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New host and country records of the Dothistroma needle blight pathogens from Europe and Asia

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Summary

Dothistroma needle blight (DNB) is a serious disease of pines (*Pinus* spp.), with a worldwide distribution. It is caused by the ascomycete fungi *Dothistroma septosporum* (teleomorph: *Mycosphaerella pini*) and *Dothistroma pini* (teleomorph unknown). Recently, DNB was found on *Pinus peuce* in Austria, *Pinus pallasiana* in Ukraine and the European part of south-western Russia, as well as on *Pinus radiata* and *Pinus wallichiana* in Bhutan. Based on DNA sequence comparisons of the internal transcribed spacer and β-tubulin gene regions, isolates from Austria and Bhutan were identified as *D. septosporum*, while isolates from Ukraine and south-western Russia were identified as *D. pini*. Additional isolates studied from *Pinus mugo* in Hungary confirmed the presence of *D. septosporum* in this country. The record of *D. septosporum* on exotic *P. peuce* in Austria represents a new host report of this needle blight pathogen in Europe. Likewise, DNB and the associated pathogen, *D. septosporum* are reported from Bhutan, eastern Himalayas, for the first time. In addition, *D. pini* was found in two European countries and on a new host, *P. pallasiana*. These European records represent the only reports of *D. pini* from outside the north-central USA. Morphological examination of selected specimens from different hosts and countries showed that *D. septosporum* and *D. pini* overlap in the length of their conidia, while the width is slightly wider in *D. pini* than in *D. septosporum*. The differences in conidial width are so small, however, that identification of the two *Dothistroma* species solely based on morphology is virtually impossible. The new host and country records provided here are consistent with the continuing trend of reports of the DNB pathogens from new hosts and new geographical areas during the last two decades, particularly in the northern hemisphere.

1 Introduction

Dothistroma needle blight (DNB), also known as red band needle blight, is a very serious needle disease of conifers. It primarily affects pine species (*Pinus* spp.), and only on occasion other conifers, with serious damage being restricted to pines. Needles of all ages are commonly affected by this disease, which can cause total defoliation and death of trees in severe cases (Gibson et al. 1964). Economic damage from DNB in forest plantations results mainly from severe growth losses (Gibson 1972; Gadgil 1984), while on shade and ornamental trees, a loss of the aesthetic value resulting from defoliation can be a problem.

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Until recently, the cause of DNB was attributed to the ascomycete pathogen, *Dothistroma septosporum* (Dorog.) Morelet (teleomorph: *Mycosphaerella pini* Rostr.), with the name *Dothistroma pini* Hulbary often used as a synonym. DNA sequence comparisons have, however, clearly shown that two phylogenetic species with very similar morphologies are responsible for DNB (Barnes et al. 2004). These two species have been designated *D. septosporum* (teleomorph: *M. pini*) and *D. pini* (teleomorph unknown), and it is now recognized that *D. pini* is not a synonym of *D. septosporum*. The two *Dothistroma* species show slight differences in conidial morphology and dimensions, however, they are best distinguished on the basis of DNA sequence comparisons of nuclear gene regions (Barnes et al. 2004). Although various hypotheses regarding the area of origin of the DNB fungi have been presented (Evans 1984; Ivory 1990), the original, natural range is still not precisely known.

To date, *D. pini* has been conclusively identified only from exotic *Pinus nigra* J. F. Arnold in certain areas of the north-central USA (Barnes et al. 2004). In contrast, *D. septosporum* is a widely distributed pathogen that has been spread to many parts of the world where pines are grown as non-natives in plantations. Initially, its notoriety was restricted to the epidemics it caused in plantations of exotic pine species, mainly *Pinus radiata* D. Don (Monterey pine), in the southern hemisphere (Ivory 1967; Gibson 1972). In the last two decades, however, there has been an increase in the incidence and severity of DNB in some parts of the northern hemisphere. Recent examples of serious epidemics of DNB include those on Corsican pine (*P. nigra* ssp. *laricio*) in the UK (Brown et al. 2003) and France (Aumonier 2002), lodgepole pine (*Pinus contorta* var. *latifolia* Dougl. ex Loud.) in British Columbia (Woods 2003; Woods et al. 2005), and Austrian pine (*P. nigra*) in the Czech Republic (Jankovský et al. 2004) and Hungary (Koltay 2001). In recent years, the disease has also been reported from a number of new hosts and from new geographical areas (Bradshaw 2004 and references therein; Bednářová et al. 2006). The concern in the northern hemisphere is that *D. septosporum* is not only causing economic losses on pines grown in intensively managed plantations, but also affects *Pinus* species in natural forests (Maschnig and Pehl 1994; Aumonier 2002; Brown et al. 2003; Woods 2003; Kehr et al. 2004; Woods et al. 2005; Kirisits and Cech 2006). With the increased awareness and importance of DNB, it has now been reported from over 70 different pine species and occasionally other conifers (Bednářová et al. 2006).

Between 2004 and 2006, DNB was found on several native and exotic pine species in various European countries and in Bhutan. The aim of this study was to identify conclusively the causal agents of the pine needle blight diseases on *Pinus peuce* Griseb. (Macedonian or Balkan pine) in Austria, on *Pinus mugo* Turra (Dwarf mountain pine) in Hungary, on *Pinus pallasiana* D. Don (Crimean pine) in Ukraine and the European part of south-western Russia, as well as on *P. radiata* and *Pinus wallichiana* A. B. Jacks. (blue pine or Himalayan blue pine) in Bhutan. *Dothistroma* isolates from these five hosts, collected in these countries were examined morphologically and compared using DNA sequence data.

2 Materials and methods

2.1 Collection sites, specimen collection and fungal isolations

Specimens for laboratory study were collected during tree disease inspections of natural forests, forest plantations and ornamental trees in various European countries and in Bhutan. Samples consisted of needles from living trees showing symptoms and signs resembling those of DNB, collected in paper bags. Needle samples were stored at −70°C for a variable amount of time (2 h to 9 months) until processing.

In Austria, collections of needles were made in April 2004 from one, approx. 80-year-old, and four, approx. 25- to 30-year-old, exotic *P. peuce* trees growing at the forest experimental garden and arboretum 'Knödelhütte' of the Institute of Silviculture, University of Natural Resources and Applied Life Sciences, Vienna (BOKU) (Mayer 1983). Needle collections from five, approx. 10- to 20-year-old, exotic, *P. mugo* trees in the botanical garden of the University of West Hungary in Sopron, were conducted in August 2005. In November 2004, needles were collected from one, approx. 25- to 30-year-old tree amongst many infected *P. pallasiana* trees in a forest plantation outside the natural range of Crimean pine in the Tsjurupinsk area, Kherson region, Ukraine. In south-western Russia, needles of exotic *P. pallasiana* were collected from forest plantations in two different areas within the Rostov region in 2006. The first collection, made in July, was from the Tarasovsky area, Gorodistchensky timber enterprise, Yefremovo-Stepanovskoye forestry, from an approx. 20-year-old tree. The second collection was made in October, from an approx. 28-year-old tree in the Kamensk area, Kamensk timber enterprise, Kamenskoye forestry, near Staraya Stanitsa village.

Collections in Bhutan were made during May and July 2005 at four different localities. One collection was obtained from western Bhutan, from an approx. 10-year-old *P. radiata* tree planted as an introduced ornamental at the Renewable Natural Resources Research Centre (RNR-RC), Yusipang (Thimphu dzongkhag). The other collections were made in central Bhutan, from native 5- to 20-year-old *P. wallichiana* trees growing in natural forests near Ura, near Tangsibi and near Lamey Goemba (all located in Bumthang dzongkhag).

Fungal cultures were obtained from infected pine needles using the method described by Barnes et al. (2004). At localities where material from only one tree was collected (i.e. *P. pallasiana* in Ukraine and *P. radiata* at Yusipang, Bhutan), several isolations from different needles were made (Table 1). All isolates obtained in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). Representative cultures and dried needles have also been deposited at the culture collection and herbarium of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and the herbarium of the V. N. Karasin National University (CWU), Kharkiv, Ukraine (Table 1).

Isolates of *D. septosporum* from Europe and of *D. pini* from the USA, previously examined by Barnes et al. (2004), were included in the DNA sequence comparisons (Table 2). Likewise, one isolate of *Dothistroma rhabdoclinis* Butin, two isolates of *Mycosphaerella dearnessii* M. E. Barr and one isolate of *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers and A.J.L. Phillips were included in the DNA sequence studies (Table 2).

2.2 DNA extraction, sequencing and phylogenetic analyses

For unambiguous identification of *Dothistroma* isolates, DNA sequence analyses were conducted. Mycelium from 2- to 3-month-old cultures of *Dothistroma* spp. from Austria, Hungary, Ukraine, south-western Russia and Bhutan was cut out from the malt extract agar medium (20 g/l malt extract, 10 g/l agar; Biolab, Midrand, South Africa) and placed into 1.5 ml Eppendorf tubes. The tubes were then freeze dried and the mycelium was further crushed into a fine powder using the Retsch GmbH MM301 mixer mill (Haan, Germany). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) regions of the rDNA (using primers ITS1 and ITS4) and a portion of the β-tubulin gene region (using the primer pair Bt2a and Bt2b) were amplified and sequenced following the method described by Barnes et al. (2004).

Sequences obtained in this study were aligned online using mafft version 5.8 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh et al. 2005) with the L-INS-I

Table 1. **Collection isolation information as well as Genbank accession numbers of *Dothistroma* isolates examined in this study**

Locality	*Dothistroma* spp.	Host	Collectors	Culture no.	Additional culture and herbarium no.	Date collected	GenBank no. ITS	GenBank no. BT2	Isolation information
Austria									
Forest experimental garden, 'Knödelhütte', Institute of Silviculture, BOKU, Vienna	*D. septosporum*	*Pinus peuce*	T. Kirisits, I. Barnes	CMW 23765	CBS 121010	April 2004	DQ926951	DQ926925	1
	D. septosporum	*P. peuce*	T. Kirisits, I. Barnes	CMW 23434		April 2004	DQ926952	DQ926926	1
	D. septosporum	*P. peuce*	T. Kirisits, I. Barnes	CMW 23766		April 2004	DQ926953	DQ926927	1
	D. septosporum	*P. peuce*	T. Kirisits, I. Barnes	CMW 23433		April 2004	DQ926954	DQ926928	1
Hungary									
Botanical garden of the University of West Hungary, Sopron	*D. septosporum*	*P. mugo*	T. Kirisits, I. Barnes	CMW 23903	CBS 121009	August 2005	DQ926956	DQ926931	1
	D. septosporum	*P. mugo*	T. Kirisits, I. Barnes	CMW 23435		August 2005	DQ926957	DQ926932	1
	D. septosporum	*P. mugo*	T. Kirisits, I. Barnes	CMW 23906		August 2005	DQ926960	DQ926935	1
	D. septosporum	*P. mugo*	T. Kirisits, I. Barnes	CMW 23904		August 2005	DQ926958	DQ926933	2
	D. septosporum	*P. mugo*	T. Kirisits, I. Barnes	CMW 23905		August 2005	DQ926959	DQ926934	2
Ukraine									
Tsjurupinsk area, Kherson region	*D. pini*	*P. pallasiana*	A. C. Usichenko	CMW 23767	CBS 121011, EX CWU (Myc) AS 1109	November 2004	DQ926964	DQ926939	2
	D. pini	*P. pallasiana*	A. C. Usichenko	CMW 23768		November 2004	DQ926965	DQ926940	2
	D. pini	*P. pallasiana*	A. C. Usichenko	CMW 23769		November 2004	DQ926966	DQ926941	2
Russia									
Kamensky area, Rostov region	*D. pini*	*P. pallasiana*	T. S. Bulgakov	CMW 24852	CBS 121005, EX CWU (Myc) AS 2086	October 2006	EF450254	EF450256	
Tarasovsky area, Rostov region	*D. pini*	*P. pallasiana*	T. S. Bulgakov	CMW 24853	EX CWU (Myc) AS 2088	July 2006	EF450255	EF450257	
Bhutan									
Yusipang, Thimphu dzongkhag	*D. septosporum*	*P. radiata*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23429	CBS 121006	July 2005	DQ926961	DQ926936	2
	D. septosporum	*P. radiata*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23430		July 2005	DQ926962	DQ926937	2
	D. septosporum	*P. radiata*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23431		July 2005	DQ926963	DQ926938	2

Table 1. **Continued**

Locality	*Dothistroma* spp.	Host	Collectors	Culture no.	Additional culture and herbarium no.	Date collected	GenBank no. ITS	GenBank no. BT2	Isolation information
Ura, Bumthang dzongkhag	*D. septosporum*	*P. wallichiana*	H. Konrad, D. B. Chhetri, I. Barnes	CMW 23432	CBS 119535	May 2005	DQ926950	DQ926924	
Tangsibi, Bumthang dzongkhag	*D. septosporum*	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23895		July 2005	DQ926944	DQ926918	1
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23896		July 2005	DQ926945	DQ926919	1
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23897		July 2005	DQ926946	DQ926920	1
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23898	CBS 121007	July 2005	DQ926947	DQ926921	1
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23899		July 2005	DQ926948	DQ926922	1
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23900		July 2005	DQ926949	DQ926923	1
Lamey Goemba, Bumthang dzongkhag	*D. septosporum*	*P. wallichiana*	T. Kirisits, N. Gyeltshen, I. Barnes	CMW 23901	CBS 121008	July 2005	DQ926942	DQ926916	2
	D. septosporum	*P. wallichiana*	T. Kirisits, N. Gyeltshen, I. Barnes	CMW 23902		July 2005	DQ926943	DQ926917	2

CMW, culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CWU, herbarium of the V. N. Karasin National University (CWU), Kharkiv, Ukraine.

[1]Isolates made from different trees.

[2]Same tree, different needles.

Table 2. **Additional isolates used in the phylogenetic analyses for which sequences were obtained from GenBank**

Fungus	Country	Host	Cultures[1]	Other Culture no.	GenBank accession numbers ITS	GenBank accession numbers β-tubulin 2
D. septosporum	Austria	*P. nigra*	CMW 23427	–	DQ926955	DQ926929
D. septosporum	Austria	*P. nigra*	CMW 23428	–	EF059972	DQ926930
D. septosporum	France	*P. coulteri*	CMW 9992	CBS 383.74	AY808290	AY808220
D. septosporum	Germany	*P. mugo*	CMW 13122	ATCC MYA604	AY808295	AY808225
D. septosporum	Poland	*P. nigra*	CMW 13004	–	AY808291	AY808221
D. septosporum	Slovakia	*P. sylvestris*	CMW 13123	ATCC MYA603	AY808294	AY808224
D. pini	USA	*P. nigra*	CMW 10951	–	AY808302	AY808232
D. pini	USA	*P. nigra*	CMW 14820	ATCC MYA-609	AY808304	AY808234
D. pini	USA	*P. nigra*	CMW 14821	ATCC MYA-606	AY808305	AY808235
D. rhabdoclinis	Germany	*Pseudotsuga menziessii*	CMW 12519	CBS 102195	AY808308	AY808239
Mycosphaerella dearnessii	China	*P. elliottii*	CMW 13119	ATCC 200602	AY808307	AY808238
M. dearnessii	France	*P. radiata*	CMW 9985	CBS 871.95	AY808306	AY808237
Neofusicoccum ribis	USA	*Ribes* sp.	CMW 7773	–	AY236936	AY236907

CMW, culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; ATCC, American Type Culture Collection, Virginia, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

strategy and the Gap opening penalty set at 1.53. Additional sequences obtained from GenBank (Table 2) were included for comparison in the phylogenetic analyses. Parsimony analyses on the alignments were performed using PAUP* version 4.0b10 (phylogenetic analysis using parsimony) (Swofford 2002). The heuristic search option with 100 random stepwise additions and tree bisection reconnection (TBR) were selected. Gaps were treated as an additional state and the tree score frequency distribution was calculated using a histogram with 20 classes to evaluate random trees. *Neofusicoccum ribis* (Table 2) was used as the paraphyletic outgroup. Bootstrap confidence levels of the branching points were determined using 1000 replicates.

2.3 Morphology

For morphological characterization, asexual fruiting structures (conidiomata) observed on the surface of needles from the respective host trees and countries were removed and mounted on glass slides containing lactic acid with cotton blue. The slides were examined with a Carl Zeiss (Carl Zeiss Ltd., Mannheim, Germany) light microscope using differential interference contrast. The lengths and widths of between 27 and 94 conidia, obtained from either one or two conidiomata from a single needle, were measured electronically using a Zeiss Axio Vision (Carl Zeiss) camera system. Measurements were made for each of the collections from *P. peuce* in Austria, *P. mugo* in Hungary, *P. pallasiana* in Ukraine and south-western Russia as well as *P. radiata* and *P. wallichiana* in Bhutan. Where possible, conidia obtained from corresponding sporulating cultures were also measured, with sample sizes ranging from 45 to 56 conidia per culture. Conidial dimensions were subjected to statistical analyses using the program SPSS for Windows, version 12.0.1 (SPSS Inc., Chicago, IL, USA). Means of conidial length and width from needles and cultures were compared separately for each *Dothistroma* collection from the respective countries and hosts using independent-samples t-tests. Differences in the conidial dimensions between *Dothistroma* collections from different countries and hosts were tested by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test. Separate analyses were performed for measurements obtained from needles and from cultures. Differences in conidial length and width between *D. septosporum* and *D. pini* were analysed by independent-sample t-tests, using the pooled values from all needle collections and isolates measured, respectively. Comparisons between *D. septosporum* and *D. pini* were also performed separately for conidial measurements obtained from needles and from cultures.

3 Results

3.1 Symptoms and signs of needle blight

Incidence of DNB on all five host trees in the respective countries was high. Disease severity varied greatly and was not precisely evaluated. However, at least some individuals of each pine species in the various countries were severely infected. Typical symptoms and signs resembling those of DNB (Pehl and Wulf 2001; Brown et al. 2003; Barnes et al. 2004; Bradshaw 2004 and references therein; Kirisits and Cech 2006) were present on all needle collections from Austria, Hungary, Ukraine, south-western Russia and Bhutan. However, symptoms and signs varied slightly on the different hosts. Unlike *P. mugo* and *P. radiata* where bright, brick-red bands occurred on infected needles, bands on needles of *P. peuce* were brownish and lacked the reddish colour that is in many situations typical for the disease. Necrotic bands on needles, necrotic needle tips or entirely necrotic needles of *P. peuce* had a rich, dark brown colour and hence appear to mask the red pigments produced by the fungal toxin, dothistromin. The black and erumpent conidiomata were therefore observed in dark brown bands or on necrotic parts of the needles devoid of bands.

Some of the *P. pallasiana* needles collected in Ukraine and south-western Russia were brown and entirely necrotic, while others were green with only the distal parts of the needle being necrotic. Conidiomata were abundant and were distributed along the whole length of necrotic parts of the needles. They also occurred singly or in groups within distinct brick-red bands. Oozing masses of white conidia could be seen discharging from almost all of the conidiomata from the needles collected from Ukraine.

Symptoms and signs of DNB on *P. wallichiana* were relatively indistinctive, which was partly because of the presence of other needle pathogens, including *Lophodermium* spp. and a *Rhizosphaera* species. Necrotic needles of *P. wallichiana* were dark brown and conidiomata of *Dothistroma* were small (<300 μm) and difficult to observe with the unaided eye. Conidiomata were occasionally located in dark brown bands, but mostly in necrotic needle parts devoid of bands. Conidiomata occurred in sparsely distributed clusters, on distal parts, particularly at the tips of needles.

3.2 Fungal isolations

Using the method described by Barnes et al. (2004), isolations were made from the needle collections and they resulted in *Dothistroma* isolates for further morphological and DNA sequence comparisons (Table 1). Isolates either originated from different trees or from different needles from the same tree (Table 1). In total, four isolates were obtained from *P. peuce* in Austria, five from *P. mugo* in Hungary, three from *P. pallasiana* in Ukraine, two from *P. pallasiana* in south-western Russia and three and nine from *P. radiata* and *P. wallichiana*, respectively, in Bhutan (Table 1). *Dothistroma* isolates were obtained from all four localities in Bhutan (Table 1). The majority of the isolates grew readily within 2–3 days and reached colony diameters of approximately 7 mm after 3 weeks of incubation at 18°C. Isolates from *P. wallichiana* were the exception, however, as conidia only started germinating 1–2 weeks after being plated out and a further 2 weeks of growth was required, before colonies could be detected with the unaided eye.

3.3 DNA extraction, sequencing and phylogenetic analyses

The DNA product extracted using the DNeasy Plant Mini Kit was of high quality and void of PCR inhibiting 'colourants' usually present when using a basic phenol/chloroform extraction protocol for *Dothistroma* spp. Subsequent PCR amplifications were thus effective and produced bands of approximately 500 bp using the ITS and 450 bp using the β-tubulin primers.

After alignments using MAFFT, 479 characters were obtained for the ITS sequence dataset, of which 117 were parsimony uninformative and 75 parsimony informative. For the β-tubulin dataset, 418 total characters were obtained, of which 31 were parsimony uninformative and 212 parsimony informative. Datasets were individually analysed and are presented in Fig. 1.

All isolates from *P. peuce* in Austria, from *P. mugo* in Hungary and from *P. radiata* and *P. wallichiana* in Bhutan were identified as *D. septosporum* based on both their ITS and β-tubulin sequences (Fig. 1). No variation between the isolates was observed in the ITS sequences. However, in the β-tubulin sequences, single base pair differences were found (Fig. 1), which induced slight changes in the topology of the four most parsimonious trees obtained. Isolates CMW 23901 and CMW 23902 from Lamey Goemba (Bhutan) and isolates CMW 23895, CMW 23898 and CMW 23899 from Tangsibi (Bhutan) shared a common substitution from a G to an A, at position 84 in the aligned sequences. An isolate from Germany, CMW 13122, contained a single transversion of a C to a G at position 34. Isolate CMW 23428 from *P. nigra* in Austria, contained a substitution from a T to a C at position 337 and an insertion of a G at position 236 not observed in any of the other isolates from Europe, but found in strains from the southern hemisphere including New Zealand, South Africa, Chile and Australia (BARNES et al. 2004). No sequence differences were detected between isolates collected from the same tree.

The isolates from *P. pallasiana* in Ukraine and south-western Russia were identified as *D. pini* based on both the ITS and the β-tubulin sequences (Fig. 1). Slight variation between these isolates and other *D. pini* isolates from the USA were observed in the ITS sequence data (Fig. 1). This was because of a base substitution in the European isolates at position 166 of the aligned sequences from an A to a G. No variation within the β-tubulin sequences was observed for *D. pini* (Fig. 1).

3.4 Morphology

Examination of conidiomata obtained from needles and cultures from all the hosts examined showed the presence of conidia that were morphologically similar to those of *Dothistroma* spp. Conidia were elongated, straight to slightly curved, hyaline and possessed between one and five septa. No teleomorph structures (pseudothecia) were seen on any of the needle collections.

Considerable differences in the lengths and widths of conidia measured from conidiomata on needles and of spores obtained in culture were observed (Table 3). In most cases, the differences between conidial dimensions on needles and in culture from the same collection were statistically significant (Table 3). There was no consistent trend, however, as to whether conidia were longer or wider on needles or in culture in all the *Dothistroma* collections examined. With regard to length, conidia of the *D. septosporum*

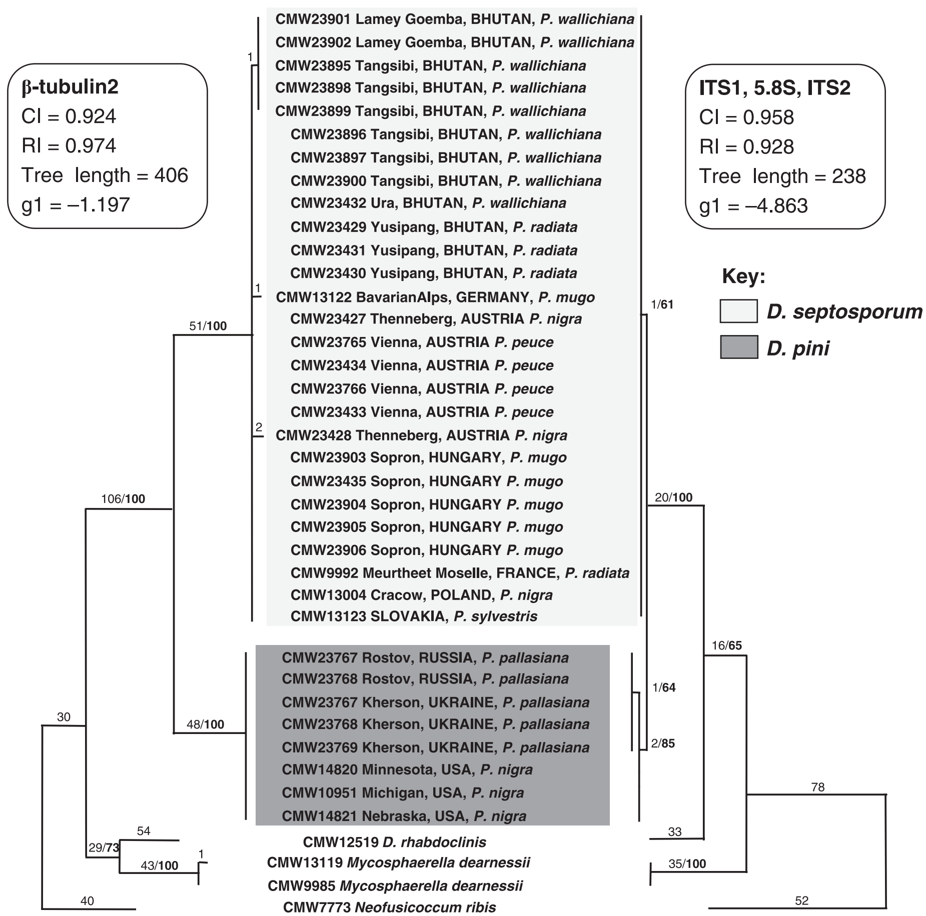

Fig. 1. Phylogenetic trees derived from maximum parsimony analysis of β-tubulin and rDNA internal transcribed spacer (ITS) sequence data. Isolates from Austria, Bhutan and Hungary all belong to *Dothistroma septosporum*, while isolates from Ukraine and south-western Russia are clearly *Dothistroma pini*. Slight variation within isolates are found within *D. septosporum* in the β-tubulin tree and within *D. pini* in the ITS tree. Tree length and bootstrap values (in bold) are indicated on the branches.

collections from *P. peuce* in Austria and from *P. wallichiana* in Bhutan were longer in culture than on needles, while the opposite was observed in the collections and corresponding isolates of *D. pini* from *P. pallasiana* in Ukraine and Russia (Table 3). With regard to the width, *D. septosporum* from *P. wallichiana* in Bhutan had wider conidia on needles than in culture, while no statistically significant differences were observed in the collection from *P. peuce* in Austria (Table 3). In all three *D. pini* collections and corresponding isolates from *P. pallasiana* in Ukraine and Russia, conidia measured from cultures were slightly wider than those measured from conidiomata on needles (Table 3).

There was substantial variation in the lengths and widths of conidia between *Dothistroma* species collected from different hosts in different countries, both for measurements taken from needles and from cultures (Table 3). For conidial length, there

Table 3. **Lengths and widths (μm) of conidia of *Dothistroma* needle collections and corresponding isolates from conidiomata obtained from needles and from cultures on 2% malt extract agar (MEA)**

					Conidial length (μm)				Conidial width (μm)			
Origin of sample	Host	*Dothistroma* spp.	Substrate	N[1]	Mean[2]	SD[3]	Range[4]	Sig.[5]	Mean[2]	SD[3]	Range[4]	Sig.[5]
Austria, Vienna	*Pinus peuce*	*D. septosporum*	Needle	93	21.4 a	3.2	14.2–31.5	***	3.0c	0.3	2.1–4.0	ns
			Culture (CMW 23766)	45	28.1 C	7.1	12.9–43.3		3.0 B	0.4	2.2–4.3	
Hungary, Sopron	*Pinus mugo*	*D. septosporum*	Needle	51	29.6 e	4.1	18.8–36.3	–	2.9 b	0.3	2.3–3.8	–
			Culture: no sporulation	–	–	–	–		–	–	–	
Bhutan, Yusipang	*Pinus radiata*	*D. septosporum*	Needle	69	23.3 b	4.4	13.9–31.2	–	2.3 a	0.3	1.8–3.1	–
			Culture: no sporulation	–	–	–	–		–	–	–	
Bhutan, Bumthang	*Pinus wallichiana*	*D. septosporum*	Needle	53	23.2 b	5.4	10.1–31.4	*	2.8 b	0.3	2.2–3.5	***
			Cultures (CMW 23432, 23898)	56	25.5 B	5.3	10.9–36.5		2.3 A	0.5	1.9–3.0	
Ukraine, Tsjurupinsk area	*Pinus pallasiana*	*D. pini*	Needle	55	27.9 d	4.7	15.8–38.4	**	3.1 c	0.3	2.3–4.0	***
			Culture (CMW 23769)	53	25.0 B	3.8	16.3–35.6		3.3 C	0.3	2.6–4.1	
Russia, Kamensk area	*Pinus pallasiana*	*D. pini*	Needle	51	26.6 cd	3.7	18.3–32.7	***	3.5 d	0.4	2.5–4.6	***
			Culture (24852)	51	20.6 A	3.1	15.3–27.8		3.9 D	0.4	3.0–4.6	
Russia, Tarasovsky area	*Pinus pallasiana*	*D. pini*	Needle	27	25.3 c	3.8	15.9–31.2	***	3.1 c	0.2	2.9–3.7	***
			Culture (24853)	55	22.1 A	3.1	17.0–30.5		4.0 D	0.4	3.0–4.7	

[1]Number of conidia measured.
[2]Values (within columns) from substrate 'needle' followed by different lower case letters and those from substrate 'culture' followed by different capital letters were significantly different ($p < 0.05$) according to one-way analysis of variance (ANOVA) followed by the LSD test.
[3]Standard deviation.
[4]Minimum–maximum.
[5]Mean values of condial length and width between substrates 'needle' and 'culture' of individual *Dothistroma* collections and isolates from different countries were compared using independent-samples t-tests (ns, not significant, *significant at $p < 0.05$, **significant at $p < 0.01$, ***significant at $p < 0.001$).

was no consistent trend whether conidia of *D. septosporum* or *D. pini* were longer. When comparing the measurements from needles, the *D. septosporum* collection from *P. peuce* in Austria had the smallest conidia (mean: 21.4 μm) of all *Dothistroma* specimens examined, followed by the *D. septosporum* collections from *P. wallichiana* and *P. radiata* in Bhutan and then the three *D. pini* collections from *P. pallasiana* in Russia and Ukraine. The *D. septosporum* collection from *P. mugo* in Hungary had significantly longer conidia (mean: 29.6 μm) than all the other specimens (Table 3). When considering conidial length in culture, ranking of isolates was different, compared with the values from needles. Here, isolates of *D. pini* from Russia had the smallest conidia (means: 20.6 and 22.1 μm), followed by the *D. pini* isolate from Ukraine and the *D. septosporum* isolate from *P. wallichiana* in Bhutan (Table 3). The *D. septosporum* isolate from *P. peuce* in Austria had significantly longer conidia (mean: 28.1 μm) than the four other isolates, although it had the smallest conidia, when conidia were measured from needles (Table 3).

For conidial width, differences between *D. septosporum* isolates and *D. pini* isolates were more consistent, although not always statistically significant. On needles, all three *D. pini* isolates had slightly wider conidia (range of means: 3.1–3.5 μm) than the three *D. septosporum* isolates (range of means: 2.3–3.0 μm). However, the means of conidial width between the *D. pini* isolates from *P. pallisiana* in Ukraine and in the Tarasovsky area in Russia were not statistically different to those of the *D. septosporum* isolate from *P. peuce* in Austria (Table 3). Moreover, in culture, all three *D. pini* isolates had wider conidia (range of means: 3.3–4.0 μm) than the two *D. septosporum* isolates measured (range of means: 2.3–3.0 μm), with statistically significant differences between these groups of isolates (Table 3).

Analyses of pooled data for *D. pini* and *D. septosporum*, from all collections and isolates, emphasized that there are no consistent differences in conidial length between the two *Dothistroma* species (Table 4). On needles, *D. pini* had significantly longer conidia than *D. septosporum*, while the opposite was observed for measurements in culture (Table 4). As indicated already in the comparisons of individual collections and isolates (Table 3), *D. pini* had significantly wider conidia than *D. septosporum*, in both measurements from needles and cultures (Table 4).

Table 4. **Lengths and widths (μm) of conidia of *Dothistroma septosporum* and *Dothistroma pini* from conidiomata obtained from needles and from cultures on 2% MEA**

Substrate	Species of *Dothistroma*	No. of collections or isolates[1]	N[2]	Conidial length (μm) Mean	SD[3]	Range[4]	Sig.[5]	Conidial width (μm) Mean	SD[3]	Range[4]	Sig.[5]
Needle	*D. septosporum*	4	266	23.8	5.1	10.1–36.3	***	2.8	0.4	1.8–4.0	***
	D. pini	3	133	26.9	4.2	15.7–38.4		3.2	0.4	2.3–4.6	
Culture	*D. septosporum*	2	101	26.6	6.2	10.9–43.3	***	2.7	0.5	1.9–4.3	***
	D. pini	3	159	22.6	3.8	15.3–35.6		3.7	0.5	2.6–4.7	

Measurements from all *D. septosporum* and *D. pini* needle collections and isolates (see Table 3) were pooled and analysed together.
[1]Number of needle collections (for conidiomata taken from needles), or isolates (for conidiomata taken from cultures), from different countries examined (see also Table 3).
[2]Number of conidia measured.
[3]Standard deviation.
[4]Minimum–maximum.
[5]Mean values of conidial length and width, within substrates 'needle' and 'culture', were significantly different between *D. septosporum* and *D. pini* (independent-samples t-test, ***significant at $p < 0.001$). Differences in condial dimensions between substrates 'needle' and 'culture' within *D. septosporum* and *D. pini* were not analysed (but see Table 3 for comparisons of conidial dimensions on needles and in cultures of individual collections/isolates from different countries).

4 Discussion

The results of this study provide interesting and important new host and country records of the DNB fungi from Europe and Asia. DNA sequence data for the rDNA ITS and β-tubulin genes verified that the isolates from Austria, Hungary and Bhutan represent *D. septosporum*. This fungus is therefore reported from a new host (*P. peuce*) in Europe, grown as an exotic in an arboretum in Vienna, Austria. Likewise, DNB and the associated pathogen, *D. septosporum*, were found for the first time on an exotic (*P. radiata*) and a native host (*P. wallichiana*) in Bhutan. In addition, *Dothistroma* isolates from Hungary were identified for the first time using DNA-based diagnostic tools and confirmed to be *D. septosporum*. In contrast, isolates from Ukraine and south-western Russia were identified as *D. pini*. This is therefore the first report of this fungus from outside the north-central USA. *Dothistroma pini* is now reported from a second continent (Europe) and its host range has been broadened to include not only *P. nigra* but also *P. pallasiana* in forest plantations outside its natural range.

The morphological studies have emphasized that conidial dimensions of the two *Dothistroma* species on pine are variable (Tables 3 and 4). Conidial dimensions differed considerably when compared *in vivo* and *in vitro*. This variation is likely influenced by many factors, including differences in host species and age, time of the year the collections were made and geographical location. *Dothistroma septosporum* and *D. pini* do not show consistent differences in the length of their conidia (Tables 3 and 4). In contrast, *D. pini* has slightly wider conidia than *D. septosporum* (Tables 3 and 4), as previously reported by Barnes et al. (2004). Differences in conidial width between the two species were generally consistent for all examined specimens, although they were not statistically significant between all *D. pini* and *D. septosporum* collections when conidia obtained from conidiomata on needles were compared (Table 3). The differences in conidial width between *D. pini* and *D. septosporum* are, however, so small that this character is of very limited, if any, practical value for species diagnosis. We therefore contend that identification based on morphology alone is ambiguous and could in many cases lead to mis-identifications. DNA sequence comparisons remain the most reliable method to conclusively distinguish between the two *Dothistroma* species on pine. Thus, a re-examination of worldwide records of the causal agents of DNB using DNA-based techniques is required to obtain more precise distribution ranges for *D. septosporum* and *D. pini*. The present data suggest that *D. septosporum* is more widespread than *D. pini* (Barnes et al. 2004), but considering the similarity of the two species, and the new records of *D. pini* from Ukraine and south-western Russia in the present study, future discoveries of *D. pini* are likely.

In Austria, DNB was recorded from *P. nigra* in the late 1950s (Petrak 1961) and this represents one of the earliest reports of the disease in Europe. Since then, DNB has been found on all pine species (*P. nigra*, *P. sylvestris* L., *P. mugo*, *P. unicinata* Mill. ex Mirb. and *Pinus cembra* L.) native in this country and it has become relatively common in recent years (Brandstetter and Cech 2003; Kirisits and Cech 2006). The discovery of *D. septosporum* infecting *P. peuce*, a non-native host in Austria, is not surprising considering its wide host range on pine and its common occurrence in central European countries. This new host record is also consistent with other recent new host records from neighbouring Czech Republic (Bednářová et al. 2006) and with continuing new host records from other regions of the world (Bradshaw 2004 and references therein).

All five individuals of *P. peuce* in the experimental garden and arboretum 'Knödelhütte' in Vienna were suffering from DNB. In contrast, other pine species, including *P. nigra* and *P. ponderosa* Laws. (Douglas), which are considered as very susceptible to the disease, were unaffected. Inspections in the experimental garden and arboretum in May 2007 revealed the presence of DNB on three other pine species, including *P. cembra* (Swiss stone pine), *P. jeffreyi* Grev. & Balf. (Jefferey's pine) and *P. uncinata* (Mountain pine). However, disease severity on the three latter hosts was much lower than on *P. peuce*. These observations on disease incidence and severity may thus indicate that *P. peuce* is highly susceptible to infection by *D. septosporum*, a suggestion that now needs to be confirmed in inoculation experiments. A few experimental plantations of *P. peuce* at high elevations in the Austrian Alps have been established, but during routine forest inventories in 2004, none of these stands were found to be affected by DNB (M. Liesebach, personal communication).

Pinus peuce is a five-needled (white) pine that occupies a relatively small, natural distribution range on the Balkans, where it primarily occurs in mountain forests at high elevations (Schreiber 1928; Nedjalkov 1963; Alexandrov et al. 2004). Natural *P. peuce* forests fulfil important ecological and economic roles. This pine species has in former times also been proposed as a replacement for eastern white pine (*Pinus strobus* L.) in central European forestry (Schreiber 1928), because of its high levels of resistance to white pine blister rust, caused by *Cronartium ribicola* J. C. Fischer (Hoff et al. 1980). However, because of its slow growth it has not achieved any significance as a forest plantation tree outside its natural range and is presently only occasionally seen in botanical gardens and arboreta or as a shade and landscape tree. Based on the report of DNB on *P. peuce* provided here, the risk posed by *D. septosporum* to this pine species within and outside its native range will require further attention and study.

Dothistroma needle blight was not recorded during surveys of conifer tree diseases in Bhutan in the 1980s (Donaubauer 1986, 1987) and in 2001 (Kirisits et al. 2002). Re-inspections of needle samples from *P. wallichiana* and *P. roxburghii* Sarg. collected by Donaubauer (1986, 1987) also failed to confirm the presence of *Dothistroma* conidiomata, but other needle pathogens [*Lophodermium* spp. and possibly *Meloderma desmazierii* (Duby) Darker] were present. Another survey of forest tree diseases conducted in Bhutan in July 2005 enabled the collection of needle samples that yielded the isolates identified as *D. septosporum* in the present investigation. This survey revealed numerous saplings and pole-sized trees of *P. wallichiana* affected by needle diseases in natural conifer forests at high elevations in central Bhutan. In most cases, symptoms were not typical of DNB and fruiting bodies of other ascomycete fungi were dominant on diseased needles. However, careful examination of collected specimens revealed the presence of tiny *Dothistroma* conidiomata on needles from many trees at various localities, shown here to belong to *D. septosporum*. From the observations made during the disease survey in 2005, it is reasonable to suggest that *D. septosporum* is the primary cause of needle blight on *P. wallichiana* in many areas in Bhutan. Other ascomycetes, either endophytes or secondary colonists, however, were more obvious on needles affected by DNB and these most likely masked the symptoms caused by the primary causal agent. *Pinus wallichiana* is an extremely important tree species in temperate conifer forests in Bhutan, and monitoring the incidence and severity of DNB on this tree species over time is recommend in order to assess its potential to cause damage.

Besides its occurrence in Bhutan and adjacent areas including Nepal, India and Pakistan (Bakshi and Singh 1968; Reddy et al. 1975; Zakaullah et al. 1987; Ivory 1990, 1994), *D. septosporum* is widespread in other parts of Asia including China, Brunei Darussalam, Georgia, Japan, South and North Korea, the Philippines and Sri Lanka (Wang et al. 1998; data sheets on quarantine pests: *Mycosphaerella dearnessi*, http://www.eppo.org/QUARANTINE/listA2.htm and *Mycosphaerella pini*, http://www.eppo.org/QUARANTINE/documented_pests.htm). In all cases, the records were based on morphological characteristics and they therefore leave some doubt as to whether they all refer to *D. septosporum* or whether they could also represent the morphologically similar *D. pini*. Thus, identification of the isolates from Bhutan as *D. septosporum* represents the first confirmation using DNA-based diagnostic methods that DNB in an Asian country is actually caused by *D. septosporum* and not by *D. pini*.

Prior to this study, *D. pini* had only been known from the north-central USA (Barnes et al. 2004). Its discovery in Ukraine and south-western Russia is thus intriguing and has important consequences, as it represents the first report of *D. pini* from Europe. Its host range now includes the exotic *P. nigra* in North America and *P. pallasiana* in Europe. *Pinus pallasiana* is similar to *P. nigra* and is considered by some authors (Bobrov et al. 1974; Dobrochaeva et al. 1987) to be a variety or subspecies of *P. nigra*: *P. nigra* ssp. *pallasiana* (D. Don in Lamb.) Holmboë, *P. nigra* var. *pallasiana* Aschers. et Graebn. and *P. nigra* var. *pallasiana* (D. Don in Lamb.) C. K. Schneid. *Pinus nigra* and *P. pallasiana* are closely related and morphologically similar to each other, and it is therefore not surprising that *P. pallasiana* is a host of *D. pini.* Symptoms of DNB on *P. nigra* and *P. pallasiana* are also very similar, especially as characteristic brick-red bands are formed on the needles of both host species.

The natural range of *P. pallasiana* covers the Crimean peninsula in Ukraine, the Balkan peninsula, the southern Carpathians, Cyprus, Crete, Anatolia and parts of the Black sea coast of Caucasus and Turkey (Bobrov et al. 1974; Dobrochaeva et al. 1987), while in the northern and central regions of Ukraine, artificial plantations of *P. sylvestris* prevail. As a result of its drought tolerance, *P. pallasiana* has been extensively used in afforestation programmes in the steppes of southern Ukraine and south-western Russia, outside its natural range (Dobrochaeva et al. 1987; Gorbok and Deryuzhkin 1987). Since 2004, DNB has become an important problem in *P. pallasiana* forests in Ukraine and south-western Russia. The disease was first found on this species in 2004 during routine inspections of forest plantations in the Kherson region (Vinogradov and Tsjurupinsk forests, Tsjurupinsk area). The severe epidemic at this site, originally suspected to be caused by *D. septosporum,* covered more than 8000 ha of forests (Usichenko and Kucherjavenko 2005). The isolates identified in our study as *D. pini* originated from this area and the epidemic there can thus be linked to this pathogen. DNB has subsequently been detected repeatedly in *P. pallasiana* plantations in the Mykolaiv and Odessa regions and in other forests in the Kherson region [herbarium samples CWU (Myc) 1228 and 1262–1265]. Presently, DNB occurs throughout southern Ukraine and its severity appears to be increasing [Usichenko et al. 2005; Usichenko and Kucherjavenko 2005 (both recorded as *D. septosporum*)].

In south-western Russia, the majority of the pine forests consist mainly of exotic plantations of *P. pallasiana* and *P. sylvestris* (Scots pine), although small natural fragments of *P. sylvestris* forests are present in the territory near to Voronezh and Lugan'sk (Ukraine) (Bobrov 1978). *Pinus pallasiana* was first introduced into the area as a highly drought-tolerant species and its wide cultivation started only in the second half of the 20th century. Here, the main purpose of initiating pine plantations was to stabilize sandy soils along the Don and Donets rivers (Shaposhnikov and Kuznetsov 1960). DNB is noticeable on *P. pallasiana* in the Rostov and Volgograd regions and its distribution spans most of the areas along the basins of the Don and Donets rivers, as well as Belaya Kalitva and Chir rivers (Bulgakov 2007; Sokolova and Fomina 2007). Highest levels of disease incidence occur on sandy hills at low elevations and areas closest to the river where air humidity is higher. In conditions where trees are growing at low densities on wind-exposed slopes at higher elevations, or occur singly alongside roads, the disease is rarely observed. Younger trees (<30 years) are also more susceptible and in plantations, trees growing inside the stands are often more infected than those at the stand margin. Although not currently a threat, epidemics of DNB could have severe consequences for the protective function that *P. pallasiana* afforestions provide for the sandy soils against extensive wind and water erosion and possible dune movements.

We have ascertained that DNB in the *P. pallasiana* forests of southern Ukraine and the Rostov region in south-western Russia is caused by *D. pini*. The possibility exists, however, that the other DNB pathogen is also present in these areas, because *D. septosporum* (as

Cytosporina septospora Dorog.) was first described by Doroguine (1911) from *P. mugo* in Russia. Unfortunately, type material from the original description no longer exists and it thus cannot be re-examined. A second collection, made in the Kiev region of Ukraine in 1914 on *Pinus sylvestris* L. by L. Kaznowski, is maintained at the St Petersburg herbarium. This material (LE 116244, herb. CBS 11381) was examined and, based on morphology only, was identified as *D. septosporum* (Barnes et al. 2004). This identification could not be verified with sequence data because of the age of the herbarium material, and it therefore remains ambiguous.

The discovery of *D. pini* in southern Ukraine and south-western Russia now raises doubts regarding the correct identity of the type specimen of *D. septosporum* and subsequent collections, making these countries intriguing regions for further studies on the DNB pathogens. Such studies could contribute substantially to the understanding of the taxonomy and origin of these fungi. Further collection and examination of *Dothistroma* isolates from *P. sylvestris* and *P. pallasiana* in the provinces of south-western Russia and southern Ukraine could clarify which DNB pathogens occur on the respective hosts in the different regions.

The discovery of *D. pini* on a native pine species in Europe also raises the question as to whether *D. pini* could have originated from Europe and was accidentally introduced into the USA. This intriguing question could be addressed by comparing populations of *D. pini* from *P. pallasiana*, preferably collected within the natural range of this pine species, with populations from the USA, using genetic markers. *Pinus pallasiana* infected with DNB within its natural range should also be examined for the occurrence of the teleomorph of *D. pini*, which has, thus far, not been found.

The new host and country records provided here for *D. septosporum* and *D. pini* are consistent with the increasing number of reports of the DNB pathogens from new hosts and new geographical areas during the last two decades, particularly in the northern hemisphere. Some of these new records might be the result of an increase in awareness and diagnostic skills of forest pathologists and foresters. However, there are also reports from many parts of the world, often in places where the disease has been present for many years that the incidence and severity of DNB is increasing. Accumulating evidence therefore suggests a real change in the DNB situation, which might be triggered by a combination of factors. These include favourable weather conditions during a number of consecutive years, planting susceptible hosts over large areas (Woods 2003; Woods et al. 2005) and a buildup of inoculum over time. The fact that two closely related fungi cause DNB, and that one of them (*D. pini*) has now been found on a second continent, complicates the situation. It especially emphasizes the need for continuing surveys of *D. septosporum* and *D. pini* in pine forests and plantations of the world. The information generated from such surveys would facilitate strategies for disease management and quarantine measures.

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