geographic range of Dothistroma needle blight. Forest Ecology and Management 257(6): 1505-1519. https://doi.org/10.1016/j.foreco.2008.12.026

White T.J., Bruns T., Lee S., Taylor J., 1990. PCR protocols: A guide to methods and applications. Part three - Genetics and Evolution. In Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (eds.), Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic press, London, pp. 315-322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Woods A.J., Martín-García J., Bulman L., Vasconcelos M.W., Boberg J., La Porta N. Peredo H., Vergara G., Ahumada R., Brown A., Diez J.J., 2016. Dothistroma needle blight, weather and possible climatic triggers behind the disease's recent emergence. Forest Pathology 46: 443-452. https://doi.org/10.1111/efp.12248