

# Characterization and Distribution of Mating Type Genes in the *Dothistroma* Needle Blight Pathogens

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## ABSTRACT

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*Dothistroma septosporum* and *D. pini* are the two causal agents of *Dothistroma* needle blight of *Pinus* spp. in natural forests and plantations. Degenerate primers amplified portions of mating type genes (*MAT1-1-1* and *MAT1-2*) and chromosome walking was applied to obtain the full-length genes in both species. The mating-type-specific primers designed in this study could distinguish between the morphologically similar *D. pini* and *D. septosporum* and between the different mating types of these species. Screening of isolates from global collections of *D. septosporum* showed that only MAT2 isolates are present in Australian and New Zea-

land collections, where only the asexual form of the fungus has been found. In contrast, both mating types of *D. septosporum* were present in collections from Canada and Europe, where the sexual state is known. Intriguingly, collections from South Africa and the United Kingdom, where the sexual state of the fungus is unknown, included both mating types. In *D. pini*, for which no teleomorph is known, both mating types were present in collections from the United States. These results provided new insights into the biology and global distribution of two of the world's most important pine pathogens and should facilitate management of the diseases caused by these fungi.

**Additional keywords:** ascomycetes, heterothallic, *Mycosphaerella*, sexual reproduction.

*Dothistroma* needle blight, also known as red band needle blight, is one of the most important diseases of *Pinus* spp., both in natural forest ecosystems and particularly in plantations of non-native pines (9,19,20,27). The disease owes its international notoriety to the fact that it has been one of the most important constraints to the development of plantation forestry in many countries of Africa as well as in New Zealand, Australia, Chile, and other South American countries (19,20,27). The disease is particularly severe on *Pinus radiata* D. Don. This species is highly desirable for its rapid growth and exceptional timber and, consequently, it was one of the first nonnative tree species established in intensively managed plantations in the tropics and Southern Hemisphere. Outbreaks of *Dothistroma* needle blight on *P. radiata* led to devastating losses and resulted in the abandonment of *P. radiata* from plantation forestry in many countries (11,31,51).

The main causal agent of *Dothistroma* needle blight has been a matter of considerable taxonomic confusion. Thus, in different parts of the world, the disease has been attributed to either a single pathogen, different species of a pathogen, or varieties of a species. This also has differed depending on whether the pathogen was considered introduced or native in areas where the disease has been studied. In a recent study based on DNA sequence comparisons, two distinct phylogenetic lineages for *Dothistroma* isolates were identified (2). These clearly separated *Dothistroma*

*septosporum*, which has a worldwide distribution, and *D. pini*, until recently found only in the north-central United States. This study also showed that the disease which devastated plantations of *P. radiata* in the Southern Hemisphere is caused by *D. septosporum*. Recently, *D. pini* has been found infecting *P. palassiana* D. Don. in the Ukraine (I. Barnes, *unpublished data*) and it clearly has a distribution much wider than was believed at the time of the study of Barnes et al. (2).

*Dothistroma* needle blight, now known to have been caused by *D. septosporum*, resulted in huge damage to *P. radiata* plantations in the Southern Hemisphere in the 1950s and 1960s (9,19,20,27). Consequently, considerable research was conducted on the disease and great efforts were made to minimize its impact (8,19, 20,41,46). These included selection of alternative species, tree breeding, agricultural practices, and the first examples of aerial applications of chemical fungicides in forest plantations (19). Although the disease has continued to be important, it generally is considered to be under reasonable control. There has, however, been a recent resurgence of the disease in various Northern Hemisphere countries and this has raised concern that a new wave of losses might occur elsewhere in the world (5,53).

Almost nothing is known regarding the genetic diversity among isolates of *D. septosporum* and *D. pini*. *D. septosporum* first was identified in New Zealand in 1964 (21). A study by Hirst et al. (26) applied random amplified polymorphic DNA (RAPD) markers to a population of *D. septosporum* (previously described as *D. pini*) from New Zealand and the results showed no genetic variation. These results support the hypothesis that it is an introduced pathogen that has been spreading asexually ever since its introduction into that country.

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The sexual state of *D. septosporum* is a species of *Mycosphaerella* known as *Mycosphaerella pini* Rostr. (17). In most countries of the Southern Hemisphere where *D. septosporum* has long been an important forest pathogen, only the anamorph has been reported (2,5,14; M. J. Wingfield, *unpublished data*). In contrast, no sexual state has ever been reported for *D. pini*. The absence or rarity of a sexual state for either of these fungi could be the result of selection pressure and a reduced need for sexual reproduction (14). Likewise, lower frequency and limited distribution of the teleomorph compared with the anamorph suggests that the primary method of dispersal of the fungus could be an asexual cycle. Here, conidia rather than ascospores would represent the inoculum of primary epidemiological importance (10,28).

Mating type genes play an important part in the biology and evolution of fungal species. Thus, knowledge of these genes can provide insight into the potential prevalence of sexual reproduction in different species. Some heterothallic Pyrenomycetes and Discomycetes can contain up to four genes at the mating type 1 idiomorph (*MAT1-1*) of the *MAT* locus (40,43,44,55). These include the *MAT1-1-1* encoding an  $\alpha$  domain protein, the *MAT1-1-2* encoding an amphipathic  $\alpha$  helix protein, the *MAT1-1-3* gene encoding a high mobility group (HMG) domain protein, and the *MAT1-1-4* gene encoding a metallothionein protein. Only one gene has been characterized for the mating type 2 idiomorph (*MAT1-2*) and it encodes a regulatory protein with an HMG domain. The DNA sequences of the idiomorphs, located at the *MAT* locus of individuals of two different mating types, are unrelated and, therefore, cannot be called alleles; however, these sequences are flanked by conserved regions (32). The formal nomenclature that is proposed for mating type genes of heterothallic ascomycetes is used here for the *MAT1-1-1* and, because only a single *MAT1-2* gene has been identified for filamentous ascomycetes, this gene is referred to as *MAT1-2* (49).

DNA and amino acid sequences of the *MAT1-1-1* and *MAT1-2* genes in fungi show no obvious similarities, although the mating type locus has common flanking regions (48). Except for the HMG and  $\alpha$  domains, the similarity of homologous mating type genes usually is very low between different species (47). The direct target genes of the mating type proteins have not yet been described, although there is evidence for the control of some genes, such as pheromone genes (4). Mating type genes have been described from various sexual and presumably asexual fungi that are close relatives of the genus *Dothistroma* (*Mycosphaerellaceae*). Detailed analyses have been done on the distribution of the mating types of the sexually reproducing *M. graminicola* (50,56) and the presumably asexual species *Septoria passerinii* (23), *Cercospora beticola*, *C. zeae-maydis*, and *C. zeina* (25). Equal distribution of the mating types was found in most of the populations from these five species sampled from different geographical scales, indicating that sexual stages probably exist for the latter four apparently asexual species.

*D. septosporum* first was described from Idaho (United States) but now is seen in many parts of the world (2). In most of the areas where this species has been introduced and causes serious disease, only the asexual state of the fungus is ever seen. This raises the interesting question as to whether this could be attributed to the introduction of only one mating type into these new environments. Thus, the aims of this study were to characterize the mating type gene or genes of the causal agents of *Dothistroma* needle blight and to ascertain which mating types are present in the different countries where diseases caused by these fungi occur. To achieve this objective, the full-length *MAT1-1-1* and *MAT1-2* genes of *D. septosporum* and *D. pini* were isolated and sequenced using polymerase chain reaction (PCR)-based techniques. This made it possible to develop a multiplex PCR method for the rapid screening of *MAT1-1-1* and *MAT1-2* in isolates of the pathogens. A global collection of isolates subsequently was screened to determine which mating types are present in these collections.

## MATERIALS AND METHODS

**Fungal isolates.** In all, 230 *Dothistroma* isolates obtained from various locations in 15 countries were chosen to represent a global distribution of *Dothistroma* spp. (Table 1). Countries for which more than one isolate was screened included Austria ( $n = 10$ ), Canada ( $n = 106$ ), Chile ( $n = 10$ ), New Zealand ( $n = 38$ ), Poland ( $n = 11$ ), South Africa ( $n = 11$ ), Ukraine ( $n = 4$ ), the United Kingdom ( $n = 10$ ), and the United States ( $n = 17$ ). Isolates were obtained from different culture collections and standard protocols were used to isolate the genomic DNA.

The initial screening of the mating type genes was undertaken for *D. septosporum* using two isolates. These included CBS 116489 obtained from *P. radiata* in Tzaneen, South Africa and American Type Culture Collection (ATCC) MYA-605 obtained from *P. radiata* in Rotorua, New Zealand. For *D. pini*, four isolates were used: CBS 116485, obtained from *P. nigra* in Crystal Township, MI; CBS 116487, obtained from *P. nigra* in Evergreen Township, MI; CBS 116483, obtained from River Township, MI; and CBS 117609, obtained from *P. palassiana* in Tsyurupinsk, Ukraine. The identities of the six isolates used for the screening of the mating types previously had been confirmed using comparisons of DNA sequence data for the internal transcribed spacer (ITS) regions of the ribosomal DNA (2; J. Z. Groenewald, *unpublished data*).

**Isolation and characterization of *MAT1-1-1* of *Dothistroma* spp.** The *MAT1-1-1*-specific degenerate primers (MgMfSpMat1-1f1 and MgMfSpMat1-1r2) (Table 2), designed by Groenewald et al. (25), were used to screen and amplify a partial region of the *MAT1-1-1* genes of the *Dothistroma* isolates.

The PCR mixtures and amplification reactions were the same as described by Groenewald et al. (25) for the amplification of the partial *MAT1-1-1* in *Cercospora* spp. The PCR products obtained were separated by electrophoresis at 80 V for 1 h on a 1% (wt/vol) agarose gel containing ethidium bromide at 0.1  $\mu$ g/ml in 1 $\times$  Tris-acetate-EDTA buffer (0.4 M Tris, 0.05 M sodium acetate, and 0.01 M EDTA, pH 7.85) and visualized under UV light. Amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, Netherlands) following the manufacturer's recommendations. The products were analyzed on an ABI Prism 3730 DNA Sequencer (Applied Biosystems, Foster City, CA). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNA-STAR, Madison, WI).

Internal primers were designed in the partially sequenced *MAT1-1-1* genes for each of the species (CBS 116489 for *D. septosporum* and CBS 116487 for *D. pini*). In order to obtain the full-length genes, these internal primers were used together with the appropriate primers from the DNA walking speedup kit (Seegene Inc., Rockville, MD) to determine additional sequences upstream and downstream of the partial *MAT1-1-1* sequences. The Blastx algorithm (1) was used to compare the sequences obtained from the two *Dothistroma* spp. with protein sequences of other fungi present in the National Center for Biotechnology Information (NCBI) nonredundant protein database. The geneid web server (v1.2; Research Unit on Biomedical Informatics of IMIM, Barcelona, Spain) was used to predict the gene and intron or exon boundaries using the genetic code of *Neurospora crassa*. The conversion of DNA sequences to putative amino acid sequences was done using the translation tool of the proteomics server ExPASy (18). The percentage of identities between the predicted *MAT1-1-1* gene sequences for the *Dothistroma* spp. was calculated using the alignment tool of ALIGN (37).

**Isolation and characterization of *MAT1-2* of *Dothistroma* spp.** The *MAT1-2*-specific degenerate primers (MgMfSpMat1-2f2 and MgMfSpMat1-2r1) (Table 2), designed by Groenewald et al. (25), were used to screen isolates of *D. septosporum* and *D. pini*

TABLE 1. Origins of the *Dothistroma septosporum* and *D. pini* strains used during this study and the distribution of their mating types

Country, area, site	Collector	Species	Number of strains	MAT1-1-1	MAT1-2
Australia					
A.C.T. Canberra	K. Old	<i>D. septosporum</i>	10	0	10
Austria					
Thenneberg	T. Kirisits	<i>D. septosporum</i>	10	6	4
Brazil					
São Paulo	T. Namekata	<i>D. septosporum</i>	1	0	1
Canada					
Northwest British Columbia (BC)					
Brown Bear Road	K. Lewis & A. Dale	<i>D. septosporum</i>	10	5	5
Bell Irving River	K. Lewis & A. Dale	<i>D. septosporum</i>	1	0	1
Bulkley Canyon	K. Lewis & A. Dale	<i>D. septosporum</i>	9	5	4
Evelyn Pasture	K. Lewis & A. Dale	<i>D. septosporum</i>	1	0	1
Jonas Creek	K. Lewis & A. Dale	<i>D. septosporum</i>	2	0	2
Kinskutch Road	K. Lewis & A. Dale	<i>D. septosporum</i>	8	7	1
Kuldo Creek	K. Lewis & A. Dale	<i>D. septosporum</i>	7	2	5
Kisgegas Canyon	K. Lewis & A. Dale	<i>D. septosporum</i>	5	2	3
Squingula River Mine	K. Lewis & A. Dale	<i>D. septosporum</i>	8	1	7
Mosque River	K. Lewis & A. Dale	<i>D. septosporum</i>	6	1	5
Mitten Road	K. Lewis & A. Dale	<i>D. septosporum</i>	7	4	3
Nangeese Road	K. Lewis & A. Dale	<i>D. septosporum</i>	8	4	4
North Kuldo Road	K. Lewis & A. Dale	<i>D. septosporum</i>	4	1	3
Sanyam River	K. Lewis & A. Dale	<i>D. septosporum</i>	1	0	1
Nash Y	K. Lewis & A. Dale	<i>D. septosporum</i>	9	7	2
Orendo	K. Lewis & A. Dale	<i>D. septosporum</i>	7	6	1
Motaze Lake & Squingula River	K. Lewis & A. Dale	<i>D. septosporum</i>	8	6	2
Sunday Lake	K. Lewis & A. Dale	<i>D. septosporum</i>	4	1	3
Goldstream River, BC	D. Morrison	<i>D. septosporum</i>	1	0	1
Chile					
Valdivia	M. J. Wingfield	<i>D. septosporum</i>	10	0	10
France					
Meurthe-et-Moselle	M. Morelet	<i>D. septosporum</i>	1	0	1
Germany					
Bavarian Alps	L. Pehl	<i>D. septosporum</i>	1	0	1
Guatemala					
Sierra de Chuacús	Unknown	<i>D. septosporum</i>	1	0	1
New Zealand					
Bay of Plenty	M. A. Dick	<i>D. septosporum</i>	1	0	1
Golden Downs sites 1/2/3	P. Hirst	<i>D. septosporum</i>	4	0	4
Kaingora Forest	M. J. Wingfield	<i>D. septosporum</i>	10	0	10
Kaingora sites 1/2/3	P. Hirst	<i>D. septosporum</i>	11	0	11
Kinleith	P. Hirst	<i>D. septosporum</i>	5	0	5
Mt. Maunganui	K. Dobbie	<i>D. septosporum</i>	1	0	1
Rotorua	M. E. Buchanan	<i>D. septosporum</i>	2	0	2
Tongariro	J. W. Gilmour	<i>D. septosporum</i>	1	0	1
West Coast South Island	B. Doherty	<i>D. septosporum</i>	1	0	1
Poland					
Miechow Forest, Cracow	T. Kowalski	<i>D. septosporum</i>	11	3	8
Slovakia	E. Foffova	<i>D. septosporum</i>	1	1	0
South Africa					
Hogsback	J. Roux	<i>D. septosporum</i>	10	3	7
Tzaneen	I. Barnes	<i>D. septosporum</i>	1	1	0
Ukraine					
Tsurupinsk	A. C. Usichenko	<i>D. pini</i>	4	4	0
United Kingdom					
West Midlands	A. Coggins	<i>D. septosporum</i>	1	0	1
South East England	A. V. Brown	<i>D. septosporum</i>	1	0	1
Forest of Dean	R. Beasley	<i>D. septosporum</i>	1	1	0
New Forest	A. V. Brown	<i>D. septosporum</i>	7	1	6
United States					
Bandon, Oregon	S. Cooley	<i>D. septosporum</i>	1	0	1
Michigan					
Crystal Township	G. Adams	<i>D. pini</i>	10	4	6
Evergreen Township	G. Adams	<i>D. pini</i>	1	1	0
River Township	G. Adams	<i>D. pini</i>	1	0	1
Central Minnesota	T. Nicholls	<i>D. pini</i>	1	1	0
Lincoln, Nebraska	G. Peterson	<i>D. pini</i>	3	2	1
Total	...	...	230	80	150

to obtain a partial region of the *MATI-2* genes. The same PCR conditions described above were used to amplify the partial *MATI-2* regions. Twelve internal primers were designed in the partially sequenced *MATI-2* sequences for both species (ATCC MYA-605 for *D. septosporum* and CBS 116485 for *D. pini*) and the chromosome walking method also was used to obtain the full-length *MATI-2* genes. The same procedure and programs described for the characterization and analyses of the *MATI-1-1* sequences were used to characterize and analyze the *Dothistroma MATI-2* sequences.

**Development and screening of *D. pini* and *D. septosporum* mating-type-specific primers.** *Dothistroma MATI-1-1*-specific primers (Table 2) were designed from the aligned *MATI-1-1* sequences of *D. pini* and *D. septosporum* (GenBank accession nos. DQ915449 and DQ915450, respectively). The forward primers were designed to be specific for *D. septosporum* (DseptoMat1f) or *D. pini* (DpiniMat1f2) and, therefore, are both species and mating type specific. The reverse primer (DotMat1r) was designed from homologous regions within the *MATI-1-1* genes and, therefore, is only mating type specific.

*Dothistroma MATI-2*-specific primers (Table 2) were designed from the aligned *MATI-2* sequences of *D. pini* and *D. septosporum* (GenBank accession nos. DQ915451 and DQ915452, respectively). The two forward primers were designed in regions of the genes that were variable between the two species. DseptoMat2f was designed to be specific for *D. septosporum* and DpiniMat2f for *D. pini*, and both, therefore, are species and mating type specific. The reverse primer (DotMat2r) was designed from homologous regions within both the *MATI-2* genes and, thus, is only mating type specific.

Multiplex PCR was used to screen for the *MATI-1-1* or the *MATI-2* of *D. pini* and *D. septosporum* in two separate reactions. The reaction mixtures had a total volume of 12.5 µl and contained 0.7 µl of diluted genomic DNA, 1× PCR buffer (Bioline, Randolph, MA), 48 µM each of the dNTPs, 4 pmol of each primer, 1 mM MgCl<sub>2</sub>, and 0.7 units of *Taq* polymerase (Bioline, Randolph, MA). The amplification reactions were done on a GeneAmp PCR System 9600 (Applied Biosystems). The initial denaturation step was done at 94°C for 5 min, followed by 40 cycles of 94°C (20 s), 65°C (20 s), and 72°C (40 s). A final elongation step at 72°C (5 min) was included in the run. The resulting PCR products were visualized as described above.

**Phylogenetic analyses.** The nucleotide sequences of the α domain (*MATI-1-1*) and HMG domain (*MATI-2*) of *D. septosporum* and *D. pini* determined in this study and additional mating type sequences for other species representing different fungal orders downloaded from NCBI's GenBank database were used for phylogenetic analyses. These sequences were analyzed using the mating type gene sequences of *Magnaporthe grisea* (GenBank accession nos. AB080672 and AB080673, respectively) as the outgroup. All phylogenetic analyses were done using Phylogenetic Analysis Using Parsimony (PAUP) v4.0b 10 (Swofford, D. L. 2003. Sinauer Associates, Sunderland, MA). Maximum parsimony analyses were conducted as described by Groenewald et al.

(24). All sequences generated were deposited in GenBank, and the alignments and trees were deposited in TreeBASE (TreeBASE accession no. SN3047).

## RESULTS

**Isolation and characterization of *MATI-1-1* in *Dothistroma* spp.** The degenerate primers MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 amplified a fragment of 914 bp for three of the six *Dothistroma* isolates tested (Fig. 1). The fragments obtained from strains CBS 116489, CBS 117609, and CBS 116487 were sequenced. The translated sequence of the fragment obtained from strain CBS 116489 (*D. septosporum*) showed 39 and 46% identity to a 229- and 63-amino-acid (aa) region of the *M. graminicola* MAT1 protein and 32% identity to a 213-aa region of the *S. passerinii* MAT1 protein using Blastx on the GenBank database. This confirmed that the 914-bp fragment is part of the *MATI-1-1* gene of *D. septosporum*.

Sequences for the fragments obtained from the *D. pini* strains (CBS 117609 and CBS 116487) showed 100% identity to each other in this region. The translated sequences showed 39% identity to a 226-aa ( $E = 2 \times 10^{-30}$ ) and 37% identity to a 78-aa region ( $E = 2 \times 10^{-30}$ ) of the *M. graminicola* mating type 1-1 protein (GenBank accession no. AAL30838). It also showed 32% identity to a 218-aa region ( $E = 5 \times 10^{-18}$ ) of the *S. passerinii* MAT1 protein (GenBank accession no. AAO49357). This confirmed that the 914-bp fragment is part of the *MATI-1-1* gene of *D. pini*.

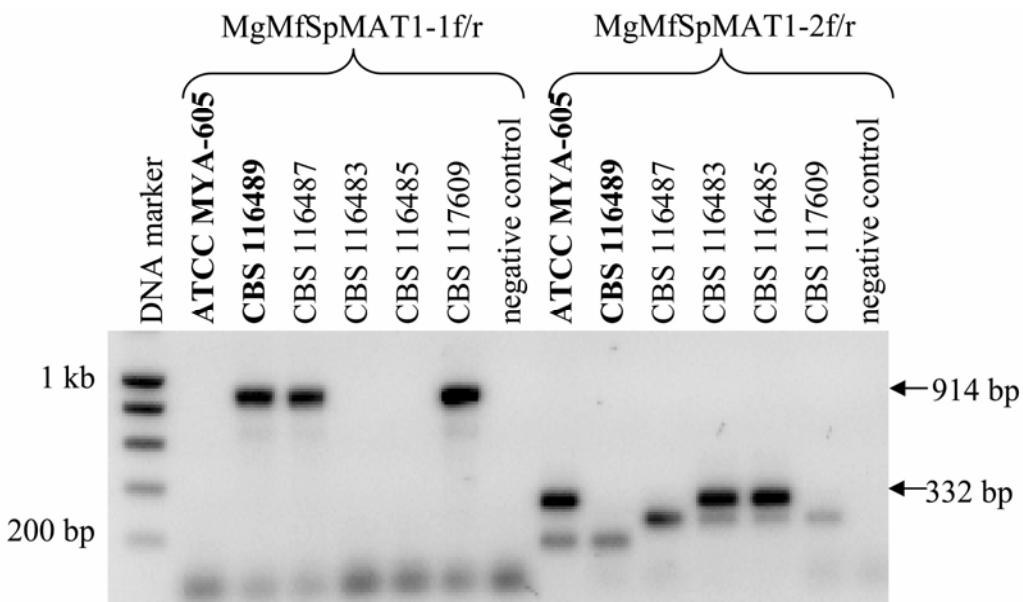
Four chromosome walking steps were used to obtain the full-length *MATI-1-1* gene sequences for *D. septosporum* and *D. pini*. The geneid software predicted that the *MATI-1-1* genes of both species contained four exons. The predicted length of the genes and the exon and intron positions are illustrated in Figure 2. Although the number of nucleotide and amino acid residues was the same for the *MATI-1-1* of *D. septosporum* and *D. pini*, an identity of 94.1 and 94.3% was found between the 1,311-nucleotide and the 387-aa residues, respectively. All introns of the *MATI-1-1* from both species contained a perfect lariat sequence (RCTRAC), except for the second intron of the *MATI-1-1* of *D. septosporum*. When this intron is included in the coding region, an early stop codon is introduced in the reading frame, indicating that this is a true intron. The positions of the three predicted introns in the *Dothistroma* spp. studied correlate with those found for *Cercospora* spp. (25). The number of predicted introns (two) in the conserved α domain of the *Dothistroma* spp. correlated with the number predicted for the same region in *M. graminicola* (50) and *S. passerinii* (23).

**Isolation and characterization of *MATI-2* of *Dothistroma* spp.** The degenerate primers MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1 amplified a fragment of 332 bp for the *Dothistroma* isolates that did not amplify the 914-bp fragment using the *MATI-1-1* degenerate primers (Fig. 1). An extra 180-bp fragment also was obtained from the two *D. septosporum* strains and an extra 280-bp fragment from the four *D. pini* strains. The 332-bp fragment obtained from strain ATCC MYA-605 (*D. septo-*

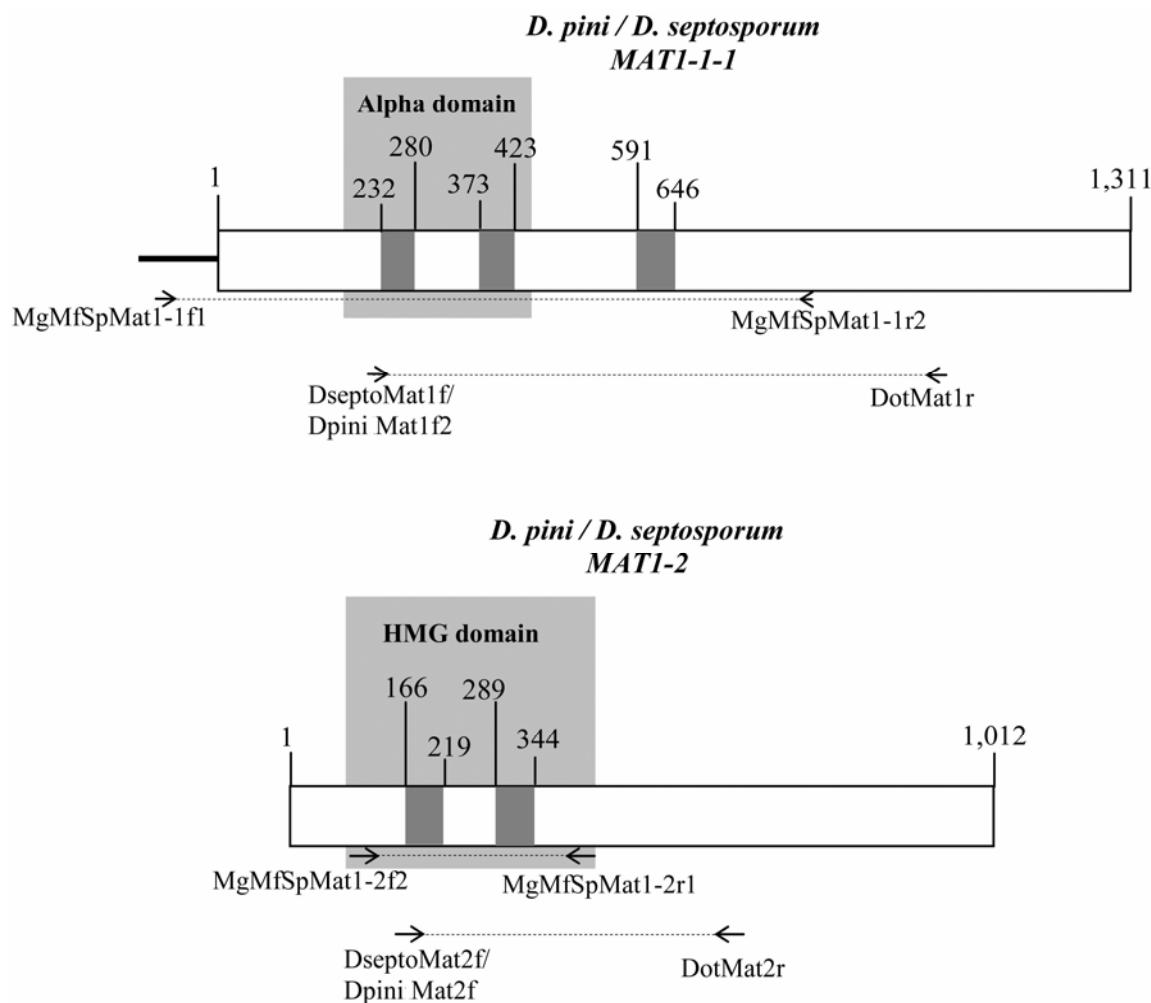
TABLE 2. Primers used during this study<sup>a</sup>

Primer	5'-3'	Description
MgMfSpMat1-1f1	CATTNGCNCATCCCTTG	<i>MATI-1-1</i> -specific degenerate primer
MgMfSpMat1-1r2	GGCTTNGANACCATGGTGAG	<i>MATI-1-1</i> -specific degenerate primer
MgMfSpMat1-2f2	CAAAGAANGCNTCNTGATCT	<i>MATI-2</i> -specific degenerate primer
MgMfSpMat1-2r1	TTCCTCTCNGATGGCTTG	<i>MATI-2</i> -specific degenerate primer
DseptoMat1f	<u>CGCAGTAAGTGATGCCCTGAC</u>	<i>Dothistroma septosporum</i> <i>MATI-1-1</i> -specific primer
DpiniMat1f2	AGTAAG <u>CGACCGCGCTCCCCATG</u>	<i>D. pini</i> <i>MATI-1-1</i> -specific primer
DotMat1r	TTGCCTGACCCGCTGCTGGTG	<i>Dothistroma</i> <i>MATI-1-1</i> -specific primer
DseptoMat2f	GT <u>GAGTGAACGCCGACATGG</u>	<i>D. septosporum</i> <i>MATI-2</i> -specific primer
DpiniMat2f	GT <u>AAGTGATCGTTGAAACATGC</u>	<i>D. pini</i> <i>MATI-2</i> -specific primer
DotMat2r	CTGGCTGTGAAGTCCATCGTC	<i>Dothistroma</i> <i>MATI-2</i> -specific primer

<sup>a</sup> Nucleotides specific to the given *Dothistroma* sp. are underlined.



**Fig. 1.** Amplification products obtained from *Dothistroma septosporum* (in bold face) and *D. pini* isolates containing the partial *MATI-1-1* (914-bp) and *MATI-2* (332-bp) genes using the degenerate primer pairs MgMfSpMAT1-1 and MgMfSpMAT1-2, respectively.



**Fig. 2.** Diagrammatic representation of the full-length *MATI-1-1* and *MATI-2* genes of *Dothistroma septosporum* and *D. pini*. The predicted sites of exons (white bars), and introns (black bars) are shown, and their locations (nucleotide position) are indicated. The areas amplified by the MgMfSpMAT1-1 and MgMfSpMAT1-2 primer sets as well as the mating-type-specific primers for each species are indicated.

*sporum*) was sequenced, and the translated sequence showed 55% identity to a 65-aa ( $E = 1 \times 10^{-19}$ ) and 70% identity to a 27-aa region ( $E = 1 \times 10^{-19}$ ) of the *M. graminicola* mating type 1-2 protein (GenBank accession no. AAL30836) as well as 50% identity to a 65-aa region ( $E = 7 \times 10^{-17}$ ) of the *S. passerinii* MAT-2 protein (GenBank accession no. AAO49358) using Blastx on the GenBank database. This confirmed that the 332-bp fragment is part of the *MATI-2* gene of *D. septosporum*. The 332-bp translated sequences for the fragments obtained from the two *D. pini* strains (CBS 116483 and CBS 116485) showed 52% identity to a 65-aa ( $E = 1 \times 10^{-19}$ ) and 68% identity to a 29-aa region ( $E = 1 \times 10^{-19}$ ) of the *M. graminicola* mating type 1-2 protein (GenBank accession no. AAL30836) as well as a 47% ( $E = 7 \times 10^{-17}$ ) and 68% identity ( $E = 7 \times 10^{-17}$ ) to the same amino acid regions of the *S. passerinii* MAT-2 protein (GenBank accession no. AAO49358). This confirmed that the 332-bp fragment is part of the *MATI-2* gene of *D. pini*. Sequences for the 180-bp (*D. septosporum*) and 280-bp (*D. pini*) fragments showed no homology to protein sequences available in GenBank.

For both of the species, four chromosome walking steps were used to obtain the full-length *MATI-2* gene sequences. The geneid software predicted that the *MATI-2* sequences of both species contain three exons. The predicted length of the genes, as well as exon and intron positions, is illustrated in Figure 2. Although the number of nucleotide and amino acid residues was the same for the *MATI-2* of the two *Dothistroma* spp., an identity of 94.4 and 92.7% was found between the 1,012-nucleotide and the 302-aa residues, respectively. All the introns found for both species contained a perfect lariat sequence. The number of predicted introns (two) of the *Dothistroma* spp. studied correlates with the number predicted for *Cercospora* spp. (25), but the specific locations of these introns within the gene differed. Only one predicted intron was found in the HMG domain of species of *Cercospora* (25), *M. graminicola* (51), and *S. passerinii* (23), whereas two predicted introns were found in the same region of the *Dothistroma* spp. studied.

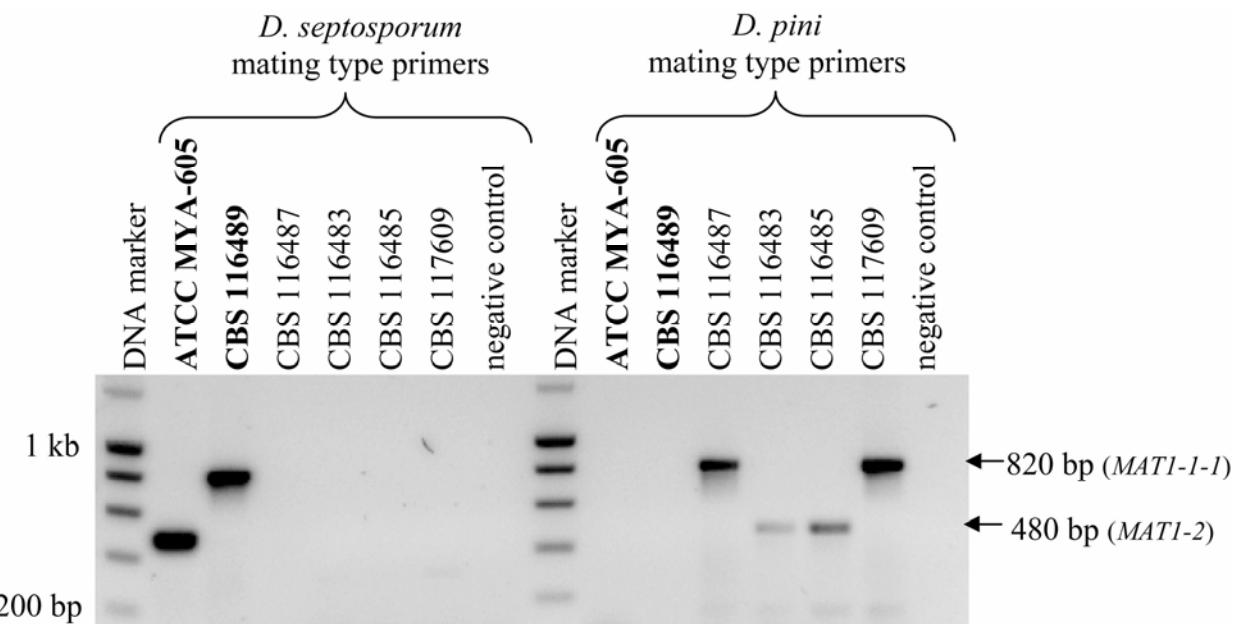
**Screening with *D. pini* and *D. septosporum* mating-type-specific primers.** In the *D. pini* MAT1 isolates, DpiniMat1f2 and DotMat1r amplified an 820-bp fragment and, in the *D. pini* MAT2 isolates, DpiniMat2f and DotMat2r amplified a 480-bp fragment (Fig. 3). Each isolate tested showed either the 820- or 480-bp fragment of the *MATI-1-1* or *MATI-2* genes, respectively. None

of the isolates contained both fragments. The *D. pini* mating-type-specific primers did not amplify the *MATI-1-1* and *MATI-2* fragments in any of the *D. septosporum* isolates (Fig. 3). The majority of the *D. pini* isolates were from areas in the United States where both mating types are known to exist. Eight isolates of each mating type were found for these *D. pini* isolates, whereas only MAT1 isolates were found for the *D. pini* collection from the Ukraine (Table 1). In the *D. septosporum* MAT1 isolates, Dsepto-Mat1f2 and DotMat1r amplified an 820-bp fragment; in the *D. septosporum* MAT2 isolates, DseptoMat2f and DotMat2r amplified a 480-bp fragment (Fig. 3). Each isolate tested showed either the 820- or 480-bp fragment of the *MATI-1-1* or *MATI-2* genes, respectively. None of the isolates amplified both fragments.

The *D. septosporum* mating-type-specific primers did not amplify the *MATI-1-1* and *MATI-2* fragments of the *D. pini* isolates (Fig. 3). In all, 20 *D. pini* and 210 *D. septosporum* isolates (Table 1) were screened with the two mating-type-specific primer sets to determine the mating type and to confirm the identity of each isolate. All *D. septosporum* isolates obtained from Chile, Australia, and New Zealand contained only the *MATI-2*. In contrast, isolates representing both mating types were present in the Austria, Canada, Poland, South Africa, and United Kingdom collections. Only one isolate was available each from Germany, Brazil, France, Guatemala, Slovakia, and the United States. All of these isolates contained the *MATI-2* gene, except for the isolate from Slovakia that contained *MATI-1-1*.

**Phylogenetic analyses.** The alignment of partial *MATI-1-1* nucleotide sequences ( $\alpha$  domain) contained 21 strains, including *M. grisea* as the outgroup, and had a total length of 174 characters. Of the 174 characters, 23 were constant, 15 were variable and uninformative, and 136 were parsimony informative. The alignment of partial *MATI-2* nucleotide sequences (HMG domain) contained 21 strains, including *M. grisea* as outgroup, and had a total length of 253 characters. Of the 249 characters, 37 were constant, 13 were variable and uninformative, and 199 were parsimony informative. Two equally parsimonious trees were obtained from each of the *MATI-1* alignments (Fig. 4A; tree length of 638 steps; CI = 0.498, RI = 0.649, RC = 0.324) and from the *MATI-2* alignment (Fig. 4B; tree length of 886 steps; CI = 0.512, RI = 0.659, RC = 0.338).

The topology of the phylogenetic trees using the  $\alpha$  domain (Fig. 4A) and HMG domain (Fig. 4B) sequences were similar.



**Fig. 3.** *Dothistroma septosporum* (bold face) and *D. pini* isolates screened using the Dsepto/Dpini/DotMat1 primer set (820-bp fragment) and the same *Dothistroma* isolates screened with the Dsepto/Dpini/DotMat2 primer set (480-bp fragment).

The Capnodiales, Hypocreales, and Pleosporales clades showed high bootstrap support (92 to 97%) in both trees. The phylogenetic analysis using the DNA sequences in the HMG-box and  $\alpha$  domain showed that *D. pini* and *D. septosporum*, respectively, are phylogenetically closely related to *Cercospora* spp., *M. graminicola*, and *S. passerinii* as illustrated by the 92% (*MATI-1-1*) and 97% (*MATI-2*) bootstrap support values.

## DISCUSSION

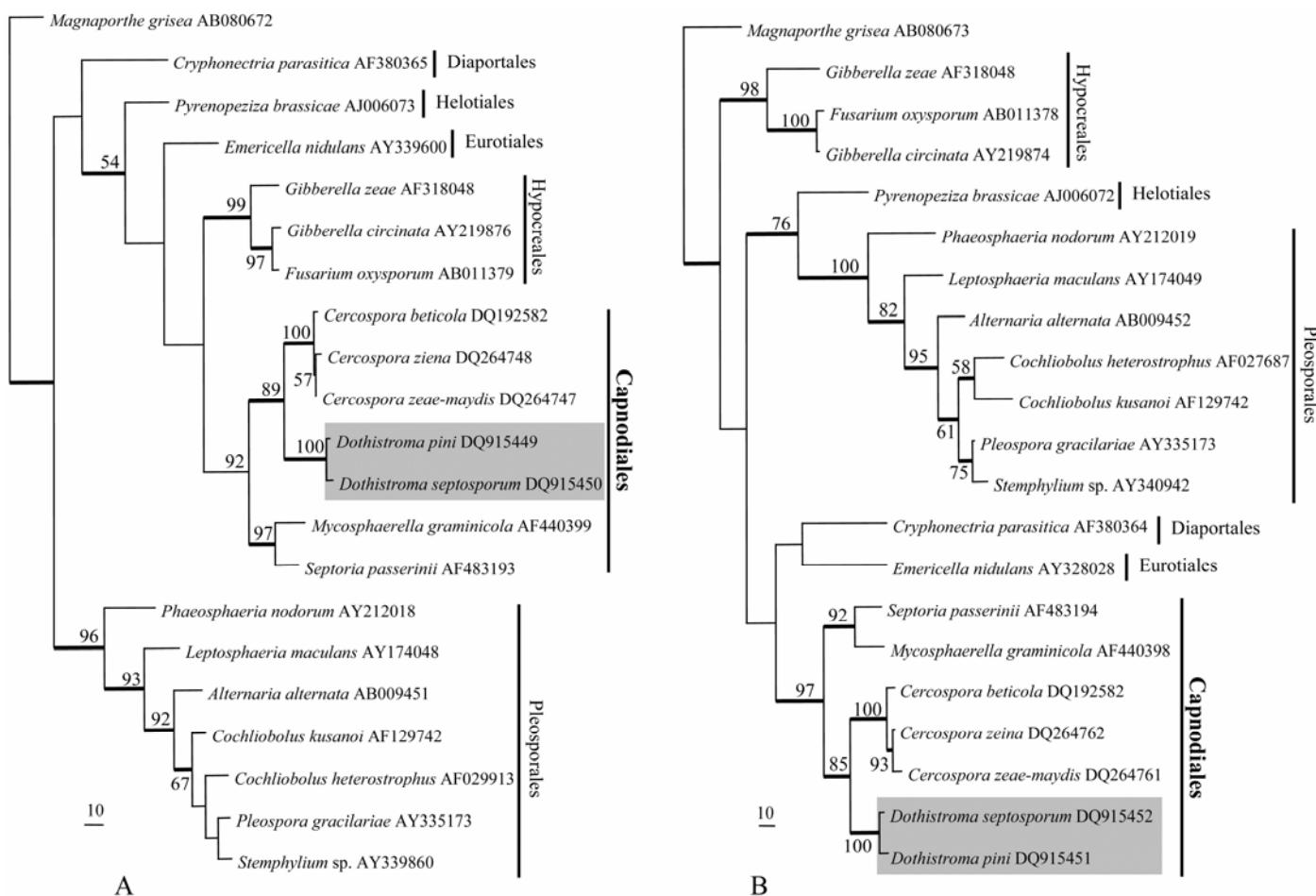
This study represents the first attempt to ascertain which mating types are present in the different countries where diseases caused by *D. septosporum* and *D. pini* occur. In this regard, emphasis is on *D. septosporum*, because it has been introduced into numerous countries, where it has caused very damaging disease problems. Thus, the degenerate primer sets MgMfSpMAT1-1 and MgMfSpMAT1-2 (25) were used successfully to amplify portions of the mating type genes of *D. septosporum* and *D. pini*. This made it possible to characterize the full-length *MATI-1-1* or *MATI-2* genes of both species.

The *MATI-1-1* and *MATI-2* genes characterized for *D. septosporum* and *D. pini* in this study contained areas that correspond to a putative  $\alpha$  domain and an HMG domain also described for the *MATI-1-1* and *MATI-2* of other ascomycetes. The two putative introns in the  $\alpha$  domains of the *Dothistroma* *MATI-1-1* also have been found in corresponding areas in *M. graminicola* (50), *S. passerinii* (23), and several *Cercospora* spp. (25). However, the third predicted intron in the downstream area flanking the  $\alpha$  domain of the *MATI-1-1* of both *Dothistroma* spp. is present only

in the *Cercospora* sp., and not in *M. graminicola* or *S. passerinii*. The number of introns found in the HMG domain of the *MATI-2* in both *Dothistroma* spp. differed from that of closely related species. The first predicted intron also is present in *M. graminicola* (50), *S. passerinii* (23), and *Cercospora* spp. (25). In contrast, the second predicted intron is present only in the *MATI-2* of the *Dothistroma* spp., and not in any other members of the Mycosphaerellaceae thus far studied. These data indicate that clear differences can be found even within the conserved regions of the corresponding genes in different *Mycosphaerella* spp.

The predicted length of the encoded proteins among different *MATI-1-1* and *MATI-2* genes of ascomycetes varies greatly (23,25,40). In most species, the MAT1 protein is much larger than the MAT2. Results of this study have shown that this also is the case for the *Dothistroma* spp., where 387 aa were found for MAT1 and 302 aa for MAT2. Expression studies have not been done on the mating type genes of any of the above-mentioned members of the Mycosphaerellaceae. Additional studies at the mRNA and protein levels would be necessary to confirm the exact length of the coding regions and the intron and exon boundaries for the mating type genes of the *Dothistroma* spp.

Results of this study showed substantial differences between the nucleotide as well as amino acid sequences of the corresponding mating type genes and proteins of *D. septosporum* and *D. pini*. Using nucleotide sequences for phylogenetic inference in these fungi is consistent with previous studies where conserved domains within the mating type genes have been used to study the phylogenetic relationships among different fungal species and families (12,25,34,35,52). Differences in mating type sequences



**Fig. 4.** One of two equally parsimonious trees obtained from each of the **A**, *MATI-1-1* sequence alignment rooted to *Magnaporthe grisea* (AB080672) and **B**, *MATI-2* sequence alignment rooted to *M. grisea* (AB080673). In both trees, bootstrap support values from 1,000 replicates are shown at the nodes, whereas thickened lines indicate strict consensus branches.

for *D. septosporum* and *D. pini* show that these species are distinct genetic entities and provides strong support for the results of Barnes et al. (2), who provided the first DNA-based evidence that the species are distinct.

Based on morphological characteristics, Barr (3) attempted to reclassify *Mycosphaerella pini* in a new genus outside of *Mycosphaerella*. However, molecular phylogenetic analyses have shown that *Mycosphaerella* is the most appropriate designation for this fungus classification (2,22). Phylogenetic analyses, based on the sequences of the HMG and  $\alpha$  domains, also confirm that *Dothistroma* spp. are members of the Mycosphaerellaceae. All remaining species also grouped within their corresponding families; however, the relationship between different families is unresolved.

The mating-type-specific primer sets developed in this study, DpiniMat1 and DpiniMat2 as well as DseptoMat1 and DseptoMat2, can be used effectively in multiplex PCR assays to amplify areas within the mating type genes for *D. pini* and *D. septosporum* populations, respectively. These primers also can be used to distinguish between the two *Dothistroma* spp., making them useful tools for rapid and accurate diagnoses of two important pathogens that are morphologically similar. Prior to this study, the only diagnostic tool available to distinguish between *D. pini* and *D. septosporum*, was to amplify the ITS of the ribosomal DNA region with universal primers and then to digest the amplicon with the restriction endonuclease *Alu*I (2). Although the latter technique is useful, the ITS amplicon of *D. pini* is digested into two fragments whereas that of *D. septosporum* is not. Therefore, to prevent a false positive result for *D. septosporum*, a prior confirmation that the fungus is a *Dothistroma* sp. is required. The mating-type-specific primer sets emerging from this study are species specific and do not require a prior view on the identity of unknown isolates. They are, therefore, multifunctional and can be used for the rapid identification of the species as well as its mating type.

Although results of this study have shown that *D. pini* is probably heterothallic with a single isolate containing only one of the two mating type genes, no teleomorph has yet been linked to this species. Where both mating types were observed for the isolates from the United States, the sexual state most likely is present, but has not been observed. In contrast, the *M. pini* teleomorph of *D. septosporum* previously has been observed in some parts of the United States (9,38,39) where *D. pini* is predominantly found. Given that the anamorphs of these fungi are morphologically similar and have been confused in the past, it is possible that teleomorph structures reported for *D. septosporum* could have been linked to *D. pini* and not to *D. septosporum*.

Although a small number of isolates were screened for most countries, this study shows that *D. septosporum* probably are heterothallic and that one mating type (MAT2) seems to be more prevalent in several of the collections studied (e.g., New Zealand). Although sexual reproduction has been confirmed in *D. septosporum*, asexual reproduction happens more frequently, and the absence or rarity of the opposite mating type (MAT1) in most of the collections can explain the common occurrence of the asexual stage. Therefore, it also is possible that the teleomorph is not as rare as first believed. We found that both mating types exist within *D. septosporum* populations from Europe (Poland and Austria) and Canada, where the sexual stage (*M. pini*) has been reported in the past (7,15,17,28,29). However, the teleomorph has never been found in countries in the Southern Hemisphere such as Chile, Australia, and New Zealand, where these pathogens have long been a major problem (14,31). These are also the countries for which only one mating type (MAT2) has been observed, and this might explain the absence or rarity of the sexual stage.

Discovery in this study of only a single mating type of *D. septosporum* in New Zealand, Australian, and Chilean collections can be explained by the fact that the fungus is an introduced

pathogen in those countries. For New Zealand, Hirst et al. (26) also found that no genetic variation exist among isolates of a *D. septosporum* population, which is strongly supported by the results of the present study. Dothistroma needle blight was introduced in Australia in the 1970s and it was suggested that this occurred by natural means, with conidia being blown across the Tasman Sea from New Zealand. This view was supported by the fact that the strict quarantine regulations in Australia would have made it unlikely that infected plant material entered the country (13,31,33). The presence of only one mating type shown in this study and the fact that no genetic diversity has been found yet for the pathogen in New Zealand (26) supports the view that only one genotype was introduced into or became established in Australia and New Zealand. Asexual reproduction evidently has perpetuated the spread of the fungus subsequently. We suspect that the same situation will have been true for Chile.

An intriguing result of this study has been the discovery that both mating types of *D. septosporum* exist in the South African and United Kingdom collections. This is especially interesting because the pathogen is non-native in these countries and it might have been expected that the situation would have been similar to that in other countries such as New Zealand, where the pathogen also is an alien invasive. In addition, the teleomorph of *D. septosporum* has never been observed in South Africa (M. J. Wingfield, *unpublished data*) and the United Kingdom (A. V. Brown, *unpublished data*), despite concerted efforts to detect it.

It is important to recognize that the presence of both mating types of *D. septosporum* in these two countries could indicate the presence of clandestine sex in the fungus. This would indicate the potential for the pathogen to evolve more effectively in these countries than would be true elsewhere in the world, where only a single mating type exists. Such change in the fungus could complicate efforts to develop trees resistant to Dothistroma needle blight infection in South Africa and the United Kingdom. In this regard, it has been shown previously that the introduction of the second mating type of a pathogen can cause rapid increase in virulence, gene transfer, and genetic variation, such as in *Phytophthora infestans* (16,30,42,45) and *Ophiostoma novo-ulmi* (36). This implies that the accidental introduction of the opposite mating type of *D. septosporum* into countries such as New Zealand, Australia, and Chile could seriously exacerbate red band needle disease in those countries. Thus, every effort must be made to ensure that new mating types of *D. septosporum* do not enter these countries.

There has been a dramatic increase in the impact of Dothistroma needle blight caused by *D. septosporum* in western Canada, the United States, and the United Kingdom in recent years (5,6,53). Possible reasons for this change in the disease situation in these countries are an abundance of host material or a directional climate change, as suggested by Woods et al. (54). The discovery that both mating types exist in these countries is another factor that can contribute to the change in the disease situation. The presence of both mating types increases the possibility for sexual reproduction. This, in turn, can lead to the exchange of genetic material between different strains, resulting in a possible increase in the viability of this species. Therefore, further investigation is necessary to determine whether the presence of both mating types, which could increase genetic diversity, a dramatic climate change, or possibly a combination of both these factors might account for the drastic increase in the severity of this disease.

Because only one mating type of *D. septosporum* appears to be present in most countries of the Southern Hemisphere, it is important to restrict the MAT1 isolates to their present locations. This can be achieved through refining quarantine regulations based on the knowledge that only one mating type of the pathogen is present in the country. The mating-type-specific PCR developed during this study could be implemented easily as a control method to

test for the presence of the mating types for *Dothistroma* spp. in pine plantations. One of the weaknesses of quarantine regulations internationally is that they typically rely on lists of names of pathogens rather than on knowledge of their biology and population genetics. Results of this study have provided valuable new insights into the distribution of mating types of *D. septosporum* and *D. pini* that should enhance the quality of quarantine regulations in the future.

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