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# Occurrence of Dothistroma needle blight in Lithuania and Belarus: The risk posed to native Scots Pine forests

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## Abstract

Dothistroma needle blight (DNB) caused by the ascomycetous fungus *Dothistroma septosporum* is an important pine disease in Europe. In this study, we determined the distribution and abundance of *D. septosporum* in local Scots pine forests and plantations in Lithuania and neighbouring Belarus by combining morphological observations and molecular tools. This is the first valid report on the occurrence of *D. septosporum* in Belarus, based on detailed morpho-cultural and molecular characteristics. Currently, in both countries only the asexual stage of *D. septosporum* is found on needles of the native Scots pine and on various ornamental exotic pines planted in botanical gardens and arboreta. Our results show that *P. sylvestris* may now be considered the most common host with varying susceptibility to *D. septosporum*. Phylogenetic analysis revealed the presence of three different ITS haplotypes among Lithuanian and Belarusian *D. septosporum* isolates. Mating type (MAT) genes analysis showed that two mating types (MAT1-1 and MAT1-2) occur in both countries, indicating a high probability of *D. septosporum* sexual reproduction. Isolates from Lithuania were represented by the idiomorph MAT1-1 and MAT1-2 in the ratio of 1:1, while isolates from Belarus were dominated by the idiomorph MAT1-1 (ratio 3:1). Our results also demonstrate the importance of DNB monitoring for prevention and control of disease outbreaks in Lithuanian and Belarusian Scots pine forests in the future.

**KEYWORDS**
*Dothistroma septosporum*, foliar pathogenic fungus, *Pinus*

## 1  |  INTRODUCTION

Dothistroma needle blight (DNB) is a widespread pine needle disease causing serious epidemics, known in many countries and found on different continents. (Barnes et al., 2008; Brown & Webber, 2008; Drenkhan et al., 2016; Evans, 1984; Gibson, 1974; Kirisits & Cech, 2006; Mullett & Brown, 2018; Mullett et al., 2018; Piotrowska, Riddell, Hoebe, & Ennos, 2017; Rodas, Wingfield, Granados, & Barnes, 2016; Watt, Kriticos, Alcaraz, Brown, & Leriche, 2009; Welsh, Lewis, & Woods, 2014; Woods, Coates, & Hamann, 2005; Woods et al., 2016; Woods et al., 2016). Two morphologically similar ascomycetous fungi *Dothistroma septosporum* (Dorog.) Morelet

(formerly *Mycoshpaerella pini* Rostr., representing sexual stage, teleomorph) and *Dothistroma pini* Hulbary (sexual stage unknown) were determined as the causal agents of DNB in pine forests and plantations and only molecular markers are capable to distinguish these two species (Barnes, Crous, Wingfield, & Wingfield, 2004; Drenkhan et al., 2016). *D. septosporum* has global distribution and a wide range of native and non-native coniferous hosts (>100 species, on 52 host species the fungus was confirmed by molecular methods) (Drenkhan et al., 2016). *Dothistroma pini* has a more limited distribution and host range, it was reported from the United States and 12 European countries, from 19 host taxa (Barnes et al., 2004, 2008; Bergová & Kryštofová, 2015; Dobreva et al., 2016; Drenkhan et al., 2016;

**TABLE 1** Data of Dothistroma septosporum distribution in Lithuania and Belarus, hosts and molecular methods used for species identity confirmation.

| Isolate/GenBank Accession Number | Coordinates | Location | Collection Date | Host | ITS/beta-tubulin 2 sequencing | ITS-RFLP | Species-specific PCR Mating Type | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | MAT1-1 | MAT1-2 |
| DOT 4A,B,C | 54.062315, 24.428942 | Lithuania (LT), Varėnos distr., Čepkeliai | 2012-05-09 | Pinus sylvestris | | + | | + |
| DOT 5A/KR995124 | 54.792824, 25.303940 | LT, Vilnius, Gulbinai lake shore | 2012-08-14 | P. sylvestris | +/− | + | + | |
| DOT 19A,B,C | 54.576672, 24.576030 | LT, Trakai distr., Tabaliukas lake shore | 2012-10-13 | P. sylvestris | | + | | + |
| DOT 23 | 54.782700, 25.335704 | LT, Vilnius, Žalieji lake shore | 2012-11-24 | P. sylvestris | | + | | + |
| DOT 24/24A KR995125 | 54.763444, 25.306310 | LT, Vilnius, Gulbinėliai str. | 2012-10-24 | P. sylvestris | +/− | + | | + |
| DOT 25 | 54.763444, 25.306310 | LT, Vilnius, Gulbinėliai str. | 2012-10-24 | P. sylvestris | | + | | + |
| DOT 25A/KR995126 | 54.763444, 25.306310 | LT, Vilnius, Gulbinėliai str. | 2012-10-24 | P. sylvestris | +/− | + | + | |
| DOT 26A/KR995127 | 54.576207, 24.515279 | LT, Trakai distr., Aukštadvaris | 2012-11-09 | P. sylvestris | +/− | + | | + |
| DOT 26 B,C | 54.576207, 24.515279 | LT, Trakai distr., Aukštadvaris | 2012-11-09 | P. sylvestris | | + | + | |
| DOT 27A | 54.612386, 23.886160 | LT, Prienai distr., Prienai | 2012-11-04 | P. sylvestris | | + | + | |
| DOT 27B/KR995128 | 54.612386, 23.886160 | LT, Prienai distr., Prienai | 2012-11-04 | P. sylvestris | +/− | + | + | + |
| DOT 28/KR995129 | 54.724976, 23.501945 | LT, Marijampolė distr., Ąžuolų būda forest | 2012-11-03 | P. sylvestris | +/− | + | + | |
| DOT 31 | 54.434801, 25.166110 | LT, Šalčininkai distr., Rūdninkai | 2013-05-01 | P. sylvestris | | + | + | |
| DOT 32 | 54.431512, 25.326769 | Lithuania, Šalčininkai distr., Jasiūnai | 2013-05-01 | P. sylvestris | | + | + | |
| DOT 33 | 54.452618, 25.117796 | LT, Šalčininkai distr., Baltoji Vokė | 2013-05-01 | P. sylvestris | | + | + | |
| DOT 34 | 54.734600, 25.405831 | LT, Vilnius, Kairėnai botanical garden | 2013-05-02 | P. nigra subsp. pallasiana | | + | + | |
| DOT 40 | 54.630733, 25.110808 | LT, Vilnius, Trakų Vokė | 2013-05-02 | P. sylvestris | | + | + | |
| DOT 47 | 54.798854, 25.396779 | LT, Vilnius distr., Antaviliai | 2013-11-01 | P. sylvestris | | + | + | |
| DOT 48 | 54.798854 25.396779 | LT, Vilnius distr., Antaviliai | 2013-11-01 | P. sylvestris | | + | | + |
| DOT 52 | 54.849467, 24.035366 | LT, Kaunas distr., Dubrava arboretum | 2013-10-29 | P. nigra | | + | ND | ND |
| DOT 53 | 54.734472, 25.407043 | LT, Vilnius, Kairėnai bot. garden | 2013-10-30 | Pinus ponderosa | | + | | + |
| DOT 54 | 55.678112, 21.105277 | LT, Curonian spit, Smiltynė | 2013-09-11 | P. mugo | | + | ND | ND |
| DOT 55 | 54.849599, 24.038517 | LT, Kaunas distr., Dubrava arboretum | 2013-10-29 | P. peuce | | + | ND | ND |

(Continues)

**TABLE 1** (Continued)

| Isolate/GenBank Accession Number | Coordinates | Location | Collection Date | Host | ITS/beta-tubulin 2 sequencing | ITS-RFLP | Species-specific PCR Mating Type | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | MAT1-1 | MAT1-2 |
| DOT 49 | 55.678112, 21.105277 | LT, Curonian spit, Smiltynė | 2013-09-11 | P. sylvestris | | + | ND | ND |
| DOT 57-KR/ MK951795 | 54.820964, 23.992338 | LT, Kaunas distr., Dubravai | 2017-09-10 | P. mugo | -/+ | ND | | + |
| DOT 58-KR | 55.072552, 25.609290 | LT, Molėtai district, Dubingiai | 2017-04-12 | P. sylvestris | | ND | | + |
| DOT59-KR/ MK951797 | 55.336237, 21.046071 | LT, Curonian spit, Bulvikis environs | 2017-10-22 | P. sylvestris | -/+ | ND | | + |
| DOT61-KR | 55.336237, 21.046071 | LT, Curonian spit, Bulvikis environs | 2017-10-22 | P. mugo | | ND | | + |
| DOT 62-KR | 55.441561, 21.072393 | LT, Curonian spit, Nagliai reserve | 2017-10-22 | P. sylvestris | | ND | | + |
| DOT 41 MT256138 | 55.139271, 29.743797 | **Belarus (BY)**, Vitebsk region, Beshankovichi distr., Luchki | 2013-10-18 | P. sylvestris | +/- | + | ND | ND |
| DOT 42 A, B MT256139 | 55.141667, 29.738611 | BY, Vitebsk region, Beshankovichi distr., Luchki | 2017-04-17 | P. sylvestris | +/- | ND | + | + |
| DOT 60 A,B/ MK951796 | 55.14000, 29.747222 | BY, Vitebsk region, Beshankovichi distr., Luchki | 2018-10-28 | P. sylvestris | -/+ | ND | + | + |
| DOT 43/MT256134/ MK951790 | 55.141944, 30.211389 | BY, Vitebsk region., Vitebsk distr., Shpili | 2017-04-24 | P. sylvestris | +/+ | ND | + | |
| DOT 44/MT256135/ MK951791 | 52.278783, 29.391534 | BY, Gomel region, Kalinkovichi distr., Bobrovichi | 2017-04-25 | P. sylvestris | +/+ | ND | + | + |
| DOT 45/ MT256136/ MK951792 | 51.492100, 30.459652 | BY, Gomel region, Narovlya distr. | 2017-04-25 | P. sylvestris | +/+ | ND | + | |
| DOT 46/ MK951793 | 55.125365, 30.227208 | BY, Vitebsk region, Vitebsk distr., Lyatokhi | 2017-04-25 | P. sylvestris | -/+ | ND | + | |
| DOT 56/ MK951794 | 55.220835, 30.129049 | BY, Vitebsk region, Vitebsk | 2017-04-24 | P. sylvestris | -/+ | ND | + | |
| DOT 63 MT256137 | 55.149204, 30.189839 | BY, Vitebsk region, Vitebsk | 2018-10-30 | P. sylvestris | +/- | ND | + | |
| DOT 64 | 55.200887, 30.212175 | BY, Vitebsk Vitebsk Bot. garden | 2018-11-02 | P. mugo | | ND | + | |
| DOT 65A, B | 55.825090, 29.916873 | BY, Vitebsk region, Vitebsk distr,, Surmino | 2018-11-04 | P. sylvestris | | ND | + | |
| DOT 66A, B | 55.269607, 27.694576 | BY, Vitebsk region, Hlybokaye distr., Glubokoye | 2018-10-27 | P. sylvestris | | ND | + | |
| DOT 67 | 55.113442, 27.633401 | BY, Vitebsk region, Hlybokaye distr., Glubokoye | 2018-12-01 | P. sylvestris | | ND | + | |

(Continues)

**TABLE 1** (Continued)

| Isolate/GenBank Accession Number | Coordinates | Location | Host | Collection Date | ITS/beta-tubulin 2 sequencing | ITS-RFLP | Species-specific PCR Mating Type | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | MAT1-1 | MAT1-2 |
| DOT 68 | 55.836907, 29.956745 | BY, Vitebsk region, Vitebsk distr., Pankry | *P. sylvestris* | 2018-11-04 | | ND | | + |
| DOT 69 | 55.838476, 29.940695 | BY, Vitebsk region, Vitebsk distr., Pankry | *P. sylvestris* | 2018-11-04 | | ND | + | |
| [a]MK622273 | 53.915635, 27.616022 | BY, Minsk Bot. garden | *P. mugo* | 2019-03-13 | +/– | | | |

Abbreviations: BY, Belarus; LT, Lithuania; ND, not detected.

[a] Additional *Dothistroma septosporum* ITS gene region sequence data (GenBank: MK622273) from Belarus used in phylogenetic analysis (Panteleev and Baranov, insert in GenBank 2019, unpublished).

EPPO, 2019; Fabre, Ioos, Piou, & Marcais, 2012; Jánošíková-Hečková et al., 2018; Matsiakh et al., 2018; Mullett et al., 2018; Ondrušková et al., 2017, 2018; Queloz, Wey, & Holdenrieder, 2014).

Scots pine (*Pinus sylvestris* L.) is widely distributed, economically important conifer in the Northern Hemisphere, native to Eurasia. In Europe, area of its stands currently exceeds 28 million hectares (Houston Durrant, de Rigo, & Caudullo, 2016). In northern and north-eastern Europe, including Lithuania and Belarus, *P. sylvestris* is the only native and widely planted pine species (Navasaitis, Ozolinčius, Smaliukas, & Balevičienė, 2003).

During the last decades, the DNB disease has significantly increased in intensity and severity across Europe and worldwide (Adamson, 2018; Boron et al., 2019; Brown & Webber, 2008; Drenkhan et al., 2016; Kranjec-Orlović et al., 2019; Woods et al., 2016). In eastern and northern parts of Europe, only *D. septosporum* is found on native host *Pinus sylvestris* L. (Adamson et al., 2018; Barnes et al., 2008; Drenkhan, Hantula, Vuorinen, Jankovský, & Müller, 2013; Drenkhan et al., 2016; Fraser, Brown, & Woodward, 2015; Millberg, Hopkins, Boberg, Davydenko, & Stenlid, 2016; Müller, Hantula, & Vuorinen, 2009; Mullett et al., 2018; Solheim, 2012; Solheim & Vuorinen, 2011). *Dothistroma septosporum* was first described in 1911, from pine needles collected in the vicinity of Saint-Petersburg, Russia (Doroguine, 1911). However, the origin of the fungus remains unclear. Recent genetic studies have shown that the most genetically diverse populations of *D. septosporum* were found in northern and eastern Europe, indicating that the fungus may be endemic in Europe and that Scots pine forests in this region may represent the natural range of *D. septosporum* (Adamson et al., 2018; Barnes, Wingfield, Carbone, Kirisits, & Wingfield, 2014; Boron et al., 2019; Drenkhan et al., 2013; Ennos, Sjökvist, Piotrowska, Riddell, & Hoebe, 2020; Mullett, Brown, Fraser, Baden, & Tubby, 2017; Piotrowska et al., 2017).

In Lithuania, the first recorded occurrence of *D. septosporum* was detected on ornamental *Pinus mugo* Turra trees near Vilnius city, in 2002 (Jovaišienė & Pavilionis, 2005). In 2008, the fungus was found on native *P. sylvestris* (Markovskaja & Treigienė, 2009). Species identity was confirmed by molecular methods in 2013 (Drenkhan et al., 2016). This pathogen is now widespread throughout the country. (Markovskaja & Raitelaitytė, 2016; Raitelaitytė et al., 2016). Comprehensive studies have shown that only *D. septosporum* is spreading in Lithuania and neighbouring countries (Poland, Latvia and Estonia) on *P. sylvestris* in natural stands and plantations (Adamson et al., 2018; Boroń, Lenart-Boroń, & Mullett, 2016; Boron et al., 2019; Drenkhan, Adamson, Jürimaa, & Hanso, 2014; Drenkhan et al., 2013, 2016; Kowalski & Jankowiak, 1998). In Europe, *D. pini* and *D. septosporum* in the same stand and on the same *P. sylvestris* host were found only in Slovenia (Piškur, Hauptman, & Jurc, 2013), Montenegro (Lazarević, Davydenko, & Millberg, 2017) and Slovakia (Jánošíková-Hečková et al., 2018).

The first record of *D. septosporum* in Belarus is quite recent (Drenkhan et al., 2016), and knowledge of DNB disease in Belarus is negligible. The first Belarusian case of DNB was discovered in Verhnedvinsk arboretum (Vitebsk region) in 2012 on young trees of

*Pinus strobus* L. and was tentatively identified as *D. septosporum* (unpublished data from phytosanitary reports). In 2013, DNB disease was allegedly detected on *P. sylvestris* in a plantation near Negoreloje (Minsk region), but the presence of *Dothistroma* spp. was not confirmed by molecular methods (V. Zviagintsev, personal communication). The first Belarusian sample of *D. septosporum* on *P. sylvestris*, which was confirmed by the species-specific ITS-RFIP procedure, was collected by P. Kolmakov in 2013 in Vitebsk region (Drenkhan et al., 2016). Later, *D. septosporum* was collected on *P. mugo* in botanical gardens (Vitebsk and Minsk) and confirmed by ITS and/or beta-tubulin 2 gene sequencing (this study and the unpublished data from Panteleev and Baranov: GenBank: MK622273).

Early pathogen detection and correct identification (including microscopy and molecular analysis) are very important for disease management.

The aim of this study was to analyse all available data on the occurrence and spread of DNB in Lithuania and Belarus, to confirm the identity of the causative agent of DNB by various molecular methods and to identify mating types of the pathogen and to assess the possible risk of the disease for native Scots pine forests in both countries.

## 2 | MATERIALS AND METHODS

Symptomatic needles were collected in Lithuania in 2012–2013 and in 2017–2018 from 10 to 40-year-old native Scots pines (*Pinus sylvestris*) and exotic pines of different ages in 20 different localities (Table 1, Figure 1). In Belarus, symptomatic needle samples were collected in 2013 and 2017–2018 from 5 to 6-year-old *P. mugo* in Vitebsk Botanical garden and from 10 to 60-year-old *P. sylvestris* trees in 12 localities of naturally regenerated and planted Scots pine forests in Vitebsk and Gomel regions. The 1-, 2- and 3-year-old infected needles ($n = 50$–100, the number depended on the infection intensity) were collected from 3 to 5 randomly selected trees in every locality. The degree of severity of the disease was assessed by the scale of crown damage (Mullet & Brown 2018, modified): 5%–20% of crown infection—low disease severity, 20%–30%—medium severity, 30%–55%—high severity, >55%—very high severity. Pine trees ($n \geq 20$) were evaluated in 1,000 $m^2$ plots in every studied forest stand. All collected needle samples were initially examined under Nikon C-PS stereo and an Olympus CX 41 dissecting microscopes using standard microscopic methods. Photomicrographs and measurements were taken with a Nikon DS-Ri2 digital camera using the NIS-elements imaging software v. 4.3. Herbarium specimens (one per host and per locality) were deposited in BILAS Herbarium (Institute of Botany, Nature Research Centre), ITS and beta-tubulin 2 gene sequencing data of selected voucher specimens were deposited at the NCBI's GenBank nucleotide database (Table 1). For morphological analysis, at least 50 randomly selected fungal sporification structures and conidia were measured. For species identity confirmation, rDNA ITS region sequencing, ITS-RFLP and mating-type-specific PCR diagnostic procedure) were employed (Barnes et al., 2004; Barnes, Kirisits, Winhfield, & Wingfield, 2011; Ioos et al., 2010).
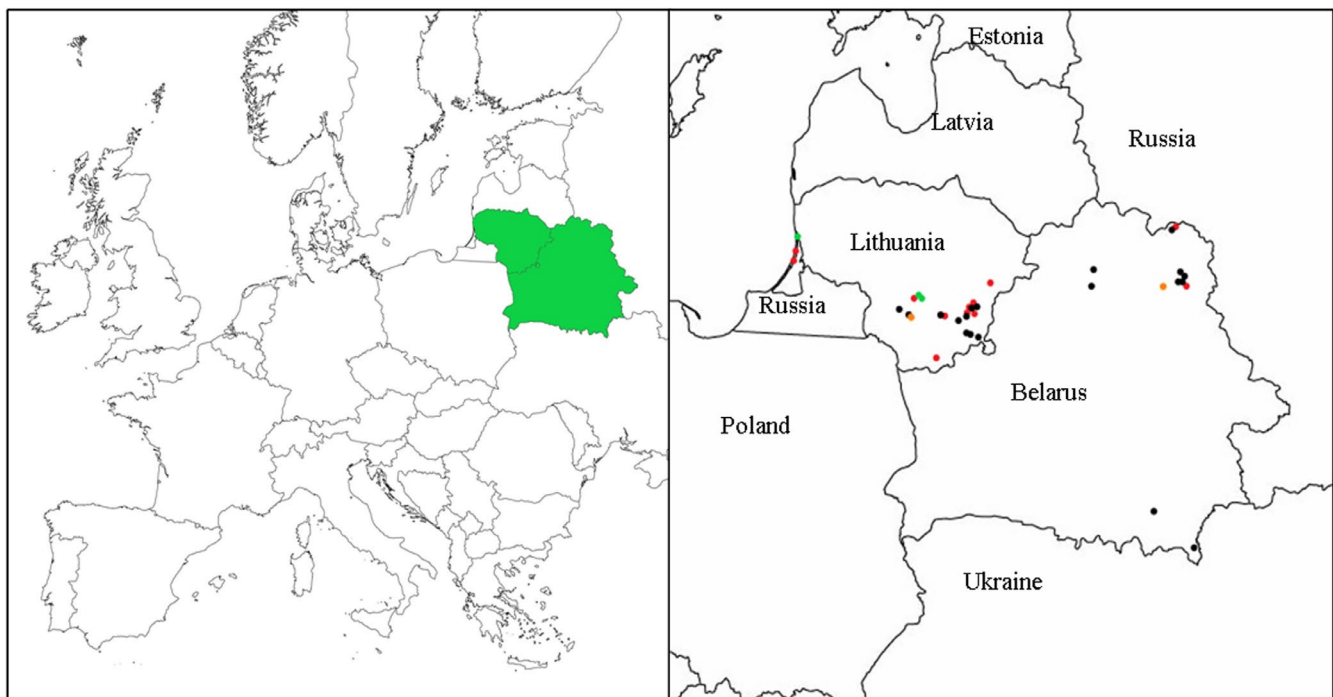


**FIGURE 1** Collection sites and spatial distribution of MAT types of *Dothistroma septosporum* isolates in Lithuania ($n = 36$) and Belarus ($n = 19$): black symbols indicate sites where MAT1-1 was found; red symbols indicate sites where MAT1-2 was found; orange symbols indicate sites where both MAT1-1 and MAT1-2 were found; green symbols indicate sites where MAT types were not detected, but pathogen was confirmed by other molecular methods
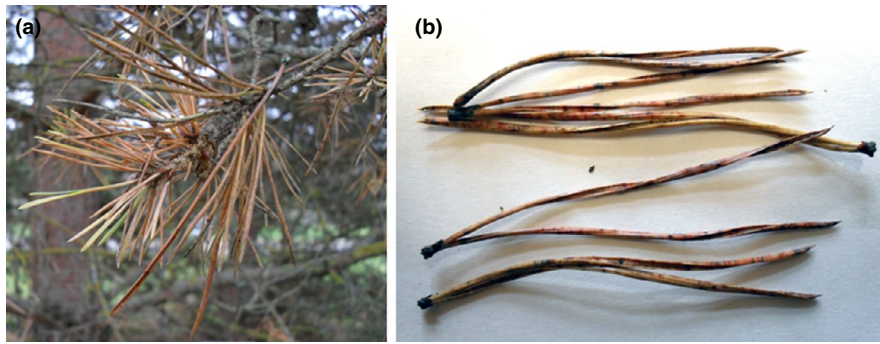
**FIGURE 2** Typical DNB symptoms on *Pinus sylvestris* (from Belarus collections): (a) infected shoot; (b) infected needles with red bands and black fruiting structures (acervular conidiomata)

Fungal genomic DNA was extracted from axenic cultures grown on 2% Malt Extract Agar (MEA, Oxoid Limited) with ZR Fungal/Bacterial DNA Miniprep™ Kit (Zymo Research Europe GmbH). The axenic cultures were isolated from mature conidiomata found on infected needles by the spore streaking method (Mullett & Barnes, 2012). The ITS1-5.8-ITS2 region of rDNA was amplified using the primers ITS 5 and ITS 4 (White, Bruns, Lee, & Taylor, 1990) under 55°C annealing temperature, and β-tubulin 2 gene region was amplified using the primers Bt2a and Bt2b (Glass & Donaldson, 1995) under 61°C annealing temperature using KAPATaq Ready Mix (KAPABIOSYSTEMS) following the manufacturer's instructions. Purified PCR products were sequenced by BaseClear B.V. The conventional PCR reactions were carried out on a 96 Universal Gradient PeqSTAR thermocycler (VWR) in 20 µl reaction volumes. PCR reaction mix: using 2 µl of template DNA, forward and reverse primers (10 pmol/µl), 2x Phire Plant PCR buffer (includes dNTPs and MgCl2; Thermo scientific), 0.4 µl of Phire Hot Start II DNA Polymerase and molecular grade water added up to 20 µl. The cycling conditions were as following: (ITS) an initial denaturation step at 98°C for 30 s; 40 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s and elongation at 72°C for 90 s; and a final extension step at 72°C for 5 min; (β-tubulin 2) an initial denaturation step at 98°C for 5 min; followed by 40 cycles of denaturation at 98°C for 5 s, annealing at 61°C for 5 s and elongation at 72°C for 20 s; with a final extension at 72°C for 60 s. PCR product was visualized on 0.5% agarose ("Agarose NEEO Ultra – Qualität") gels under UV light. The resulting PCR products were visualized using the E.A.S.Y. Win 32 (Herolab). Two different PCR products from each specimen and four repeats in total for each sequence from both ends (5′ and 3′) were sequenced to confirm the sequence. The rDNA homology searches (BLAST) were performed through the Internet at the National Center for Biotechnology Information (National Institutes of Health).

The phylogenetic analysis of investigated *D. septosporum* isolates and related taxa (*Dothistroma pini*, *Lecanosticta* spp.) was conducted in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). The maximum parsimony (MP) tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence (Nei

& Kumar, 2000). There were a total of 780 positions in the final dataset. *Teratosphaeria nubilosa* strain CPC 12243 was used as an outgroup. The consistency index is (0.9597), the retention index is (0.9890), and the composite index is 0.952695 (0.9491) for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein, 1985).

The species-specific ITS-RFLP procedure was generated by restricting the ITS amplicons with the restriction enzyme Alu I and then visualized by electrophoresis on 3% agarose gel. According to Barnes et al. (2004), samples of *D. pini* produce two fragments of approximately 200 and 350 bp in size, meantime samples of *D. septosporum* are not digested and produce only one fragment about 600 bp.

Mating-type-specific multiplex PCR diagnostic procedure was carried out using *D. pini* and *D. septosporum* specific primers DpiniMat1f2, DpiniMat2f, DseptoMat1f, DseptoMat2f, DotMat1r and DotMat2r (Groenewald et al., 2007). The species-specific primers of both species amplify regions of approximately 820 bp for MAT1-1 and 480 bp for MAT1-2. All mixes for mating-type-specific multiplex PCR reactions were prepared using KAPATaq Ready Mix (KAPABIOSYSTEMS) under 65°C annealing temperature following the manufacturer's instructions.

## 3 | RESULTS

Diseased pine trees with Dothistroma needle blight (DNB) symptoms were found in various habitats, including natural and planted Scots pine forests in Lithuania and two regions (Vitebsk and Gomel) of Belarus (Table 1). Infection usually starts in the basal areas of pine trees with more strongly affected 2 to 3-year-old needles of lower branches. Based on a polyphasic taxonomic approach, including morphological features of fungus asexual stage and various genetic approaches, the pathogen was identified as *Dothistroma septosporum* (Dorog.) Morelet. The pathogen was detected on native *Pinus sylvestris* L. in forest stands in their natural environment in both Lithuania and Belarus and on four non-native pine species: *Pinus mugo* Turra, *P. nigra* J. F. Arnold subsp. *pallasiana* (Lamb.), *P. peuce* Griseb. and *P. ponderosa* Dougl. ex P. et C. Laws. in Lithuania and on *P. mugo* in Belarus (Table 1).

## 3.1 | Disease symptoms

DNB-affected Scots pine needles had similar symptoms in both countries: on the infected brownish parts of needles developed clearly visible red or reddish brown bands of various intensity with deeply erumpent black fruiting structures. Sometimes the tips of the needles were brown with basal parts, which were still green or vice versa (Figure 2). *D. septosporum* was represented only by asexual stage (anamorph) and produced dark brown to black acervular conidiomata up to 1 mm in diameter with greyish mass of conidia inside. Conidiomata were abundant ($n = 15$–$34$) on strongly infected needles, occurring singly or gregarious, usually very unevenly distributed over the length of necrotic parts of the needles.

## 3.2 | Short morphological and cultural characteristics

Conidiophores were hyaline to light brown, branched or simple, with 1–3 septa, 15–34 × 2–3.5 µm; conidiogenous cells ampulliform, percurrently proliferating; conidia hyaline, 1–2(3) septate, rarely 4–5 septate, subcylindrical to obclavate, straight or curved, 12.5–40.5 × (1.5) 2–3 µm, smooth and thin-walled (Figure 3). The sexual stage (teleomorph) was not found.

Thirty-six axenic cultures were isolated from conidiomata on the needles of *P. sylvestris*, *P. mugo*, *P. nigra*, *P. ponderosa* and *P. peuce* collected in Lithuania. Seventeen axenic cultures were isolated from conidiomata developed on needles of *P. sylvestris* and one on *P. mugo* collected in Belarus (Vitebsk and Gomel regions and Vitebsk city, respectively). On Malt Extract Agar media, the red or reddish brown colour typical of *Dothistroma* colonies usually appeared, but sometimes the fungus also produced unusual blue coloration (Figure 4). Our study has shown that the fungal isolates producing blue coloration in vitro usually produced reddish brown or brown but not red bands on the infected needles. Morphologically, conidia did not differ in shape or size between cultures producing red or blue pigmentation.

## 3.3 | Molecular diagnostics

Different molecular methods were used as the next step for species identity confirmation (Table 1). All obtained ITS gene region sequences of Lithuanian and Belarusian isolates (GenBank KR995124–KR995129 and MT256134–MT256139) shared 99%–100% identity between each other and had up to 100% similarity with Hungarian *D. septosporum* strain (GenBank MH864656) from CBS culture collection (Vu et al., 2019) and with strain (GenBank KU948398) from CNW culture collection (Barnes et al., 2016). The phylogeny of investigated *Dothistroma* isolates based on rDNR operon (ITS1-5.8S-ITS2) locus was performed using maximum parsimony (MP) to confirm these isolates as *D. septosporum* and to separate its different haplotypes. The MP tree out of three most parsimonious trees (length = 518) is shown in Figure 5. The performed phylogenetic analysis grouped all Lithuanian and the majority of Belarusian isolates into two subclades of the separate *D. septosporum* clade and one Belarusian isolate from Gomel region (Dot45/GenBank MT256136) nested into another *D. septosporum* clade distinct from *D. pini* and *Lecanosticta* spp. clades (Figure 5). Two first *D. septosporum* subclades represent two different haplotypes: Ds_HAP.1 and Ds_HAP.2, respectively; isolate from Gomel region represents the third haplotype Ds_HAP.3.

All obtained β-tubulin 2 gene sequences of Lithuanian and Belarusian isolates (GenBank MK951790–MK951797) demonstrated 99.5% to 100% identity with *D. septosporum* neotype isolate (GenBank KX364411) from Russia (Barnes et al., 2016) and confirmed the identity of these isolates as *D. septosporum*.

Together with rDNR ITS and β-tubulin 2 gene region sequencing of selected voucher specimens, the simple molecular diagnostic species-specific ITS-RFLP procedure was used for the identity confirmation of different isolates. According to the data in silico, created by software ApE-A plasmid Editor v2.0.47, Alu I restriction enzyme cut ITS gene region of *D. pini* (sample of *D. pini* Nr.10 from Ukraine was used in this study for comparison with Lithuanian and Belarusian isolates) into two fragments: ~373 and ~220 bp, the same ITS regions of *D. septosporum* from Lithuania and Belarus had no sites
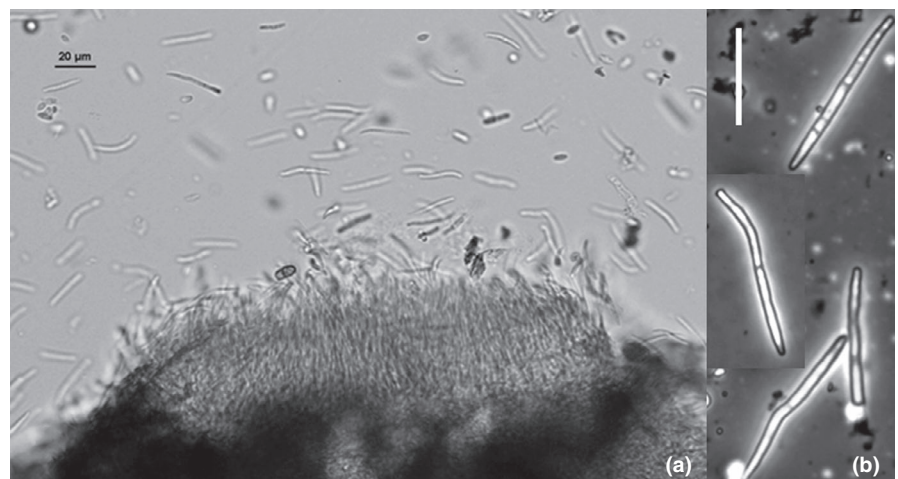


**FIGURE 3** *Dothistroma septosporum*: (a) opened fruitbody (acervulus) with conidia; (b) 1–2 septate hyaline conidia (scale bar = 20 µm)
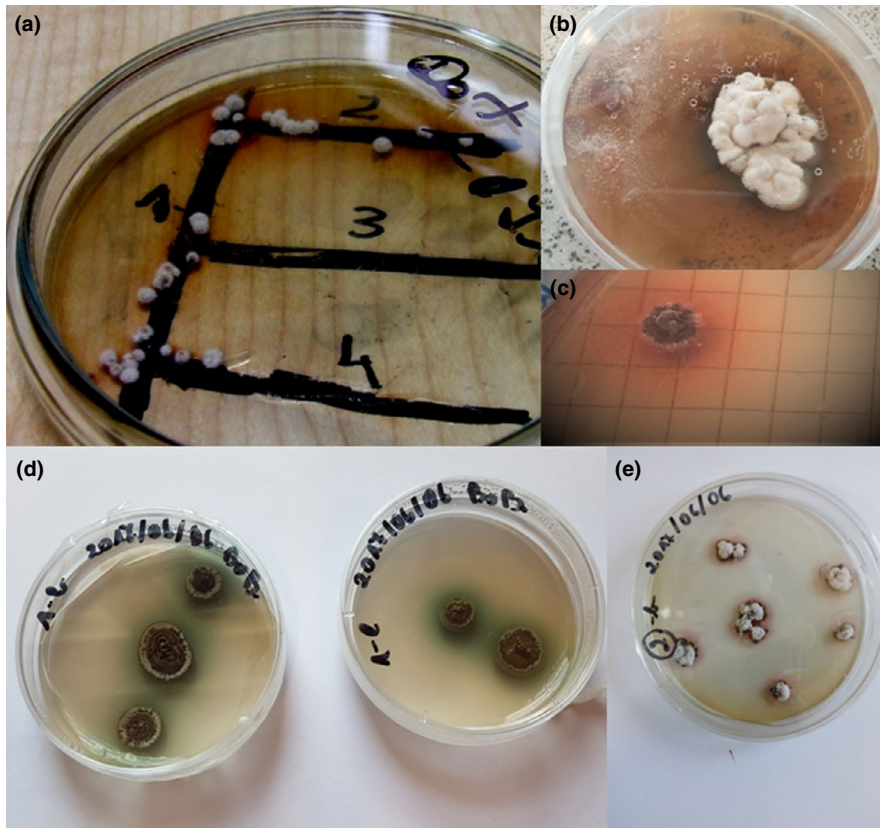
**FIGURE 4** Pigment production of *Dothistroma septosporum* in culture: (a, c, e) characteristic red or reddish brown agar media (2% MEA) coloration; (b) a brown agar coloration; (d) a blue agar coloration

for restriction of particular enzyme and was not cut (Figure 6). None of the rDNA from any of the examined Lithuanian and Belarusian isolates was cut by AluI during this analysis. Thus, this method also showed that in Lithuania and Belarus only *D. septosporum* was the cause of DNB and that *D. pini* isolates were not obtained.

The mating-type species-specific PCR diagnostic procedure of Lithuanian and Belarusian samples obtained specific amplicons of 820 bp for MAT1-1 and 480 bp for MAT1-2 of *D. septosporum* (Figure 7).

## 3.4 | DNB distribution and severity

*Dothistroma septosporum* in Lithuania is commonly found on ornamental pines in Dubrava Arboretum, Vilnius and Kaunas Botanical gardens. As a forest pathogen, it was detected on *P. mugo* in Curonian Spit (Kutorga et al., 2012), where mountain pine has been widely used since the 19th century for reforestation of dunes and stabilization of drifting sand (Olšauskas, 2009), and on *P. sylvestris* in both natural forests and commercial plantations throughout the country (17 localities, Table 1). In Belarus, *D. septosporum* was detected on *P. mugo* from Vitebsk Botanical garden and on native *P. sylvestris* in naturally regenerated and planted Scots pine forests in 12 localities in northern (Vitebsk region) and southern (Gomel region) parts of the country. Analysing the spread of DNB in both countries, different degrees of disease severity were found on local *P. sylvestris* trees in Scots pine forests and plantations. In Lithuania, the severity of *P. sylvestris* disease was usually not very high, in the most surveyed

plantations (13 from 17) only the lower branches of trees were usually infected with DNB, and the average severity of the disease did not exceed 10%–30% of the canopy. The lowest severity (5%–10%) was observed in the north-western part of the country, while the medium severity (20%–30%) was found in most sites in the central and south-eastern part of the country, except for some densely planted pine stands in Kaunas (Dubravai environs, 54.820964, 23.992338) and Trakai (Aukštadvaris environs, 54.576207, 24.515279) districts, where the severity was quite high and reached 40%–50%. Very high severity of the disease (about 55%–60%) was found only on two Scots pine plantations, one of which is located in southern Lithuania, in Marijampolė district (Ažuolų Būda Forest, 54.724976, 23.501945) and the other in eastern Lithuania, in Molėtai district (Dubingiai Forest, 55.072552, 25.609290). However, the highest degree of DNB severity, reaching up to 70%, was found on ornamental, exotic pine trees: *P. nigra* subsp. *pallasiana*, *P. heldreichii*, *P. peuce*, *P. ponderosa* and *P. sibirica* in Vilnius and Kaunas Botanical gardens and in Dubrava Arboretum (Kaunas district). On *P. heldreichii* and *P. sibirica*, DNB was identified only by symptoms and morphological characteristics, and on other hosts, the identity of the pathogen was confirmed by morphological and molecular methods. It is noteworthy that the DNB severity in the *P. mugo* stands planted over a hundred years ago on the Curonian Spit (Lithuanian Baltic Sea coast, western part of the country) was low (5%–10%), on some trees it rarely reached 10%–20%. The severity of the disease in naturally regenerated Scots pine forests in Belarus was also not high; in most localities, it was usually low or medium, reaching 10%–30% of the tree crown, and only in two plantations (one in Beshankovichi district,
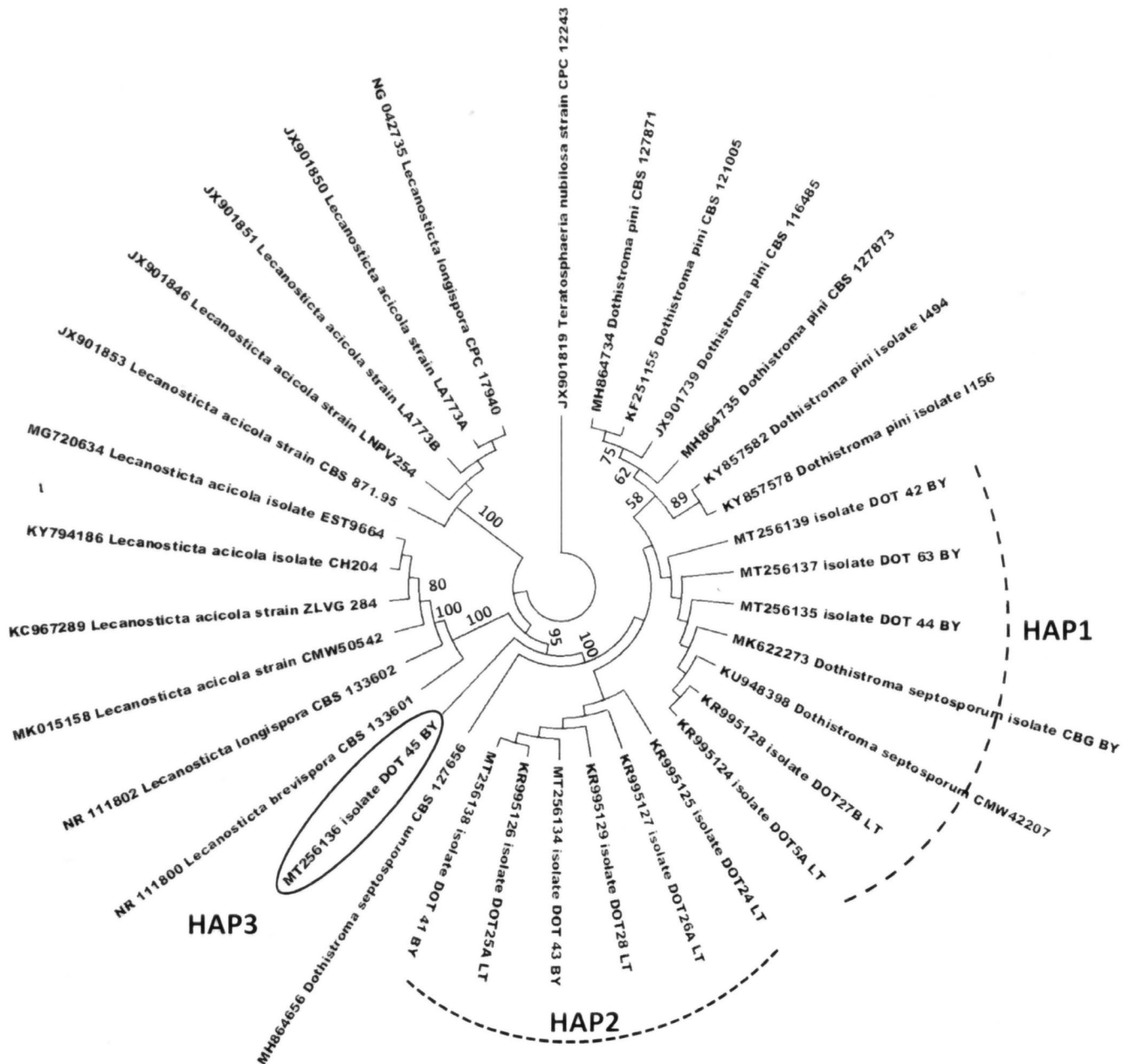
**FIGURE 5** Phylogeny of investigated *Dothistroma septosporum* isolates and related taxa, inferred from the rRNR operon (ITS1-5.8S-ITS2) locus sequence data using maximum parsimony (MP). *Teratosphaeria nubilosa* strain CPC 12,243 was used as the outgroup

Luchki environs, 55.141667, 29.738611 and one in Vitebsk district, Pankry environs, 55.838476, 29.940695), it was higher, reaching 30%–40%. In Vitebsk Botanical garden, the degree of severity of DNB on *P. mugo* trees was low (about 10%–15%). Our results show that DNB is spreading in both countries in different biotopes where native *P. sylvestris* and/or exotic pines are present.

## 3.5 | Occurrence of mating types

Analysis of the mating-type (MAT) genes of *D. septosporum* showed the presence of both mating-type idiomorphs among Lithuanian and Belarusian isolates. In Lithuania, MAT1-1 and MAT1-2 idiomorphs were found in the ratio 1:1, while in Belarus MAT1-1 idiomorph prevailed (ratio 3:1). Occurrences of mating types differed between localities: only one mating type was found in most of the surveyed localities, while the occurrence of both mating types together was rare, present in four localities in Lithuania (Vilnius, Trakai, Prienai and Kaunas districts) and two localities in Belarus (Beshankovichi and Vitebsk districts, Vitebsk region) (Table 1, Figure 1).

## 4 | DISCUSSION

The molecular data obtained during our study confirmed the presence and spread of *D. septosporum* rather than *D. pini* as a
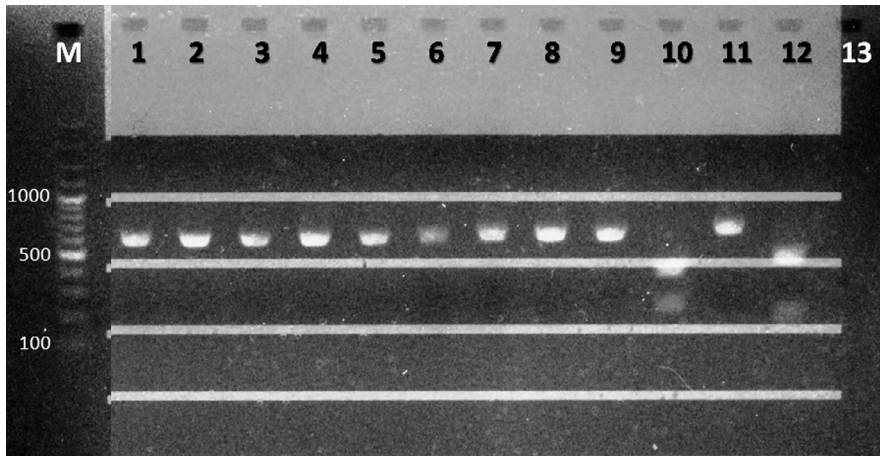
**FIGURE 6** PCR-RFLP patterns of some investigated isolates, ITS region digested by restriction enzyme Alu I. M – DNA marker GeneRuler 100 bp Plus DNA Ladder (100–3,000 bp); 1—DOT5A; 2—DOT19; 3—DOT24; 4—DOT27; 5—DOT31; 6—DOT32; 7—DOT41(Belarus); 8—DOT42A (Belarus); 9—DOT53(Lithuania); 10—DOTpini (Ukraine, *Dothistroma pini*); 11—LA (Lithuania, *Lecanosticta acicola*); 12—CAT (Lithuania, *Neocatenulostroma germanicum*); 13—NEGATIVE CONTROL
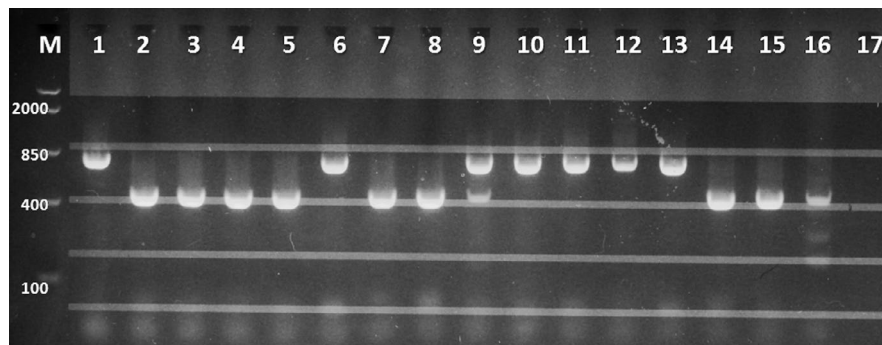


**FIGURE 7** Mating-type-specific multiplex amplicons obtained from some investigated isolates rDNA. M – DNA marker FastRuler Middle Range DNA Ladder (5,000, 2,000, 850, 400, 100 bp); 850 bp—MAT1-1 ; 400 bp—MAT1-2; 1—DOT 5A; 2—DOT 19; 3 – DOT 24; 4—DOT 24A; 5—DOT 25; 6—DOT 25A; 7—DOT 26A; 8—DOT 27A; 9—DOT 27B; 10—DOT 28; 11—DOT 31; 12—DOT 32; 13—DOT 42A(Belarus); 14—DOT 42B (Belarus); 15—DOT 58(Lithuania); 16—DOTpini (Ukraine, *Dothistroma pini*); 17—NEGATIVE CONTROL

causal agent of DNB in the neighbouring countries, Belarus and Lithuania. It also showed that the native Scots pine is susceptible to this fungal pathogen. In this study, differences in colour of the secreted pigment were observed on infected needles and on agar media (characteristic red or reddish brown bands on needles, red agar discoloration from the production of dothistromin and appearance of blue pigment in some cultures). The variation in agar coloration during *Dothistroma* spp. growth in culture has been mentioned by other authors, too (Kowalski, Nawrot-Chorabik, Grad, & Leszczyński, 2016; Mullett & Barnes, 2012). The colour of red bands in necrotic needle lesions depends mainly on the production and accumulation of red toxin, dothistromine, but can sometimes also occur due to the metabolic products of other fungi inhabiting the same needles (Kowalski et al., 2016). During in vitro experiments, it was found that the colour of the secreted pigment is not a constant character of the fungal isolate, but usually depends on the growth temperature and pH of the medium (Kowalski et al., 2016). Lower temperature and lower pH stimulated the production of the blue pigment.

Three different ITS haplotypes (Ds_HAP.1, Ds_HAP.2 and Ds_HAP.3) were identified among Lithuanian and Belarusian

*D. septosporum* isolates (Figure 5). Two of them, Ds_HAP.1 and Ds_HAP.2, are spread both in Lithuania and in northern Belarus (Vitebsk region). The first ITS haplotype (Ds_HAP.1) was most common in northern Belarus, and the second haplotype (Ds_HAP.2), vice versa, was more common in Lithuania (Figure 5). The third haplotype (Ds_HAP.3) belongs to the rare ITS haplotypes and is represented by one isolate (DOT45/GenBank MT256136), which was found only in the southern part of Belarus (Gomel region). This haplotype was not found in Lithuania. The performed mating-type (MAT) genes analysis indicated clonal character of pathogen populations with dominance of MAT1-1 idiomorph in Belarus (Table 1). The Lithuanian *D. septosporum* isolates were characterized by an distribution equilibrium of MAT1-1 and MAT1-2 idiomorphs, but in most localities, only one mating type was found, which also indicates clonal populations. Co-occurrence of both mating types in the same localities and sometimes on the same pine trees in Lithuania and Belarus (Table 1) demonstrate a high probability of sexual reproduction of *D. septosporum* in both countries, although so far only asexual reproduction and spread via conidia have been registered (Drenkhan et al., 2016; Markovskaja & Raitelaitytė, 2016; Markovskaja & Treigienė, 2009; Raitelaitytė et al., 2016). It is noteworthy that both mating types

in the same DNA sample were found in one of Lithuanian isolates (DOT27B, on *P. sylvestris*, Prienai forest) (Figure 7), which indicates a particularly high probability of sexual stage development.

Many authors suggest that the equal presence of both mating types and the high genetic diversity of European *D. septosporum* populations increases the likelihood of sexual reproduction of the fungus and genetic recombination (Adamson et al., 2018; Barnes et al., 2008; Boron et al., 2019; Drenkhan et al., 2013; Groenewald et al., 2007; Mullett, Brown, & Barnes, 2015), as well as increases the possibility of the pathogen to co-evolve with the native pine hosts (Barnes et al., 2011, 2014; Boroń et al., 2016; Drenkhan et al., 2014, 2016; Kowalski & Jankowiak, 1998; McDonald & Linde, 2002; Perry, Brown, Cavers, Cottrell, & Ennos, 2016; Perry, Wachowiak, et al., 2016; Piotrowska et al., 2017). Recent studies in the UK have shown a high variability in the aggressiveness of the pathogen and in the susceptibility of Scots pine populations to *D. septosporum*, including the different adaptive capacity of trees to survive and co-exist with the pathogen (Fraser et al., 2015; Perry, Brown, et al., 2016). It is likely that a variation in the pathogen's aggressiveness led to a variation in the host's response. In any case, the impact of *D. septosporum* on pine tree health depends not only on differences in host resistance and pathogen aggressiveness, but also on disease monitoring and forest management practices in different countries (Bulman et al., 2016; Perry, Wachowiak, et al., 2016; Piotrowska et al., 2017).

The wide distribution and high genetic diversity of *D. septosporum* populations in Scots pine forests and plantations in the Baltic and Nordic countries (Adamson et al., 2018; Drenkhan et al., 2013, 2016; Jánošíková-Hečková et al., 2018; Millberg et al., 2016; Müller et al., 2009; Mullett et al., 2017, 2018; Solheim & Vuorinen, 2011) indicates a high probability of its long-term presence in north-eastern Europe in the latent stage and its co-evolution with the native host *P. sylvestris*. The wide distribution of *D. septosporum* and the relatively low severity of the disease in most naturally regenerated Scots pine forests in Lithuania and Belarus (usually 10%–30% of the tree crown) confirm the theory of co-evolution of the pathogen and its native host. In both countries, *P. sylvestris* may now be considered the most common host with varying susceptibility to *D. septosporum*. Another common host, *P. mugo*, also demonstrated varying susceptibility to *D. septosporum* in Lithuania: from low or moderate in the forest plantations in the Curonian Spit and high in urban parks, botanical gardens and arboretum, although in Belarus its susceptibility was low everywhere. Other ornamental pines growing in urban areas, botanical gardens and arboreta, such as *P. nigra* subsp. *pallasiana*, *P. heldreichii*, *P. peuce*, *P. ponderosa* and *P. sibirica*, which are new hosts of DNB in Lithuania, were highly susceptible to this pathogen. Drenkhan et al. (2016) list *P. nigra*, *P. nigra* subsp. *pallasiana* and *P. ponderosa* as highly susceptible to *D. septosporum*. Meanwhile, *P. heldreichii*, *P. peuce* and *P. sibirica* are considered as only slightly susceptible hosts worldwide (Barnes et al., 2008; Bednářová et al., 2006; Drenkhan et al., 2016; Jankovský, Bednářová et al., 2004). However, these three species have been severely affected by DNB (disease severity about 50%–70%) in Lithuania (Dubrava arboretum, Kaunas and Vilnius Botanical gardens).

The current increase in the incidence, range and severity of DNB and other pine needle diseases in Europe may be caused by global climate change (Adamson et al., 2018; Drenkhan et al., 2016; Jánošíková-Hečková et al., 2018; Mesanza et al., 2019; Mullett et al., 2018; Ondrušková et al., 2017). Over the past two decades, climatic conditions seem to become increasingly favourable for DNB outbreaks in the Northern Hemisphere, and the risk of this disease to Scots pine forests in the boreal region may increase significantly in the future (Möykkynen, Fraser, Woodward, Brown, & Pukkala, 2017; Sturrock et al., 2011; Watt, Ganley, Kriticos, & Manning, 2011; Watt et al., 2009; Woods et al., 2016). Dothistroma needle blight is particularly sensitive to temperature and water availability, the optimal conditions for the development and sporulation of the pathogen is a combination of temperature 15–20/10–12°C (days/nights) with constant humidity and an average daily relative humidity above 90% (Dvorak, Drapela, & Jankovský, 2012; Woods et al., 2016). An increase in the frequency of prolonged rains during the warm season has been identified as a key factor in the successful spread of DNB and the progression of infection in the temperate zone (Woods et al., 2005, 2016). Over the last century, the Baltic Sea region has seen a statistically significant increase in mean air temperature (around 0.8°C/decade) (HELCOM, 2013), which together with the increase in precipitation may have a positive impact on DNB distribution. Most of the recently proposed multi-models of global climate predict increased annual temperature in the 21st century in the Baltic Sea region and average warming with a greater increase in winter temperature, including extremely warm summers (Räisänen, 2017), as well as an increase in extreme daily precipitation both in winter and in summer (Christensen, Kjellström, & Zorita, 2015). A model of the possible impact of climate change on the composition of tree species in Lithuania for 2061–2090 (Ozolinčius et al., 2014) has shown that by the end of this century the climate may become less suitable for *Pinus sylvestris* and other conifers, and warming of the climate will negatively affect their resistance to diseases and pests. Models of *D. septosporum* distribution for 2030 in Europe (Möykkynen et al., 2017) also showed that new DNB outbreaks may occur in the future in the Nordic and Baltic countries, Scotland and Ireland, including northern Germany, Poland and Belarus, in regions covered by extensive areas of Scots pine forests, especially where *P. sylvestris* is planted in monocultures.

The present data show that in northern and eastern Europe *D. septosporum* is a common and widespread pathogen on various native and exotic pine hosts, and it is *D. septosporum* that is currently found in Lithuania and Belarus as a DNB agent on native *P. sylvestris*. Another causal agent of DNB, *D. pini*, is not so widespread as *D. septosporum* and was not detected in northern Europe. This pathogen usually occurs in western, central and southern European countries with warmer climate and in the USA, usually on *P. mugo*, *P. nigra*, *P. ponderosa* and *P. radiata* (Barnes et al., 2004, 2008, 2011; Fabre et al., 2012; Matsiakh et al., 2018; Mullett et al., 2018; Ondrušková et al., 2018; Piou & Ioos, 2014; Queloz et al., 2014; Siziba et al., 2016) and only rarely on *P. sylvestris* (Jánošíková-Hečková et al., 2018; Lazarević et al., 2017; Piškur et al., 2013). The closest countries to Belarus and Lithuania, where *D. pini* was detected, are Russia

(southwestern regions) and Ukraine (Barnes et al., 2008; Drenkhan et al., 2016; Matsiakh et al., 2018; Siziba et al., 2016). In Ukraine (Kherson region), both mating types of *D. pini* were detected on *P. nigra* subsp. *pallasiana*, and meantime, in Russia (Rostov region) only the MAT1-1 idiomorph was obtained on *P. mugo*, *P. nigra* subsp. *nigra* and *P. nigra* subsp. *pallasiana* (Siziba et al., 2016). The presence of only one mating type indicates that *D. pini* has been introduced into the southwestern regions of Russia and is of clonal nature. Only *D. septosporum* was found on *P. sylvestris* in Ukraine and Russia so far (Barnes et al., 2004, 2016; Davydenko, 2014; Musolin et al., 2014), but it is possible that after a while *P. pini* will also appear on *P. sylvestris*. Recent detections of *D. pini* along with *D. septosporum* on *P. sylvestris* in naturally regenerated and planted stands in northern Slovakia close to Polish territory demonstrate an increased risk of *D. pini* spreading to neighbouring Poland (Jánošíková-Hečková et al., 2018) and potentially reaching Lithuania and Belarus in future.

## 5 | CONCLUSIONS

The results of this study confirm the expansion of the *D. septosporum* range in the northern and eastern directions in Europe and confirm the forecast of possible DNB disease outbreaks in the Baltic region and Belarus in the future. The sexual stage of *D. septosporum* has never been observed either in Lithuania or Belarus, so the reproduction of the pathogen is considered predominantly asexual and distribution clonal. Analysis of mating-type genes showed the presence of idiomorphs of both mating types (MAT1-1 and MAT1-2) in both countries, but one mating type usually dominated in the individual localities. In Belarus, MAT1-1 idiomorph prevailed in most localities. However, the presence of both mating types in some populations at frequencies only slightly different from the 1:1 ratio confirms the presumption of the presence of the sexual cycle in fungus life. Continuous disease monitoring, careful inspection of seedlings in nurseries before planting (*D. septosporum* may be present in the latent stage in asymptomatic needles), control of transportation of infected plant material, breeding programmes for Scots pine and other conifers based on genetic selection to ensure resistance to DNB, and the use of appropriate forest management can be highly effective methods to prevent the transmission of *D. septosporum* and DNB outbreaks in Lithuania, Belarus and other countries.

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