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Distribution and characterization of Dothistroma needle blight pathogens on *Pinus mugo* in Slovakia

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Abstract The occurrence and distribution of Dothistroma needle blight (DNB) on *Pinus mugo* was studied in 2014–2015 around the Slovakia. In total, 42 localities were investigated both native and planted ones. Symptoms of DNB were observed on 35 localities only on planted shrubs. All these 35 localities are new *P. mugo* DNB stands. No DNB symptoms were observed in natural and naturally regenerated plantations. DNA was extracted from a total of 236 isolates and eight needle samples. Based on the ITS-rDNA comparisons and using species specific primers, both pathogenic *Dothistroma* species were detected: *D. septosporum* and *D. pini*. Isolates of *D. septosporum* had ITS sequences identical to *D. septosporum* from Europe and both mating types were identified with slight predominance of MAT2. The ratio of *D. septosporum* mating types varies significantly between sites, ranging from an equal proportion of each mating type to single mating type populations. *D. pini* ITS sequence grouped with *D. pini* from Ukraine, Russia and Switzerland and only MAT2 was found.

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Introduction

Dothistroma needle blight (DNB), also known as red band needle blight, is a very serious needle disease of conifers that primarily affects pine species (*Pinus* spp.). The disease is characterized by red bands surrounding black, erumpent conidiomata that split the epidermal layers of infected pine needles. These needles become necrotic, are cast, and after successive defoliation, the disease can result in stunted tree growth (Gibson 1972; van der Pas 1981). Needles of all ages are commonly affected (Gibson et al. 1964; Kowalski and Jankowiak 1998).

Two very similar ascomycete fungi are known to cause DNB: *Dothistroma septosporum* (Dorog.) Morelet and *Dothistroma pini* Hulbary (Barnes et al. 2004). *D. septosporum* is the pathogen responsible for the epidemics mentioned during the past 50 years; it has successfully invaded, becoming established in many European countries. During this time, the geographic distribution of the pathogen has expanded and serious disease epidemics have emerged (Barnes et al. 2014). *D. septosporum* has not only a worldwide geographical distribution but also a wide host range (Maschning and Pehl 1994; Woods 2003; Bradshaw 2004; Kehr et al. 2004; Kirisits and Cech 2006). In contrast, *D. pini* has a partly limited distribution and host range (Barnes et al. 2004, 2008; Ioos et al. 2010; Queloz et al. 2014).

D. septosporum was recorded for the first time in Slovakia in the locality of Modrý Kameň in 1996 (Kunca and Foffová 2000), located in Central South Slovakia close to the Hungarian border. Consequently, it might have spread naturally from Hungary (Kunca and Foffová 2000; Zúbrik et al. 2006; Barnes et al. 2011). Later, the area of its distribution gradually extended further into Slovakia. It can presently be found in forests on trees aged from 5 to 100 years in all the regions of Slovakia (Zúbrik et al. 2006; Pastirčáková et al. 2014).

Pinus nigra Arnold (Austrian pine), a species considered to be exotic to Slovakia, is the main host species of DNB in Slovakia (Zúbrik et al. 2006). It was planted widely in the 1980s, but now its use is decreasing. The disease mainly affects *P. nigra* Christmas-tree plantations; but, there are some young *P. nigra* plantations in extreme environmental conditions, such as with shallow soil, south slope exposure and dry climate, where severe *D. septosporum* infection has caused mortality (Zúbrik et al. 2006).

There are no published data about DNB infection on *Pinus sylvestris* L. (Zúbrik et al. 2006; Kunca et al. 2010; Foffová and Foff 2007; Pastirčáková et al. 2014). Barnes et al. (2004) used in their phylogeny study one isolate of *D. septosporum* isolated from *P. sylvestris* (Scots pine) with origin from Slovakia without any extra information about locality, nor about date of collection.

So far there is no evidence of the occurrence of the fungus on seedlings and transplants in forest nurseries. This may be caused by the intensive fungicidal control of needle cast diseases on *P. nigra* as well as *P. sylvestris* (Zúbrik et al. 2006).

As well as the susceptible *P. nigra*, microscopic identification of *Dothistroma* sp. was confirmed on introduced pine species *Pinus jeffreyi* Grev. & Balf. and *Pinus aristata* Engelm., and on mountain pine *Pinus mugo* Turra in urban greenery in Slovakia (Foffová and Foff 2007).

Mating type genes play an important part in the biology and evolution of fungal species. Thus, knowledge of these genes can provide insight into the potential of sexual reproduction in different species (Groenewald et al. 2007). *D. septosporum* and *D. pini* are heterothallic ascomycetes for which both mating type genes have been identified by Groenewald et al. (2007). Two studies investigating the mating types of *Dothistroma* sp. have included isolates from Slovakia, by Groenewald et al. (2007) and by Barnes et al. (2014) with 1 and 24 isolates analysed, respectively.

The aims of the present study were: i) to determine the occurrence and distribution of DNB on *P. mugo*, ii) to compare the infection of *P. mugo* native and planted stands, iii) to identify the *Dothistroma* species on *P. mugo*, and iv) to characterize the fungus population with respect to mating types. The current study makes an important contribution to DNB population studies in this part of Europe, where the fungal population data were missing.

Materials and methods

Study area and sampling

The investigation was carried out in the years 2014–2015. Localities with *Pinus mugo* situated around Slovakia were selected and examined. The descriptions of evaluated localities are listed in Table 1. Both natural, naturally regenerated sites and planted individuals of *P. mugo* in urban greenery were selected.

Samples were collected from *P. mugo* visually classified as affected by DNB according EPPO descriptions. The needles demonstrated the following symptoms were collected: needle tip dieback, dead tip of needles in brown/ red in colour, red or brown banding on needle, small black fruit bodies in affected part of needle. One sample was collected from one tree. In case of five and less trees presented in a stand, needles from each tree were collected as separate samples. When more than five trees were present in a stand, randomly selected trees were sampled, but a minimum of five. Number of collected needles per samples depended on the infection intensity and the size of the evaluated tree. The needles were collected randomly from different parts of the tree crown. Every sample consisted of at least 30 needles. Preferentially the needles showing presence of mature conidiomata were selected. Infected needles collected from the field were deposited in freezers until processing. Herbarium specimens are deposited in the Plant Pathology Herbarium of the Institute of Forest Ecology of Slovak Academy of Sciences (NR).

Fungal isolation

The needles were gently surface-sterilized by wiping with 96 % ethyl alcohol-soaked tissue and placed in the damp

Table 1 List of evaluated localities with stand description and identified *Dothistroma* species on locality

Name of locality	No	Latitude (N); Longitude (E)	Sampling date	Type of stand	S	NoS	*Dothistroma* identity	Herbarium item	GeneBank accession number
Arboretum Mlyňany-Vieska nad Žitavou	1	N48.34108616; E18.36203645	21.11.2014	planted	+	4	*D. septosporum*	NR5406, NR5407	KX058157
Bachledova Dolina	2	N49.27226106; E20.30856261	5.01.2015	planted	+	3	*D. septosporum, D. pini*	NR5264, NR5265, NR5400	KX058155
Brusno	3	N48.83465398; E19.35636527	3.03.2015	planted	+	1	*D. septosporum*	NR5260	KX058154
Bystrá	4	N48.88796418; E19.61809361	24.04.2014	planted	+	1	*D. septosporum*	NR5396	–
Detvianska Huta	5	N48.57144464; E19.59615813	29.06.2015	planted	+	1	*D. septosporum*	NR5410	–
Dlhá nad Oravou	6	N49.27678368; E19.45167471	9.10.2015	planted	+	1	*D. septosporum*	NR5413	–
Dolný Kubín	7	N49.24973306; E19.29303421	11.04.2015	planted	+	2	*D. septosporum*	NR5259, NR5397	KX058160
Donovaly	8	N48.87372412; E19.23372086	5.04.2015	planted	+	1	*D. septosporum*	NR5258	KX058158
Duchonka	9	N48.66220387; E18.08767086	17.03.2015	planted	+	2	*D. septosporum*	NR5378, NR5379	–
Galanta	10	N48.19151299; E17.73108599	8.09.2015	planted	−	0	–	–	–
Heľpa	11	N48.85668941; E19.96226368	3.03.2015	planted	+	1	*D. septosporum*	NR5394	KX058152
Horný Smokovec	12	N49.13994734; E20.23685727	24.04.2014	planted	+	3	*D. septosporum*	NR5241, NR5392, NR5393	KX058141
Hybe	13	N49.05217565; E19.836612	27.02.2014	planted	+	4	*D. septosporum*	NR5237, NR5238, NR5239, NR5240	KX058145
Korytnica	14	N48.8765964; E19.23503179	25.03.2014	planted	+	3	*D. septosporum*	NR5225, 5226, 5227	KX058151
Košice	15	N48.70992711; E21.26526182	1.07.2015	planted	+	1	*D. septosporum*	NR5412	–
Krompachy	16	N48.90474159; E20.87725276	1.07.2015	planted	+	2	*D. septosporum*	NR5408, NR5409	–
Levoča	17	N49.02789163; E20.58092956	27.07.2014	planted	+	1	*D. septosporum*	NR5256	KX058147
Lipníky	18	N49.0485968; E21.41228474	25.04.2014	planted	+	1	*D. septosporum*	NR5403	KX058156
Nemecká	19	N48.81200234; E19.42865198	24.04.2014	planted	+	3	*D. septosporum*	NR5261, NR5262, NR5263	KX058142
Nitra	20	N48.300233; E18.098009	30.03.2015	planted	+	1	*D. pini*	–	–
Nový Smokovec	21	N49.13802303; E20.21851105	24.04.2014	planted	+	3	*D. septosporum*	NR5235, NR5236, NR5402	KX058144
Párnica	22	N49.19739414; E19.19285826	14.06.2015	planted	+	1	*D. septosporum*	NR5411	–
Pezinok	23	N48.27618548; E17.25548342	9.07.2015	planted	−	0	–	–	–
Rožňava	24	N48.65937514; E20.53546772	20.09.2014	planted	+	1	*D. septosporum*	NR5398	KX058153
Ružomberok - Biely Potok	25	N49.02920657; E19.2734351	9.10.2015	planted	+	1	*D. septosporum*	NR5414	–
Sečovce	26	N48.699766; E21.65647998	10.05.2015	planted	+	1	*D. septosporum, D. pini*	NR5399	KX058161, KX058162
Stará Lesná	27	N49.13671403; E20.30620226	25.02.2014, 26.02.2014	planted	+	11	*D. septosporum*	NR5243, NR5245, NR5246, NR5247, NR5248, NR5249,	KX058146

Table 1 (continued)

Name of locality	No	Latitude (N); Longitude (E)	Sampling date	Type of stand	S	NoS	*Dothistroma* identity	Herbarium item	GeneBank accession number
								NR5250, NR5251, NR5252, NR5253, NR5254	
Staré Hory	28	N48.83990423; E19.1201086	25.03.2014	planted	+	1	*D. septosporum*	NR5255	KX058143
Starý Smokovec	29	N49.14002497; E20.221342	24.04.2014	planted	+	2	*D. septosporum*	NR5233, NR5234	KX058140
Šajdíkove Humence	30	N48.6527421; E17.27448052	13.05.2015	planted	+	7	*D. septosporum*	NR5388, NR5389, NR5390, NR5391	KX058163
Tatranská Lesná	31	N49.1508613; E20.25775002	24.04.2014, 6.03.2015	planted	+	3	*D. septosporum*	NR5386, NR5387, NR5385	KX058150
Tatranská Lomnica	32	N49.16669148; E20.28011435	24.02.2014, 3.01.2015, 6.03.2015	planted	+	8	*D. septosporum*	NR5228, NR5224, NR5230, NR5231, NR5232, NR5223	KX058149
Telgárt	33	N48.84871164; E20.18753347	3.03.2015	planted	+	1	*D. septosporum*	NR5405	–
Vaľkovňa	34	N48.83482; E20.050101	3.03.2015	planted	+	1	–	–	–
Víglaš	35	N48.55603918; E19.28524937	26.06.2015	planted	+	2	*D. septosporum*	–	–
Vysoké Tatry - Hrebienok	36	N49.15953911; E20.22499269	25.02.2014	nat regen	–	0	–	–	–
Vysoké Tatry – Mlynická dolina	37	N49.13541451; E20.05553823	25.02.2014	native	–	0	–	–	–
Vysoké Tary - Skalnaté pleso	38	N49.18193085; E20.2394072	24.02.2014	native	–	0	–	–	–
Vysoké Tatry - Štrbské pleso	39	N49.12133506; E20.06039591	25.02.2014	nat regen	–	0	–	–	–
Vysoké Tatry – Zelené pleso	40	N49.21251332; E20.23230586	26.02.2014	native	–	0	–	–	–
Závadka nad Hronom	41	N48.85018563; E19.9112745	3.03.2015	planted	+	1	*D. septosporum*	NR5395	KX058148
Zvolen	42	N48.57488129; E19.12374361	20.03.2014, 31.03.2015	planted	+	6	*D. septosporum*	NR5266, NR5267, NR5381, NR5382, NR5383, NR5380	KX058159

No – number of evaluated locality

S - Symptoms: + = present, − = absent

NoS – number of collected samples per locality

Type of stand: planted – artificially planted shrubs in urban greenery, private gardens or parks, native – natural, native stands, nat regen - naturally regenerated stands of *P. mugo*

chamber for 24 h. From five randomly selected needles single conidiomata per needle were selected. Conidiomata were excised under a binocular microscope using a scalpel, placed in sterile distilled water on the glass slide, gently crushed with a scalpel and transferred with water using a micropipette onto the surface of water agar (15 g/1 l) supplemented with antibiotic (100 mg l^{-1} streptomycin sulphate) in Petri plates.

Blocks of agar were cut from the plates in areas where germinating conidia occurred but no contaminating debris. These blocks were then transferred to new plates with water agar and subsequently sub-cultured on 3 % MEA. Cultures were incubated at 20 °C until colonies formed. Cultures used in this study are stored in the culture collection of Institute of Forest Ecology SAS Zvolen, Branch for Woody Plants Biology Nitra. Dothistroma isolates were used for DNA extractions and further analyses (sequencing, mating type assay).

Isolation of DNA and sequencing

DNA was extracted from pure fungal cultures after incubation for ca. 3 weeks at temperature 20 °C in 3 % MEA. In samples where the isolation of the fungus in pure culture was not successful, the DNA was extracted directly from the infected needles (in total, eight samples from localities Bystrá, Dlhá nad Oravou, Horný Smokovec 2 samples, Nitra, Párnica, Ružomberok-Biely Potok, Valkovňa; Table 1, No's 4, 6, 12, 20, 22, 25, 34 respectively). 3–8 conidiomata / sample were excised from the infected needles after surface sterilization with 96 % ethyl alcohol. DNA extraction was done using the E.Z.N.A.® Fungal DNA Mini-Kit (Omega Bio-Tek Inc., Norcross, GA, USA), following the manufacturer's instructions.

5× HOT FIREPol® Blend Master Mix (Solis BioDyne) consists of HOT FIREPol® DNA polymerase, proofreading enzyme, 5× Blend Master Mix Buffer, 10 mM $MgCl_2$, 2 mM dNTPs, BSA, blue and yellow dye was used for all PCR reactions in this study.

Identification to species level was done by conventional PCR using species specific primers according to Groenewald et al. (2007), where the targeted genes are α domain (*MAT1–1-1*) and HMG domain (*MAT1–2*). Amplification of DNA was performed in PCR reaction mix using approximately 2 ng/μl of template DNA, forward and reverse primers (10 pmol/μl), 5× HOT FIREPol® Blend Master Mix (Solis BioDyne) 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 was different for *D. pini* at 65 °C and for *D. septosporum* at 63 °C 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.

In case of negative results in *Dothistroma* species identification following Groenewald et al. (2007), species specific primers according to Ioos et al. (2010) were used. The conventional PCR reactions were carried out in 20 μl reaction volumes. The cycling conditions were the same for both *Dothistroma* species, included an initial denaturation step at 95 °C for 15 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 60 s; with a final extension at 72 °C for 10 min.

The internal transcribed spacer (ITS) region of ribosomal DNA was amplified for the randomly selected samples (23 *D. septosporum* and 1 *D. pini* sample) with primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993) to sequence the amplicons in both directions. Amplification of DNA was performed in PCR reaction mix consists of approximately 2 ng/ μl of template DNA, 5× HOT FIREPol® Blend Master Mix (Solis BioDyne), forward and reverse primers (10 pmol/ μl) and molecular-grade water added up to 20 μl. Molecular-grade water was used as negative amplification control during preparation of the reaction mix. Reaction conditions included an initial denaturation at 95 °C for 14 min, followed by 13 cycles of denaturation at 95 °C for 35 s, annealing at 55 °C for 55 s, and elongation at 72 °C for 45 s; followed by a further 13 cycles of denaturation at 95 °C for 35 s, annealing at 55 °C for 55 s, and elongation at 72 °C for 2 min, and then lastly 9 cycles with the same condition for denaturation and annealing, with a longer elongation 3 min. A final extension was carried out at 72 °C for 10 min. Prior to sequencing, target fragments were directly purified using an PCR Purification Kit (Qiagen, Hilden, Germany). Each amplified product was diluted with 30 μl H_2O. Sequence reactions were run an ABI3130xl sequencer (Applied Biosystems) at SEQme s.r.o. (Dobříš, Czech Republic). The retrieved sequences were compared by BLAST (Basic Local Alignment Search Tool, available at http:// www.ncbi.nlm.nih.gov/genbank/) against DNA sequences deposited in GenBank for *Mycosphaerella pini* (AY808294) and *Dothistroma pini* (AY808302, DQ926964, KJ878557, KJ878558). ITS sequences were deposited in NCBI GenBank.

Mating type assays

The DNB species and mating type specific primers (Groenewald et al. 2007) were used to confirm the mating type of all obtained fungal isolates. They were also used for *Dothistroma* species identification, PCR mixture and conditions are described above. The species-specific primers amplify regions of approximately 820 bp and 480 bp for MAT1 and MAT2 in *D. pini* and 823 bp and 480 bp for MAT1 and MAT2 in *D. septosporum*.

An exact binomial test using two-tailed *P*-values (software R version 3.1.3.) was used to determine whether populations deviated from the null hypothesis of 1:1 ratio of mating types. Localities with low sample size were excluded from this analysis ($N \leq 4$).

Results

Disease incidence

Forty-two localities with *P. mugo* were investigated around Slovakia. From these locations, five were natural or naturally regenerated *P. mugo* stands, all situated in the Tatry Mountains where natural plantations of mountain pine are situated. The rest of the evaluated localities were planted stands.

Symptoms of Dothistroma needle blight (DNB) were observed at 35 localities. All these 35 localities were new *P. mugo* DNB stands, demonstrating that the disease has spread across the country. No DNB symptoms were observed on *P. mugo* shrubs in natural and naturally regenerated plantations, all were disease free. Shrubs were only infected in planted, ornamental stands (94.6 % of them, two of them were without symptoms). Although incidence was high, disease severity varied and was not precisely evaluated. The younger shrubs were less infected than the older annually-repeatedly infected. In some cases the defoliation was observed, only the last year needles retain. Symptoms were present on the previous years' needles, with typical red or brownish bands on needles bearing conidiomata.

Fungal isolation

Isolates originated from different needles from the same trees and from different trees from the same locality. In total 236 isolates were obtained from the host trees. Isolation of the fungus was not successful in six localities: Bystrá, Dlhá nad Oravou, Nitra, Párnica, Ružomberok- Biely Potok, Valkovňa (Table 1, No's 4, 6, 20, 22, 25, 34 respectively).

The fungal mycelium was firstly white and aerial in culture, which turned greyish-brown in colonies, then orange brown to grey-black with black stromata. The colonies grew slowly and agar was mostly coloured light reddish-brown by diffusates from the colonies.

DNA extraction, sequencing

DNA was extracted from all 236 fungal isolates. From the total DNA extraction in subsequent PCR amplifications 96.70 % produced bands for at least one species-specific primer combination. No positive DNB result was obtained for one locality Valkovňa (Table 1, No 34), in spite of the red band symptoms and conidiomata being present in the sample.

All DNB positive samples, except two, were identified as *D. septosporum* based on PCR test using species-specific primers. *D. septosporum* was detected in each evaluated locality, except one Nitra (Table 1, No 20). In this locality (Nitra) only *D. pini* was detected from needle samples.

Three isolates and one sample from needles from three different localities (Nitra - needles, Bachledova Dolina – isolate, Sečovce – two isolates; Table 1, No's 20, 2, 26 respectively) were identified as *D. pini* based on the PCR test using species-specific primers. Two isolates from different localities Bachledova Dolina and Sečovce (Table 1, No's 2, 26 respectively) gave positive results for *D. septosporum* and *D. pini* simultaneously.

The ITS sequences obtained with primers ITS1/ITS4 for 23 *D. septosporum* samples were identical, sequence identity was 100 %. They showed a high degree of similarity to the DNA sequence deposited in GenBank for *Mycosphaerella pini* with accession number AY808294 (Barnes et al. 2008).

The ITS sequence obtained for the *D. pini* isolate showed the 100 % homology to the sequence DQ926964 from Ukraine and KJ878558 from Switzerland (Queloz et al. 2014; Barnes et al. 2008).

The ITS sequences obtained in this study were deposited in the GenBank (for GenBank accession numbers see Table 1).

Mating type

The mating type analysis was carried out for a total of 232 DNA samples of *D. septosporum*. For nine isolates the mating type identification failed (isolates from localities Bachledova Dolina, Duchonka, Hybe, Nemecká, Sečovce, Tatranská Lomnica and Zvolen; Table 1, No's 2, 9, 13, 19, 26, 27, 32, 42 respectively); both mating types were identified for five isolates from localities Hybe, Stará Lesná and Tatranská Lomnica (Table 2, No's 13, 27, 32 respectively). The results of mating type analysis

Table 2 *Dothistroma septosporum* mating types at 33 localities in Slovakia on *Pinus mugo* host tree

No	Name of locality	Num	Exact binomial tests			
			Observed		Expected MAT1/MAT2	p - value[1]
			MAT1	MAT2		
1	Arboretum Mlyňany -Vieska n/Ž.	5	2	3	2.5	1
2	Bachledova Dolina	11	5	5	5	1
3	Brusno	3	0	3	1.5	
4	Bystrá	1**	0	1	0,5	
5	Detvianska Huta	2	0	2	1	
6	Dlhá nad Oravou	1**	0	1	0.5	
7	Dolný Kubín	8	3	5	4	0.727
8	Donovaly	3	3	0	1.5	
9	Duchonka	5	4	0	2	0.125
11	Heľpa	3	0	3	1.5	
12	Horný Smokovec	3	0	3	1.5	
13	Hybe	13	6	8	7	0.791
14	Korytnica	9	0	9	4.5	0.004*
15	Košice	1	0	1	0.5	
16	Krompachy	3	2	1	1.5	
17	Levoča	3	3	0	1.5	
18	Lipníky	2	1	1	1	
19	Nemecká	13	5	7	6	0.774
21	Nový Smokovec	9	8	1	4.5	0.039*
22	Párnica	1**	0	1	0.5	
24	Rožňava	2	0	2	1	
25	Ružomberok - Biely Potok	1**	0	1	0.5	
26	Sečovce	2	1	0	0.5	
27	Stará Lesná	34	22	13	17.5	0.176
28	Staré Hory	3	3	0	1.5	
29	Starý Smokovec	9	1	8	4.5	0.07
30	Šajdíkove Humence	15	7	8	7.5	1
31	Tatranská Lesná	8	5	3	4	0.727
32	Tatranská Lomnica	29	6	22	14	0.004*
33	Telgárt	3	0	3	1.5	
35	Víglaš	6	0	6	3	0.031*
41	Závadka nad Hronom	3	2	1	1.5	
42	Zvolen	18	12	5	8.5	0.144
	Total	232	101	127	114	0.097

No = number of locality corresponds to Table 1 and Fig. 1

Num = number of samples analysed per locality

[1]= *p*-value two-tailed test, localities with low sample size were excluded from this analysis ($N \leq 4$)

*Mating type ratio deviated from the null hypothesis of a 1:1 ratio at $p < 0.05$

**Identification of mating types made from needles

are showed in Table 2 and the mating type distribution over Slovakia in Fig. 1.

Generally, in Slovakia, both mating types of *D. septosporum* were identified within all the samples from the evaluated localities. Both mating types were identified in 15 localities, only MAT1 in five localities (15 %) and only MAT 2 in 13 localities (39.4 %).

A *P*-value two-tailed test was made for 15 population (15 localities), where the number of samples was more than four. Four populations, from localities Korytnica, Nový Smokovec, Tatranská Lomnica and Víglaš (Table 2, No's 14, 21, 32, 35 respectively), deviated significantly from the 1:1 ratio of random mating with the MAT2 idiomorph being significantly more common in three of them (localities Korytnica, Tatranská Lomnica and Víglaš, Table 2, No's 14, 32 and 35 respectively) while in other one Nový Smokovec (Table 2 No 21) the MAT1 was dominated. All other localities showed evidence of a randomly mating type population. When the whole Slovakian dataset was taken into account, the mating type did not significantly differed from a 1:1 ratio, in spite of the slight dominance of MAT2 idiomorph.

The obtained *D. pini* isolate from locality Sečovce (Table 1, No 26) presented the mating type MAT2.

Discussion

The first report of DNB caused by *D. septosporum* in Slovakia comes from 1996 (Kunca and Foffová 2000; Zúbrik et al. 2006) when the symptoms were observed on a *P. nigra* host tree. Both *P. nigra* and *P. sylvestris* have been reported as confirmed host trees of *D. septosporum* in Slovakia (Barnes et al. 2004, 2014) but the information about *Dothistroma* spp. in Slovakia is limited.

The results of this study provide the first detailed country report of DNB in Slovakia on *P. mugo,* a new host species for Slovakia, while DNA based diagnostic tools confirmed the presence of both Dothistroma

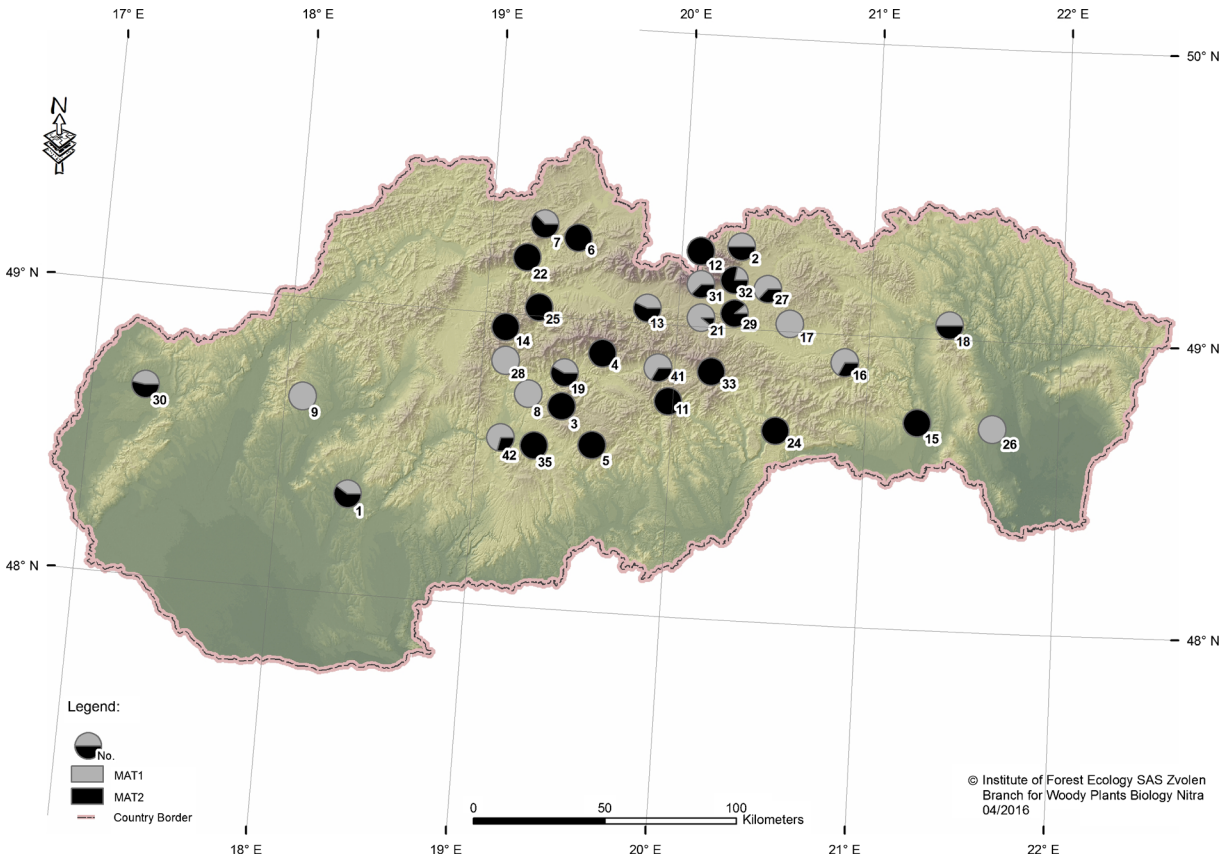

Fig. 1. *Dothistroma septosporum* mating type distribution on *Pinus mugo* in Slovakia, the numbers refer to the localities numbers and names in Tables 1 and 2, the grey colour in dots represents the presence of mating type MAT1 and black colour of the mating type MAT2

species. This was not unexpected as this host tree has been reported as moderately susceptible (Watt et al. 2009) and as a host tree for DNB in surrounding countries: the Czech Republic (Bednářová et al. 2006), Poland (Boroń et al. 2016), Austria (Kirisits and Cech 2006) and Hungary (Barnes et al. 2007), but the reports of *D. pini* on *P. mugo* are rare.

In this study, 35 new localities with DNB were discovered for Slovakia. They are located all over the country with a major concentration in the northernmost central part. According to results of Watt et al. (2009) using an Ecoclimatic Index (EI) classification (Kriticos et al. 2003a, 2003b), this part of the country was classified as an optimal area for *Dothistroma* spp. (EI > 25, CLIMEX™ model integrates potential for population growth of host and fungus, soil moisture and temperature, length of growing season, stress index). All five evaluated natural and naturally regenerated localities of *P. mugo* are situated in the High Tatra Mountains at altitudes between 1255 and 1781 m. All the natural and naturally regenerated localities were DNB free, in spite of that they are situated in optimal ecoclimatic condition for DNB (Watt et al. 2009) and in the surrounding villages the ornamental ones were highly infected. In Poland also, the ornamental type of *P. mugo* trees were infected (Boroń et al. 2016), in Slovenia and Switzerland trees in gardens and parks in urban areas were infected as well (Queloz et al. 2014; Piskur et al. 2013). Damage has been also detected on *P. mugo* in Serbia, but only on park trees, it was never attacked by *M. pini* in natural habitats (Karadžič 2004). There is a different situation in Germany where both types of *P. mugo* localities (native and ornamental) are infected (Maschning and Pehl 1994; Pehl and Butin 1992). DNB was first noticed in south Germany in 1983 (Butin and Richter 1983) and from 1994 was observed in the native areas of *P. mugo* at altitudes between 1200 and 1600 m (Maschning and Pehl 1994).

D. septosporum (as *Cytosporina septospora* Dorog.) was first described by Doroguine (1911) from *P. mugo* in Russia. In Hungary, the first DNB collections from *P. mugo* trees (five trees, approx. 10- to 20-year-old) came from ornamental trees in the botanical garden of the University of West Hungary in Sopron; where *D. septosporum* was identified as the causal agent (Barnes et al. 2008). Similarly in Slovenia, where both *Dothistroma* species were identified, only *D. septosporum* has been confirmed on *P. mugo* (Piskur et al. 2013). In the Czech Republic, *P. mugo* is the most frequent host trees besides *P. nigra*, and only *D. septosporum* has been identified (Bednářová et al. 2006). Only recently, in Poland, *D. septosporum* was identified on this host trees (Boroń et al. 2016). In 2002, *D. septosporum* was detected for the first time in Lithuania on *P. mugo* (Jovaišienė and Pavilionis 2005), which was the only record of this fungus for several years afterward. Later in 2007, *D. septosporum* was found on several ornamental *P. mugo* in Estonia (Hanso and Drenkhan 2008).

In the first report about *D. septosporum* mating types from Slovakia, only one isolate was analysed and reported as MAT1 (Groenewald et al. 2007). Later (samples collected in 2006), one larger population (23 isolates from one *P. nigra* location) was analysed and the mating types were considered to be in equilibrium in that population (Barnes et al. 2011).

The occurrence of both mating types of *D. septosporum* found in the current study supports the results of Groenewald et al. (2007), Barnes et al. (2011), Tomšovský et al. (2013), and Boroń et al. (2016), who studied the mating type system of *D. septosporum* and also found both mating types among their set of isolates from the neighbouring countries: Austria, Hungary, the Czech Republic, and Poland. The close proximity of isolates of opposite mating type would increase the chance of sexual reproduction and the development of the teleomorphic stage (Barnes et al. 2011). The teleomorph of *D. septosporum* has not been observed in the Slovak Republic yet, and reports in other European countries are unusual (Butin 1985; Karadžič 1989; Kowalski and Jankowiak 1998). The asci and ascospores have only rarely been observed, although an extensive observation was performed (Kowalski and Jankowiak 1998). Additionally, Groenewald et al. (2007) confirmed the presence of both mating types in Austria, United Kingdom and Poland, Barnes et al. (2011) in Hungary, and Tomšovský et al. (2013) in the Czech Republic. Nevertheless, the occurrence of the teleopmorph was occasionally recorded in three European countries: Germany, Serbia and Poland (Butin 1985; Karadžič 1989; Kowalski and Jankowiak 1998), with similar environmental conditions. The results presented in this study confirm the occurrence of both mating types at 15 studied localities (more than 45 % of all evaluated localities) what increase the possibility for sexual reproduction of the pathogen. However, further investigation is necessary to determine the genetic affinity of isolates.

D. pini has been reported from France, Belgium, Czech Republic and Switzerland, but from different host trees, not from *P. mugo* (Fabre et al. 2012; Piou and Ioos 2014; Queloz et al. 2014). The only published report for *D. pini* isolated from *P. mugo* originated from south west Russia (Barnes et al. 2011).

One sequenced *D. pini* isolate (KX058162) showed 100 % homology to the sequence DQ926964 from Ukraine and KJ878558 from Switzerland (Queloz et al. 2014; Barnes et al. 2011). The detection of this species at locations in Slovakia supports the views of Barnes et al. (2011) that the fungus might be also present in other parts of Europe. This isolate groups with isolates of *D. pini* originated from Ukraine (Tsjurupinsk area) and Russia (south west part: Tarasovsky and Krasnosulinski districts) isolated from three host pine species: *P. mugo*, *P. pallasiana* and *P. nigra*. The *D. pini* of the same group was identified also in Switzerland from *P. nigra* (Queloz et al. 2014). The locality where the *D. pini* isolate came from is situated in the south-east part of Slovakia, close to the Ukrainian and Hungarian borders. The *D. pini* isolates from Hungary with known sequences all originated from west of the country and they do not group with the examined Slovakian *D. pini* isolate (Barnes et al. 2011). Introduction of *D. pini* into Slovakia could have been from Ukraine. The mating type of the Slovak *D. pini* isolate presented the MAT2 mating type gene. In the major part of Europe, the same mating type gene was reported for *D. pini*. Only MAT2 was identified in all screened *D. pini* isolates from Hungary, Russia (Barnes et al. 2011) and Slovenia (Piskur et al. 2013). In Switzerland, within *D. pini* both mating types were present and sometimes in the same needle of *P. nigra* in a forest site (Queloz et al. 2014). In Ukraine also, both mating types of *D. pini* were identified in Tsyurupinsk area: MAT1 (Groenewald et al. 2007) and MAT2 (Barnes et al. 2011).

DNA extraction from two samples (two isolates) from localities Bachledova Dolina and Sečovce (Table 1, No's 2 and 26) gave positive results for *D. septosporum* and *D. pini* simultaneously, a 231-bp fragment using *D. septosporum* –specific primers and a 193-bp fragment using *D. pini*-specific primers (Ioos et al. 2010) were amplified using DNA extracted from culture. These results indicate concurrent infections of both species on the same host and in the same needle. This work supports that of Ioos et al. (2010), Barnes et al. (2011) and Piskur et al. (2013) by documenting the overlap of geographical ranges and the co-existence of both species within the single needle.

Results of this study are consistent with the increasing number of new reports of *Dothistroma* pathogens from new areas and hosts. Recently, the occurrence of DNB symptoms, hosts and *Dothistroma* species characterization has been intensified in Slovakia.

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