Chapter 6

Needle blight caused by two species of Dothistroma in Hungary

ABSTRACT

Dothistroma septosporum and *D. pini* cause the serious pine needle disease, Dothistroma needle blight (DNB). Of these, *D. septosporum* has a global distribution, while *D. pini* is known only from the U.S.A., Russia and Ukraine. During a study of *D. septosporum* isolates from *Pinus nigra* in Hungary, microsatellite markers revealed the presence of a second Dothistroma species. The aim of this study was to identify the DNB pathogens occurring within Hungary using four different molecular techniques. These included sequencing of the rDNA ITS region, a species-specific ITS-RFLP, mating type primers and a diagnostic microsatellite marker, Doth_A. Results showed that both DNB pathogens can occur on the same tree and the same needle and co-inhabit the same conidioma. Both mating types of *D. septosporum* were shown to be present in Hungary, but only the MAT2 of *D. pini* was found. This represents the first report of *D. pini* in Central Europe. It also represents the first report of both DNB pathogens occurring in the same area. In addition, examination of needles with DNB symptoms from Russia revealed the presence of *D. pini* on a new host, *Pinus mugo*. The molecular markers applied in this study proved to be sufficiently robust to identify and differentiate between the two DNB pathogens, both in culture and directly from needles. They will consequently be useful to document the geographical range and monitor the spread of *D. septosporum* and *D. pini* in future studies.

INTRODUCTION

The disease known as Dothistroma needle blight (DNB) is characterised by red bands surrounding black, erumpent conidiomata that split the epidermal layers of infected pine needles. These needles become necrotic and are then cast which results in stunted tree growth (Gibson 1972, van der Pas 1981). When infection is severe, high levels of mortality occur in susceptible *Pinus* spp. (Karadzić 1988, Woods 2003). The disease has caused major epidemics on *P. radiata* D. Don (Monterey pine) in the Southern Hemisphere, particularly in Central Africa, Chile, New Zealand and Australia (Gibson 1972, Bradshaw 2004). In recent decades it has also been increasing in incidence and severity in the Northern Hemisphere. Currently, serious disease epidemics on *P. contorta* var. *latifolia* Dougl. Loud. (Lodgepole pine) are occurring in British Colombia (Woods 2003, Woods *et al.* 2005) and on *P. nigra* Arnold subsp. *laricio* (Poiret) Maire (Corsican pine) in Britain (Brown & Webber 2008).

Two very similar ascomycete fungi are known to cause DNB (Barnes *et al.* 2004). *Dothistroma septosporum* (Dorog.) Morelet (teleomorph: *Mycosphaerella pini* Rostr.) is the pathogen responsible for the epidemics mentioned above and has a worldwide distribution. It infects over 70 different pine species (Bednářová *et al.* 2006) in their native and non-native ranges (Maschning & Pehl 1994, Aumonier 2002, Woods 2003, Bradshaw 2004, Kehr *et al.* 2004, Kirisits & Cech 2006). In contrast to *D. septosporum*, *Dothistroma pini* Hulbary (telemorph: unknown) has been found only in the North-Central U.S.A. on *P. nigra* J. F. Arnold and in the bordering countries of Ukraine and South-Western Russia, on *P. pallasiana* D. Don (Barnes *et al.* 2004, Barnes *et al.* 2008a). In both these cases, the infected trees are planted outside their natural range.

The morphological characteristics of the two DNB fungi are very similar with the only credible difference being the width of the conidia (Barnes *et al.* 2004, Barnes *et al.* 2008a). On both infected needles and in culture, conidia of *D. pini* are on average slightly wider than those of *D. septosporum* (Barnes *et al.* 2008a). Because of their almost identical morphology and ability to cause the same disease, DNA-based methods provide the only unambiguous route to distinguish between the two species.

Sequencing of gene regions such as the rDNA ITS, β-tubulin and the translation elongation factor (EF1-α) has shown that *D. septosporum* and *D. pini* differ phylogenetically and reside in two distinct lineages (Barnes *et al.* 2004). These species can also be identified using an

ITS-RFLP diagnostic procedure (Barnes *et al.* 2004) and two sets of species-specific mating type primers designed by Groenewald *et al.* (2007). Another approach that is useful for large populations of isolates utilises the microsatellite primer pair, Doth_A (Barnes *et al.* 2008b). This primer can be used as a diagnostic marker during genescan analyses as the locus amplified is monomorphic for allele 124 in *D. septosporum* and 114 in *D. pini* (Barnes *et al.* 2008b). These alleles also differ in their sequences (see GenBank accession nos; EF591826 for *D. septosporum* and EF591827 for *D. pini*).

Recently, DNB has been steadily increasing in distribution and intensity across pine plantations in Hungary. In a preliminary screening of isolates collected from infected *Pinus nigra* trees in this country, the microsatellite diagnostic marker, Doth_A, revealed the presence of a different allele of 109 bp, possibly that of *D. pini*, in addition to the 124 bp allele of *D. septosporum* (Barnes 2009, Barnes *et al.* 2008b). The first aim of the present investigation was to confirm the identity of *D. pini* in Hungary using ITS sequence data. Subsequent aims were to (i) determine whether *D. pini* is also present in a second population of isolates collected at a different location in Hungary to that where *D. pini* was first noticed; (ii) determine whether both species potentially occur on the same trees, the same needles and in the same conidiomata; and (iii) determine the mating types of *D. pini* isolates from Hungary. All three diagnostic methods described above were utilized in this study with an additional objective to determine whether isolates could be identified as either *D. septosporum* or *D. pini* without the need to sequence. Furthermore, infected needles with typical DNB symptoms from two locations in Russia were included in this study to identify the causal agent of these infections.

MATERIALS AND METHODS

Sampling

Pinus nigra needles infected by DNB were collected in May 2007 from two areas on dolomite sites in the Highlands of Lake Balaton, Hungary (Figure 1). One collection site was a small pine stand planted alongside the road near the village Diszel. Here, trees were 12 to 15 years old and needles were collected from every second tree. The second collection site was in Csabrendek (Bakonyerdő, Forestry and Timber Industry Co. unit Devecser), where 18-year-old trees growing in a plantation were sampled. A handful of needles were collected from every second tree along vertical and horizontal transects. All collected needles were placed

separately into paper bags, transported to the laboratory and stored at -80˚C until they were further processed.

Needles from Russia were collected from two different districts and from three different host species including *P. mugo* Turra (Dwarf mountain pine), *P. pallasiana* (Crimean pine) and *P. nigra* (Austrian pine) (Table 1). They were sent to the Forestry and Agricultural Biotechnology Institute for species identification and were included in this study.

Isolations and DNA extractions

Diszel and Russia

Isolations and DNA extraction from the material collected near Diszel were carried out as part of a previous study (Barnes 2009). Cultures linked to this material as well as those for the Russian material were obtained by isolating single conidia from one mature conidioma on a needle, per tree, and growing these on 2% malt extract agar (MEA, Biolab, Midrand, Johannesburg) plates at 18˚C for 4-6 weeks. Fungal colonies for DNA extraction were scraped clean of excess agar, freeze-dried and crushed using the Retsch GmbH MM301 mixer mill (Haan, Germany). Total DNA was extracted from the crushed mycelium using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Csabrendek

For the collection made in Csabrendek, a more structured isolation strategy was adopted. Two needles were randomly selected from each of 10 trees (tree 1 to 10). From the first needle, single conidia were isolated from a single conidioma and plated onto MEA plates. From the second needle, single conidia were isolated from each of two different conidiomata. For tree 4 and tree 8, additional isolations were made from conidiomata on the needles (Table 2). Single conidia (approximately three to four from each conidioma) were grown at 18˚C until their colony sizes had reached 2 to 3 mm in diameter. The colonies were crushed manually and the DNA extracted using 100 µl PrepMan Ultra Sample Preparation reagent following the manufacturers protocol (Applied Biosystems, Foster City, USA).

In addition to isolating DNA from single conidial isolates, DNA was extracted directly from complete conidioma on the needles. In this case, two entire conidiomata were excised from different needles obtained from tree 4 and a further five from needles on tree 8 (Table 3). After removing excess plant material, the entire conidioma was added directly to 10 µl PrepMan Ultra reagent, mixed well in a vortex mixer and heated for 10 min at 100˚C. From

these crude and fast extractions, 0.5 µl of the supernatant was used in subsequent PCR reactions.

DNA analyses

Sequencing

For the Diszel material, the ITS region of 13 isolates that contained the unknown 109 bp allele and four that contained the 124 bp *D. septosporum* allele, as determined in a previous study (Barnes 2009), were sequenced to determine, or confirm, their identities. Four isolates obtained from the Csabrendek material and all five isolates from Russia were sequenced as well (Table 1).

The ITS1, 5.8S and ITS2 regions of the ribosomal RNA operon were amplified using the primers ITS1 and ITS4 (White *et al.* 1990) as described in Barnes *et al.* 2004. These products were purified through G-50 sephadex (SIGMA-Aldrich, Steinheim, Germany) in Centri-sep Spin Columns (Princeton separations Inc., Adelphia, U.S.A). Sequencing reactions were prepared using Big Dye v 3.1 (Applied Biosystems) and run on an ABI PRISM™ 3100 capillary autosequencer (Applied Biosystems). Sequences were analysed using Vector NTI 10.3.0 Advance software (Invitrogen, California, U.S.A.) and aligned online, using MAFFT version 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). Default settings were used with the strategy set at L-INS-I (Katoh *et al.* 2005). Parsimony analyses using the heuristic search option with tree bisection-reconnection (TBR) and 100 random stepwise-additions were selected in PAUP* Version 4.0b10 (Phylogenetic Analysis Using Parsimony) (Swofford 2002). Bootstrap confidence limits for the branching nodes were determined using 1000 replicates. *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers and A.J.L. Phillips was used as the outgroup in the phylogenetic analyses.

Primer Doth_A

For all DNA samples, the microsatellite locus DMS_A (GenBank accession no; EF591826) was amplified using the forward primer Doth_A(F)-(FAM): 5'CGG CAT CAC TGT TCA CCA CGC3' and reverse primer Doth_A(R): 5'GAA GCC GCA AGT GCC AAT GGC3' as described by Barnes *et al.* (2008b). The annealing temperature was set at 58˚C. Fluorescent PCR products were run on an ABI PRISM™ 3100 capillary autosequencer (Applied Biosystems) and allele sizes were determined using GENEMAPPER™ software version 3.0 (Applied Biosystems).

RFLP

The species-specific RFLP patterns for DNB (Barnes *et al.* 2004) were generated by restricting the purified ITS amplicons with the restriction enzyme *Alu*I following the protocol recommended by the supplier (Fermentas Life Science, MD, U.S.A.). The resultant DNA fragments were subjected to 3% agarose gel electrophoresis. *Dothistroma pini* was considered present if samples produced two fragments of approximately 170 bp and 350 bp in size. Amplicons that were not digested by the enzyme represented *D. septosporum*. This ITS-RFLP species identification method was utilised on all the single conidial isolates obtained from Csabrendek. It was not utilised for isolations from whole conidiomata due to the possible amplification of other contaminating plant and/or fungal material.

Mating type

The DNB species-specific mating type primers (Groenewald *et al.* 2007) were utilised to confirm the identity of the DNB isolates as determined using the ITS-RFLP patterns. They were also used to determine the mating type of the isolates. 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*. PCR reactions and conditions were the same as those described by Groenewald *et al.* (2007).

RESULTS

Isolations and DNA analyses

Diszel and Russia

Four isolates with the 124 bp allele and ITS sequences identical to those of *D. septosporum* from Europe emerged from the Diszel collections (Figure 2). All 13 of the isolates that produced the 109 bp allele grouped within the *D. pini* clade and produced ITS sequences identical to those found in the U.S.A. on *P. nigra* (Figure 2). Similarly, the isolates from *P. mugo*, *P. nigra* and *P. pallasiana* from Russia had identical ITS sequences to those from Ukraine on *P. pallasiana* and were identified as *D. pini* (Figure 2).

Csabrendek

A total of 32 isolates were obtained from the 10 trees sampled at Csabrendek (Table 2). In three cases, single conidia isolated from a single conidioma produced colonies on the same MEA plate that differed significantly in colour. These isolates were thus treated separately

(N1.1 and N1.2 from tree 4; N2A.1 and N2A.2 from tree 8; N3B.1, N3B.2 and N3B.3 from tree 8; Table 2; Figure 3). Twenty-five isolates contained the 124 bp allele for primer pair Doth_A and all the ITS-RFLP products were of the same size as the undigested controls. This confirmed that these isolates were all *D. septosporum* (Table 2). The seven remaining isolates from tree 4 and tree 8 each produced a 109 bp allele at the locus Doth_A. In addition, the ITS amplicons from these isolates were digested by the restriction enzyme *Alu*I providing further confidence that these isolates represent *D. pini* (Table 2).

Sequences of the ITS region confirmed the results obtained with the diagnostic markers (Doth_A and ITS-RFLP). Isolates N1.2 from tree 4 (CMW 29374) and N2B from tree 8 (CMW 29371) grouped within the *D. pini* clade from U.S.A. and isolates N1 from tree 5 (CMW 29372) and N2A from tree 7 (CMW 29373) (Table 2) grouped within the *D. septosporum* clade (Figure 2).

For the whole conidiomata studies (Table 3), application of the mating type primers showed that six of the seven examined conidiomata produced amplicons of 480 bp with the *D. pini* specific primers. This indicated that *D. pini* was present and that they were all MAT2. This result was confirmed with the Doth_A primer, where the same isolates produced the 109 bp allele, indicating *D. pini*. The species-specific mating type primers also showed that *D. septosporum* was present in all seven conidiomata with amplicons of 480 bp representing MAT1 and 823 bp representing MAT2 being produced. The presence of *D. septosporum* was confirmed in these samples using the microsatellite marker Doth_A that amplified the 124 bp allele. Both mating type genes of *D. septosporum* were found in a single conidioma from tree 8 (conidioma 2, Table 3).

Mating type

All 20 isolates (Table 1, Figure 2) and the six DNA extracts from needles (Table 3) identified as *D. pini* in this study, from both localities in Hungary and Russia contained only the MAT2 mating type gene. Additionally, isolates from Russia (CMW 24852 and CMW 24853) and Ukraine (CMW 23767, CMW 23768 and CMW 23769), already identified as *D. pini* by Barnes *et al.* (2008b) were screened and were all of the MAT2 type.

DISCUSSION

This study represents the first report of the pine needle pathogen, *D. pini,* in Hungary and Central Europe, where the pathogen was present at two collection sites. In addition, *D. septosporum* was also found at the same locations, on the same trees, on the same needles and in some cases, in the same conidiomata. Amongst isolates from Russia included for identification purposes, *D. pini* was found for the first time at two new locations, confirming the previous report of this fungus from that country (Barnes *et al.* 2008a). The Russian isolate from *P. mugo* represents a new host record for *D. pini* and this pathogen is also reported for the first time from *P. nigra* in Central Europe and Russia.

Results of this study emphasise the utility of various newly developed DNA-based tools (Barnes *et al.* 2004, Groenewald *et al.* 2007, Barnes *et al.* 2008b) to discriminate between the two morphologically and ecologically similar DNB pathogens. They also show that it is possible to screen for these pathogens directly from conidiomata without first sub-culturing, using the species-specific mating type primers and providing a confirmation of the result with the microsatellite primer set Doth_A. While this provides a useful approach for diagnosis, utilisation of the Doth_A microsatellite primers alone is not recommended. While this marker can distinguish between the DNB pathogens based on allele sizes, it is not species-specific and has been shown to amplify DNA in other closely related species (Barnes *et al.* 2008b).

The DNB pathogens enter pine needles via the stomata (Gadgil 1967, Peterson & Walla 1978). The fact that both *D. pini* and *D. septosporum* were detected within single conidiomata indicates that several individuals of a single species can enter the stomata simultaneously and co-infect or co-inhabit the same area of tissue. The formation and co-habitation of single conidiomata by these two species was confirmed by the fact that both mating types of the heterothallic *D. septosporum* were found in a single conidioma. The occurrence of both mating types of *D. septosporum* in Hungary supports the results of Groenewald *et al.* (2007), who also found both mating types in *D. septosporum* isolates from Austria and Poland. The close proximity of isolates with the opposite mating type would increase the chance of sexual reproduction. The teleomorph of *D. septosporum*, *M. pini*, was, however, not observed in this study.

It is unlikely that sexual reproduction is occurring in *D. pini* in parts of Europe where the fungus has been detected. All the isolates of *D. pini* collected in Hungary, Russia and Ukraine

are of the same mating type (MAT2). The second mating type has thus-far not been found in Europe. This suggests that either a single introduction of this pathogen has occurred or that limited introductions of only MAT2 isolates have happened in various parts of Europe. The presence of *D. pini* on *P. nigra* in Hungary might suggest that the source area of introduction into Europe could be the U.S.A., where both mating types of the pathogen are present on *P. nigra* (Groenewald *et al.* 2007). However, if *D. pini* is native to the U.S.A., its native hosts remain unknown, as the fungus has thus-far only been found on *P. nigra,* which is not indigenous to this region.

The introduction of the DNB fungi into Hungary is thought to have originated in neighbouring Serbia (A. Koltay, *personal communication*), where it has been known since 1955 (Krstić 1958). Marincović & Strajković (1969), and Karadzić (1988) noticed the disease appearing on trees growing in sandy areas in parts of Serbia located near the southern part of Hungary. In 1990, the pathogen (as *D. septosporum*) was identified in an 11-year-old *P. nigra* stand near the town of Veszprém (I. Szabó, *personal communication*) and a few years later, during 1994-1995, large outbreaks of the disease began to occur throughout the country (Szabó 1997). Today, *P. nigra,* mainly as the subspecies or variety *austriaca* Aschers. & Graebn (Austrian pine), occurs widely in Hungary and occupies an area of 69,000 hectares, corresponding to 4.1% of the forested area (ÁESZ 2002). DNB is present in most of these stands (Koltay 2007). From Hungary, the disease spread northwards into Slovakia and was first reported in this country in 1996 (Zúbrik *et al.* 2006). Today, the disease is present in all regions of Slovakia. Because there are few, strict geographic barriers in Europe, pathogen spread between countries is typically difficult to control. The discovery of *D. pini* at two locations within Hungary in this study, suggests that it might also be present in other parts of Europe. It is thus highly likely that if *D. pini* is not already present in countries neighbouring Hungary, its spread to those areas is inevitable. The introduction of *D. pini* into Hungary could have originated in neighbouring Ukraine and it will presumably show a pattern of spread in Europe similar to that of *D. septosporum* in the past.

In addition to providing new geographic and host reports of *D. pini* in Europe, we have shown that both DNB pathogens can occur in the same area. During the last two decades DNB has become a serious emerging forest disease and it is presently of concern to the custodians of pine plantations and pine forests globally. Particularly within Europe, the disease appears to be spreading rapidly and increasingly occurring at epidemic levels with the newest reports of *D. septosporum* coming from Estonia on *P. nigra* (Hanso & Drenkhan 2008), Finland on *P.*

taeda L., *P. sylvestris* L. and *Picea abies* (L.) Karst., as well as from Sweden (J. Huntula, *personal communication*).

Surveying and monitoring of DNB in Europe is likely to become increasingly important in the future. In this regard, accurate identification of the two pathogens causing this disease will be essential. Identifications in the past have mainly been based on morphology and many reports are considered to be of dubious value. The molecular markers applied in this study and shown to be sufficiently robust to identify and discriminate between the two DNB pathogens, should thus be useful tools for future scientific studies and disease surveys.

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Table 1. Description of the collection areas where isolates were obtained and the isolates used for sequencing.

Country	Location	Host	Host age	Fungal species	Isolate n
Hungary	Csabrendek (Bakonyerdő, Forestry and Timber Industry Co. unit Devecser)	*Pinus nigra*	18 years	*D. pini*	CMW 29
		P. nigra	18 years	*D. septosporum*	CMW 29
Hungary	Near Diszel: trees alongside the road	*P. nigra*	12-15 years	*D. pini*	CMW 26 CMW 26 CMW 26 CMW 26
		P. nigra	12-15 years	*D. septosporum*	CMW 26
Russia	Tarasovsky district, Gorodishchensky timber enterprise, Gorodishchenskoye forestry	*P. pallasiana*	10 years	*D. pini*	CMW 29
Russia	Krasnosulinsky district, Donskoye forestry	*P. nigra*	30 years	*D. pini*	CMW 29
		P. pallasiana	25 years	*D. pini*	CMW 29
		P. mugo	7 years	*D. pini*	CMW 29

[1]CMW numbers refers to the isolates which were sequenced in this study and which are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, 0002.

Table 2. Characterisation and mating type of Dothistroma isolates obtained from the needle collection at Csabrendek based on ITS-RFLP banding patterns, the allele size (in base pairs) obtained using the microsatellite marker Doth_A and species-specific mating type primers.

Tree	Isolate[1]	ITS-RFLP	Doth_A allele	Species	Mating type
Tree 1	N1	-[3]	-	-	
	N2A	1 band	124	*D. septosporum*	
	N2B	1 band	124	*D. septosporum*	
Tree 2	N1	1 band	124	*D. septosporum*	
	N2A	-	-	-	
	N2B	-	-	-	
Tree 3	N1	1 band	124	*D. septosporum*	
	N2A	1 band	124	*D. septosporum*	
	N2B	1 band	124	*D. septosporum*	
Tree 4	**N1.1*[2,5]**	**1 band**	**124**	***D. septosporum***	
	N1.2 (CMW 29374)[4]	**2 bands**	**109**	***D. pini***	**MAT-2**
	N2A	**-**	**-**	**-**	
	N2B	**1 band**	**124**	***D. septosporum***	
	N3A	**1 band**	**124**	***D. septosporum***	**MAT 1**
	N3B	**1 band**	**124**	***D. septosporum***	**MAT 2**
	N4A	**2 bands**	**109**	***D. pini***	**MAT 2**
	N4B	**1 band**	**124**	***D. septosporum***	**MAT 2**
Tree 5	N1 (CMW 29372)*	1 band	124	*D. septosporum*	
	N2A	1 band	124	*D. septosporum*	
	N2B	1 band	124	*D. septosporum*	
Tree 6	N1	1 band	124	*D. septosporum*	
	N2A	-	-	-	
	N2B	1 band	124	*D. septosporum*	
Tree 7	N1	1 band	124	*D. septosporum*	
	N2A (CMW 29373)*	1 band	124	*D. septosporum*	
	N2B	1 band	124	*D. septosporum*	
Tree 8	**N1**	**2 bands**	**109**	***D. pini***	**MAT-2**
	N2A.1*	**2 bands**	**109**	***D. pini***	**MAT-2**
	N2A.2*	**1 band**	**124**	***D. septosporum***	
	N2B (CMW 29371)*	**2 bands**	**109**	***D. pini***	**MAT-2**
	N3A	**2 bands**	**109**	***D. pini***	**MAT 2**
	N3B.1*	**1 band**	**124**	***D. septosporum***	**MAT 1**
	N3B.2*	**2 bands**	**109**	***D. pini***	**MAT 2**
	N3B.3*	**1 band**	**124**	***D. septosporum***	**MAT 1**
Tree 9	N1.1	1 band	124	*D. septosporum*	
	N2A	-	-	-	
	N2B	-	-	-	
Tree 10	N1	1 band	124	*D. septosporum*	
	N2A	1 band	124	*D. septosporum*	
	N2B	-	-	-	

[1] From each of 10 trees, single conidia were isolated from a conidioma from one needle ("N1") and from two conidiomata from a second needle ("N2A" and "N2B"). Exceptions are tree 4 and tree 8 where isolations were made from additional needles.

[2] Single conidia originating from the same conidioma that produced different coloured colonies (marked with '*' in the table), were treated as separate isolates (tree 4, N1.1 and N1.2, tree 8, N2A.1 and N2A.2 and tree 8, N3B.1, N3B.2 and N3B.3 – see Figure 3).

[3] "-" Isolations were not successful either due to contamination or non-viable conidia.

[4] Two isolates of each *D. pini* and *D. septosporum* (those with CMW numbers, see Figure 2) were sequenced to validate the robustness of the diagnostic markers.

[5] The trees that contain both DNB species are indicated in bold.

Table 3. The identity and the mating type of the DNB fungi present within conidiomata taken directly from the needles of tree 4 and tree 8 from Csabrendek (see Table 2) as determined using the Dothistroma species-specific mating type primers and the microsatellite marker Doth_A.

Tree	Conidioma	Species-specific mating type primers		Doth_A allele[#]	
		D. pini	*D. septosporum*	Allele 1	Allele 2
Tree 4	1	-	MAT2	-	124
	2	MAT2	MAT1 (faint band)	109	-
Tree 8	1	MAT2	MAT1	109	124
	2	MAT2	**MAT1 and MAT2***	109	124
	3	MAT2	-	109	124
	4	MAT2	MAT2 (faint band)	109	-
	5	MAT2	MAT1	109	124

[*]The text in bold indicates the presence of both mating types of *D. septosporum* within one single conidioma (tree 8, conidiomata 2).

[#]Allele 109 confirms the presence of *D. pini* and allele 124 that of *D. septosporum*.

Figure 1. Dothistroma needle blight on *Pinus nigra* in Hungary: a) a severely infected tree at the Csabrendek collection site (Bakonyerdő, Forestry and Timber Industry Co.); b) 18 year-old, infected trees in the same compartment; c) a small, young tree showing mortality; d) infected two-year-old needles will soon be cast and provide the source of inoculum for infection of the one-year-old needles; e) typical red band symptoms with black asexual fructifications (conidiomata) of the DNB fungi; f) 12-15 year-old trees at the collection site near the village of Diszel.


a


d


e


b


c


f

Figure 2. Phylogram of the Dothistroma needle blight isolates from Hungary and Russia based on maximum parsimony analyses of the ITS sequences. Of 475 total characters, 312 were constant, 101 variable characters were parsimony-uninformative and 62 were parsimony-informative. Isolates from Hungary, from both collection areas (Diszel and Csabrendek), contain isolates that group into the *D. septosporum* and *D. pini* (in bold) clades. All the Russian isolates are *D. pini*. The isolates sequenced in this study had 100 % similarity to the representative isolates from GenBank for each clade. Isolates sequenced from Romania, Hungary and Russia with CMW numbers are maintained in the culture collection of FABI. Tree lengths (above) and bootstrap values (below) are indicated on the branches.

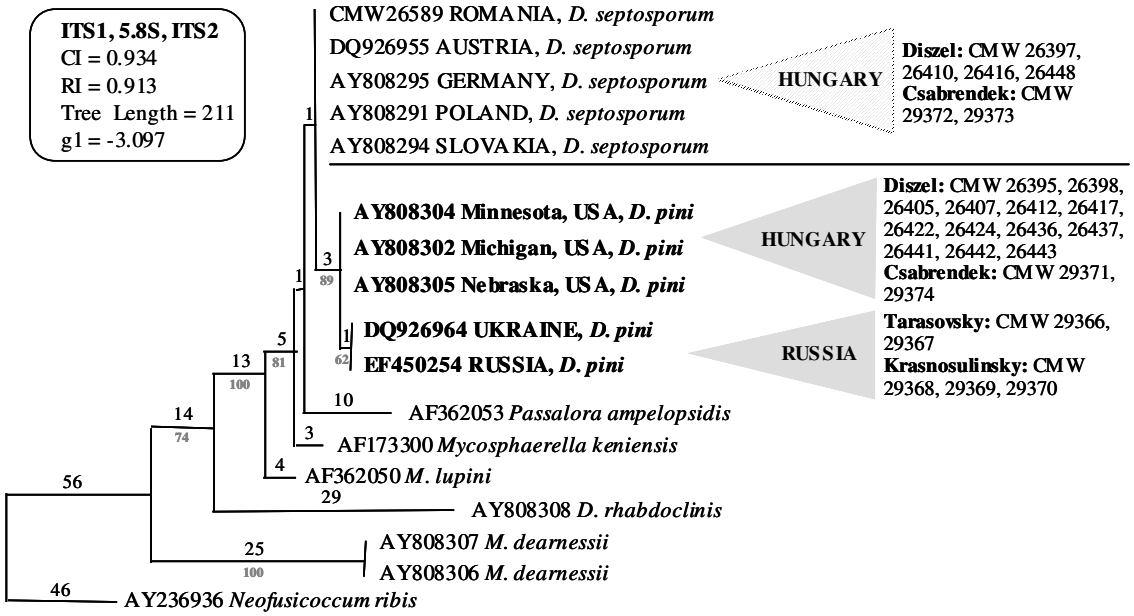
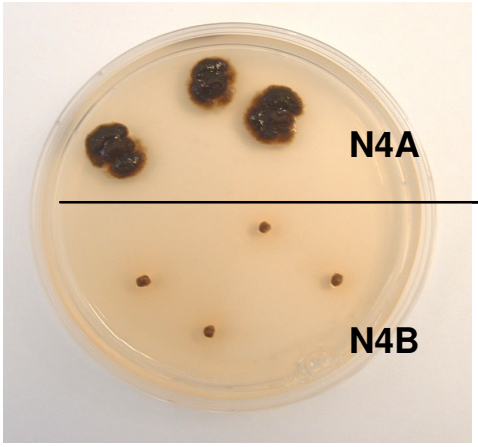

ITS1, 5.8S, ITS2
CI = 0.934
RI = 0.913
Tree Length = 211
g1 = -3.097
CMW26589 ROMANIA, D. septosporum
DQ926955 AUSTRIA, D. septosporum
AY808295 GERMANY, D. septosporum
AY808291 POLAND, D. septosporum
AY808294 SLOVAKIA, D. septosporum
HUNGARY
Diszel: CMW 26397, 26410, 26416, 26448
Csabrendek: CMW 29372, 29373
AY808304 Minnesota, USA, D. pini
AY808302 Michigan, USA, D. pini
AY808305 Nebraska, USA, D. pini
HUNGARY
Diszel: CMW 26395, 26398, 26405, 26407, 26412, 26417, 26422, 26424, 26436, 26437, 26441, 26442, 26443
Csabrendek: CMW 29371, 29374
DQ926964 UKRAINE, D. pini
EF450254 RUSSIA, D. pini
RUSSIA
Tarasovsky: CMW 29366, 29367
Krasnosulinsky: CMW 29368, 29369, 29370
AF362053 Passalora ampelopsidis
AF173300 Mycosphaerella keniensis
AF362050 M. lupini
AY808308 D. rhabdoclinis
AY808307 M. dearnessii
AY808306 M. dearnessii
AY236936 Neofusicoccum ribis
1
3
89
1
1
62
5
81
13
100
10
3
4
29
14
74
56
25
100
46

Figure 3. Different cultural characteristics observed when single-conidial isolates were made from different conidiomata on the same needle (tree 4, needle 4, conidiomata 4A and 4B) and from the same conidioma (tree 8, needle 3, conidioma 3). In the latter case, cultures that differed in morphology were treated as separate isolates in the study. See Table 2.

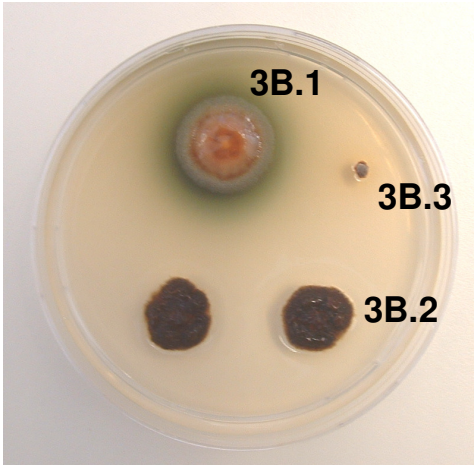
Tree 4, Needle 4



3 single-conidial isolates from the same conidioma 4A = *D. pini*

4 single-conidial isolates from the same conidioma 4B = *D. septosporum*

Tree 8, Needle 3



4 single-conidial isolates from conidioma 3B with different colony morphologies

3B.1 = *D. septosporum*

3B.2 = *D. pini*

3B.3 = *D. septosporum*

SUMMARY

Student: Irene Barnes

Thesis title: Taxonomy, phylogeny and population biology of the red band needle blight pathogens and related species

Supervisors: Professor Brenda Diana Wingfield
Professor Michael John Wingfield

Department: Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI)

Degree: Ph.D

Dothistroma needle blight is caused by the haploid ascomycete fungus *Dothistroma septosporum* and is one of the most serious diseases known on *Pinus* spp. The disease is characterised by red bands surrounding black, erumpent conidiomata on the needles and needle necrosis leading to successive defoliation that can seriously affect tree growth and in some cases, cause tree death. The ecology, biology, pathogenicity and physiology of the pathogen has been extensively studied in the past. However, there is a distinct lack of knowledge pertaining to the genetic aspects of *D. septosporum*. In this thesis, the taxonomy, phylogeny and population biology of the pathogen has been studied. Multigene phylogenies revealed that two fungal species, which are morphologically similar, are responsible for causing Dothistroma needle blight and the names *Dothistroma septosporum* and *D. pini* were made available for the two taxa. A complete taxonomic description of these species based on morphology and sequence data was also provided. In order to facilitate rapid and accurate identification of these two important species, various diagnostic procedures were developed. These methods make it possible to identify the pathogens directly from the symptoms on the needles without first having to isolate the fungus. Furthermore, a set of 12 polymorphic microsatellite markers were developed, using an enrichment technique, to study the population dynamics of *D. septosporum.* It is this species which has successfully invaded areas in both the Northern Hemisphere where pines are native, and the Southern Hemisphere where pines have been introduced. This is also the species which is responsible for the current global disease epidemics occurring primarily on monoculture plantations of susceptible hosts. Population genetic studies revealed that the populations in the Northern Hemisphere are generally in a state of panmixia with high levels of genetic diversity, geneflow and regular sexual reproduction, while at the same time expanding their geographical range. The patterns

of genetic diversity reflected the human mediated movement of pine, and the pathogen, into the Southern Hemisphere. Evidence was found for repeated introduction events in Africa while a few virulent, clonal individuals, perpetuated by asexual reproduction, are maintained in South America and Australasia. These clonal isolates cause severe epidemics, particularly in the extensive plantations of *P. radiata* in those countries. Research making up this thesis is presented in three manuscripts and has substantially increased the body of knowledge relating to the biology, distribution and occurrence, population genetics and evolutionary potential of the serious and important pathogens that cause DNB.