Taxonomy, phylogeny and population biology of the red band needle blight pathogen and related species

BY

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M.Sc. (U. P.)

Submitted in partial fulfillment of the requirements for the degree

PHILOSOPHIAE DOCTOR

in the Faculty of Natural and Agricultural Sciences, Department of Genetics, University of Pretoria, Pretoria, South Africa

January 2009

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DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree *Philosophiae Doctor* to the University of Pretoria, contains my own independent work and has hitherto not been submitted for any degree at any other University.

Irene Barnes

January 2009

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I dedicate this thesis to my beloved children Sandra and Byron who were "born" with this work and who became my additional research chapters

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to the following people:

Mike and Brenda Wingfield for giving me the opportunity to build my castle with golden bricks. Mine is all on the "love" list of FABI.

Thomas Kirisits: our chance meeting at a conference gala dinner was the start of a wonderful collaboration. Much of the work in this thesis was as a result of your interest in my work, collections that you made and even field trips that you organised. You have become an incredible mentor and friend, and your advice has been invaluable. Thank you.

Joha Grobbelaar and Maria Noél Cortinas: It was great to have your company to travel this long, sometimes hard but mostly fun, journey together. We have suffered through the fiasco of Fiasco and others, but we persevered and prevailed. I am honoured and pleased that we, as friends and colleagues, have had the opportunity to publish together.

To Grace Nakabonge, Dina Paciura, Alvaro Durán and Ryan Nadel for making the X^2 lab a really fun, inspirational and dynamic place to be.

To Bernard Slippers, whose debates and discussions I have enjoyed and whose insights into my work are appreciated. To Renate Zipfel for the incredible service you have provided.

To my husband, Kevin Barnes, who is the light of my life. Thank you for your endless encouragement, understanding and support.

To my sister and friend, Karen Vincent, who has always been my solid, supportive rock in every possible way. Thank you for your tremendous help at work and at home.

To the Lord, for making this all possible.

PREFACE

Chapter one of this thesis presents the literature pertaining to biological invasions and the different stages of invasions in terms of plant pathogens. The review focuses on determining areas where studying the population genetics of a pathogen might complement the knowledge of the ecology of the pathogen in order to gain a better understanding of their evolutionary and invasive potential. *Dothistroma septosporum,* which causes Dothistroma needle blight (DNB), is one of the better known examples of an invasive plant pathogen and is the focus of this thesis. After presenting an overview of the pathogen, it is concluded that a thorough knowledge of the ecological aspects of the pathogen has been gained by many intensive studies but that there is a distinct, and perhaps dangerous, lack of knowledge pertaining to the global population genetics of this pathogen.

Throughout the years of this study, DNB has become a global concern as more epidemics were being reported, not only in the Southern Hemisphere where the disease has been problematic for over 50 years, but also in the Northern Hemisphere in both native and non-native forests. An "International Dothistroma Alliance Committee" was established in 2004 among researchers world-wide with the aim being to share and integrate knowledge about the pathogen and the disease it causes. The main focus of this group incorporates aspects such as diagnostics and monitoring, assessing pathogen risk (pest-risk), disease impact, management and pathogen variability as well as population diversity. The research in this thesis greatly assists in the latter two aims of the committee.

In the second chapter, multigene phylogenies were constructed from *Dothistroma septosporum* isolates obtained worldwide. These studies revealed that the disease is not caused by one pathogen with varying morphotypes, but that two, very closely related sibling species are responsible. A thorough description of the two species, named *Dothistroma septosporum* and *D. pini,* is provided. It is also shown in Chapter six that both species are capable of infecting the same needle and can co-exist within the same conidioma. There is thus a distinct potential for hybridisation to occur between these two species.

Coincident with the initiation of this research is an increase globally in the incidence of DNB. This increased the knowledge base about the pathogens around the world and also made it easier to obtain cultures and isolates for this study from different sources. As a consequence, an understanding of the distribution and occurrence of each species was possible and is

documented in Chapters three and six. The molecular techniques developed in this work have enabled the development of a robust technique to distinguish between the two pathogens causing DNB. A combination of the species-specific mating type markers designed by Groenewald *et al.* (2007), the diagnostic Primer_A, and an effective species-specific RFLP test, provide a quick and effective means of identifying the DNB pathogens, directly or indirectly, from conidiomata on infected needles.

In order to study the population diversity of the DNB fungi, twelve microsatellite markers were developed in Chapter three. In Chapter four, the preliminary applications of these markers provide a first glimpse of the global diversity of *D. septosporum* which has caused the most devastation world-wide. From this research it is also obvious that the pattern of diversity reflects the movement of its host (pine) from its native Northern Hemisphere to various countries within the Southern Hemisphere.

Dothistroma septosporum is an important disease of both plantation grown pines and native forests. Climate change and the continual movement of infected plant material pose great threats to existing forests. The fact that a sibling species has been discovered shows the evolutionary potential of the species to adapt to changing environments. Successful quarantine and monitoring will aid in curbing the further spread of the disease into areas where it could, potentially, be more devastating.

The chapters in this thesis correspond to different research projects and are represented in the text in the format of a publication. Due to the nature of this style, however, there is some unavoidable repetition in the text, especially within the introduction of each chapter. Three of the chapters within this thesis have been published in internationally recognised ISI rated journals.

Chapter 1

Biological invasions: A pine pathogen perspective with special reference to Dothistroma needle blight

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1.0 INTRODUCTION

Biological invasions and their potential impact on the environment have been recognised and studied for many years (Elton 1958, Richardson & Pyšek 2008, Lockwood *et al.* 2008). However, during the course of the last two decades, there has been a surge of interest, and number of publications, on this topic (Figure 1). Entire journals are now dedicated to this area of science and books have been written relating to biological invasions, invasion biology, alien invasives and emerging pests and diseases etc. (Table 1). The central theme of this recent focus is the introduction of non-native species into new environments and the subsequent impact that they have had on native species, communities and ecosystems.

In addition to the proliferation of literature, many committees have been established to deliberate on issues pertaining to biological invasions. Websites with general information and databases have been constructed with the intention of increasing public awareness and establishing the ecological and economic impact that invasive species have on environments and economies (Table 1). For example, the newly established DAISIE (Delivering Alien Invasive Species Inventories for Europe: www.europe-aliens.org), estimates that over 11,000 alien invasives have been introduced into Europe. Over 50,000 aliens are reportedly established in the United States causing an estimated economic loss of U.S. $125 billion per year (Pimentel *et al.* 2000). A global estimate of the damage caused by invasive species amounts to £1.5 trillion per year; approximately 5 % of the global GDP (CABI: http://www.cabi.org/datapage.asp?iDocID=173).

Invasive or alien species are defined as plants, animals or micro-organisms (fungi, bacteria, viruses) that are present in a new environment (Drake *et al.* 1989). They include competitors, predators, pathogens and parasites and are present in almost all native ecosystems. Generally, invasive species have a profound, often irreversible, impact on agriculture, recreation, and natural resources and they thus pose a serious threat to global biodiversity, food security, habitat quality, ecosystem functionality and economic development (Sakai *et al.* 2001, Allendorf & Lundquist 2003). The key factors associated with the increase in biological invasions during the last half century have been the growth of globalization through commercial trade and travel, resulting in an escalating introduction of organisms into new environments, either intentionally or accidentally (Mack & Lonsdale 2001, Rossman 2001, Wingfield *et al.* 2001, Brasier 2008). In addition, climate change is believed to have an

additive effect to the impact of invasives (Ayres & Lombardero 2000, Lebarbenchon *et al.* 2008).

Various life history traits and characteristics of organisms contribute strongly to the invasiveness of species. These characteristics have been very well studied in plants and animals but less so in terms of fungi and other micro-organisms (Desprez-Loustau *et al.* 2007). Invasive traits in plants, for example, include their ability to become "weedy" due to early germination with rapid growth from seedling to sexual maturity, the potential to reproduce both sexually and asexually, high tolerance to environmental heterogeneity and adaptation to environmental stress (Heywood 1989, Rejmanek & Richardson 1996). For micro-organisms, effective survival and dispersal mechanisms would be important life-history traits, as well as their ability to infect hosts and reproduce rapidly. For example, some fungi produce clamydospores that are resistant to environmental stresses and that provide an important survival strategy for the pathogen, especially during movement or transportation (Desprez-Loustau *et al.* 2007). Likewise, a common factor favouring spread is the fact that fungi produce large numbers of spores that are easily dispersed by wind and water (Agrios 1997).

Although the ecology of invasives has been well studied and documented (Lockwood *et al.* 2008, Richardson & Pyšek 2008), the genetic and evolutionary factors contributing to biological invasions are increasingly being seen to play an even more important role in understanding these invasions (Kirkpatrick & Barton 1997, Barrett *et al.* 2008). Understanding the population biology of a species makes it possible to predict aspects related to the invasive potential of the species (Allendorf & Lundquist 2003, Richardson *et al.* 2005). For example, introduced species can encounter novel stresses and selection pressures to which they might be poorly adapted (Gilbert 2002, Prentis *et al.* 2008). They might, however, be able to overcome these selection pressures by some form of adaptation or evolution due to increased genetic diversity or evolution in quantitative traits, giving them the ability to then become invasive. Studying potentially invasive species at a population level can provide an indication of the genetic factors that predisposes a species to adapt and become invasive (McDonald & Linde 2002). Thus, studies of population structure, genetic diversity, geographic patterns, range expansion, lag times and the potential for rapid evolution of invasive organisms may offer a unique understanding of the dynamics involved in the colonisation and spread of invasive species (Sakai *et al.* 2001, Prentis *et al.* 2008). This knowledge could also contribute to the management of these species by providing insight into

the stage of the invasion process that an organism has reached. This in turn could highlight whether quarantine measures are likely to be effective or whether other actions such as eradication, would be more practical (Allendorf & Lundquist 2003). Therefore, it is important to combine both the knowledge of ecology and the population genetics of organisms when attempting to predict invasions or control the invasives that cause them.

In comparison to their plant and animal counterparts, plant pathogens have not been widely studied as invasives. This is surprising given the fact that their impact has been felt for many centuries. Indeed, the rust God Robigo, who was created by the Romans a few hundred years B.C., was offered sacrifices of red dogs and sheep during the special spring holiday known as Robigalia in order to prevent infection of their grain crops by rusts (Agrios 1997).

Perhaps the two best known biological invasions caused by plant pathogens, and which have had huge impacts, are those of late blight of potatoes and chestnut blight. Late blight, caused by *Phytophthora infestans*, destroyed the entire potato crop in Ireland in 1845 leading to a large-scale famine that resulted in the deaths of hundreds of thousands of people and prompted over 1.5 million people to emigrate from Ireland to the United States (Large 1940, Bourke 1964). This epidemic was caused by a single asexually reproducing strain of *P. infestans* that was introduced from Mexico via the United States (Fry *et al.* 1993, Goodwin 1997). Similarly, *Cryphonectria parasitica* was introduced into North America from Asia around 1900 and destroyed almost all of the American chestnut throughout its native range (Elton 1958, Hepting 1974, Anagnostakis 1987).

Since the first alien invasive plant pathogens were described, there has been an exponential increase of invasive plant pathogens reported globally (Brasier 2008). In France for example, it has been documented that the rate of introduction of invasive fungi has included, on average, two species per year since the 1970's, compared to 0.5 species per year before 1930 (Desprez-Loustau 2009). The disturbing realisation that less than 10 % of the world fungi are known (Hawksworth 2001) is of particular concern, considering the potential that some of these organisms might have to cause major diseases.

The invasion process resulting from non-native species being introduced into a new area includes a sequence of events that can be divided into several definitive stages (Figure 2): These are (a) The introduction or arrival into a new habitat; (b) Initial colonisation and survival; (c) Establishment and (d) Subsequent dispersal or spread of the pathogen. During all

of these stages, there is great potential for genetic change to occur. Studying population genetic parameters such as genetic drift, gene flow, hybridisation, natural selection and adaptation could be useful in predicting whether or not a non-native or introduced species will have the potential to ultimately become invasive (Sakai *et al.* 2001, Allendorf & Lundquist 2003).

This review is arranged in three main sections. The first section provides a discussion of the different stages of invasiveness, particularly in the case of fungal plant pathogens. For each of these stages, a few examples are given where population genetic studies have provided a better understanding of the biology of the plant pathogen and aspects related to the components of its invasiveness (Table 2). There are different categories of invasiveness related to whether the host or pathogen is native or introduced and vice-versa (Figure 2). In the second section, these situations are described, specifically using examples of pine species and their fungal pathogens. Fungal pathogens chosen to illustrate these situations are either well known or those that have been particularly damaging. The third section of this review presents a case study based on the pine pathogen *Dothistroma septosporum,* which is known to be invasive in many parts of the world.

2.0 STAGES IN THE FUNGAL PLANT PATHOGEN INVASION PROCESS

2.1 Introduction of the pathogen

The first stage in the invasion process is marked by the arrival of the pathogen in a new environment. This occurs either through short distance dispersal, discussed later in this review, or long distance movement (Brown & Hovmøller 2002, Stukenbrock *et al.* 2006). Long distance transport of invasive species is usually considered to be a consequence of human activities, either directly or indirectly, and resulting from trade and travel (Mack & Lonsdale 2001, Rossman 2001, Wingfield *et al.* 2001, Brasier 2008, Sakai *et al.* 2001).

For many centuries, plants and plant parts have been moved around for agricultural, forestry and ornamental purposes, usually without quarantine to guard against the movement of pathogenic organisms (Burgess & Wingfield 2001). Thus, numerous pathogens associated with the plants or plant parts have been moved extensively. The pathogens easily avoid detection, in part due to their microscopic nature. Many also exist as symptom-less

endophytes within the plants (Schoeneweiss 1983, Stanosz *et al.* 1997), and in other cases infections are latent with symptoms only developing later (Bassett & Fenn 1984, Desprez-Loustau *et al.* 2007).

In some rusts, latency can last up to two years before symptoms develop (Diekmann *et al.* 2002). *Cronartium ribicola*, for example, is a fungal stem rust pathogen that was introduced into Western North America from Europe with shipments of eastern white pine seedlings, grown in France in 1910 (Allen & Humble 2002). In another pine example, *Diplodia pinea* (syn. *Sphaeropsis sapinea*), an endophyte and latent pathogen of pines (Smith *et al.* 1996, Flowers *et al.* 2003), was probably introduced into the Southern Hemisphere along with its host (Burgess *et al.* 2001b). Likewise, the grape pathogen *Plasmopara viticola*, a downy mildew oomycete, was introduced into Europe in 1878 with pylloxera-resistant wild American rootstocks from North America (Gobbin *et al.* 2006, Hesler 2008).

Introductions of plant pathogens are not always accidental. Fungi are for example introduced for biological control purposes (Kok *et al.* 2000, Desprez-Loustau *et al.* 2007, Goettel 1995). Likewise, beneficial mycorrhizal species have been widely moved to facilitate the establishment of commercial forestry plantations (Richardson 1998).

Long distance dispersal of pathogens can occur naturally by means of wind blown spores. This means of dispersal occurs in some fungi such as rusts that can spread on a continental and global scale (Brown & Hovmøller 2002). Aylor (2003), for example, calculated that spores of the oomycete *Peronospora tabacina* (tobacco blue mould disease) can move on average between 9 and 18 km per day via wind currents and that it has the capacity to cover distances of 500 – 1000 km. Similarly, the urediniospores of the wheat stem rust pathogen *Puccinia graminis* f. sp. *tritici* can move between 16 and 76 km per day and could spread anywhere between 1500 – 2500 km via wind currents in the upper atmosphere. In one striking case, distinct pathotypes of *Puccinia graminis* f.sp. *tritici* were shown to have migrated from Southern Africa, across the Indian Ocean, to Australia in a single flight (Burdon *et al.* 1982). Meteorological data captured with high-altitude balloons showed that at this time, circular patterns were responsible for this long-distance dispersal. These amazingly effective dispersal mechanisms clearly illustrate a substantial threat to agriculture and forestry because the nature of the spread is natural, substantially complicating disease prediction and control.

The origin or source population of invasive pathogens can be elucidated by studying the levels of diversity between pathogen populations, co-evolution between host and pathogen, and by determining the historical source and sink patterns of migration amongst populations (Sakai *et al.* 2001, McDonald & Linde 2002, Burdon *et al.* 2006, Desprez-Loustau *et al.* 2007). For example, the highest levels of genetic variability observed in Central Asian isolates of the apple scab pathogen, V*enturia inaequalis,* showed that the geographic origin of the pathogen is the same as that of the host (Gladieux *et al.* 2008). Similarly, North America is considered to be the centre of origin for *Plasmopara viticola* given that American grape varieties show resistance to the pathogen (Gobbin *et al.* 2006). Banke *et al.* (2004), used phylogeographical studies to show that the origin of the wheat pathogen, *Mycosphaerella graminicola* was in the Middle East, which is considered to be the centre of origin of wheat (Harlan 1971). This was based on high sequence diversity found in these populations. Low levels of sequence divergence were found in the "new world" (North and South America and Australia) populations and thus suggested that recent migrations of this pathogen into these regions occurred as a direct consequence of the international wheat trade (Banke & McDonald 2005).

2.2 Host colonisation

After a pathogen has entered a new environment, the invasion process proceeds with a susceptible host plant being successfully infected (Sakai *et al.* 2001). Several genetic indicators can be used to distinguish between populations in their initial stages of colonisation and those that are already established.

2.2.1 Reduced genetic diversity

The main characteristic of a population that has successfully colonised its host plant is found in its reduced genetic diversity (Sakai *et al.* 2001). This generally arises from founder effects or genetic bottlenecks (McDonald & Linde 2002). Thus, a limited number of organisms are able to establish themselves and the genetic diversity of the invasive population is typically lower than that of the source population (Nei *et al.* 1975, Hartl & Clark 1989).

Founder effects result in the presence of only a few alleles of a pathogen population due to the small number of initial colonists (Sakai *et al.* 2001, McDonald & Linde 2002). Thus introduced populations have lower levels of diversity compared to those from which they were derived (Sakai *et al.* 2001, Dlugosch & Parker 2008). For example, the pine pitch canker

pathogen *Fusarium circinatum,* which causes pitch canker of pine, has limited genetic variation in its areas of introduction compared to that in its native geographic range (Wikler & Gordon 2000). Likewise, Carlier *et al.* (1996) showed that *Mycosphaerella fijiensis,* the causal agent of Black leaf streak disease of banana, has high genetic diversity and large numbers of private alleles at low frequency in its area of origin within South-East Asia. Reduced levels of genetic diversity were, however, observed in recently established populations in Latin America, the Pacific Islands and Africa. The majority of alleles observed for the pathogen in these countries were also present in South-East Asia (Carlier *et al.* 1996).

Reduced genetic diversity is thought to have a negative effect on populations because they would be more susceptible to genetic drift (McDonald & Linde 2002). Genetic drift increases the risk of losing alleles that might confer adaptive abilities for organisms to survive in their new environment (Sakai *et al.* 2001, Allendorf & Lundquist 2003). This is, however, not a limiting factor for some plant pathogenic fungi, especially where they are able to reproduce asexually or where clonal lineages of one mating type commonly dominate an area. In such cases, substantial disease occurs together with an expansion in the geographic range of the pathogen (Goodwin 1997, Milgroom *et al.* 2008). *Cryphonectria parasitica,* for example, has recently been shown to have colonized south-eastern Europe and it is spreading through many adjacent countries. These populations are all considered clonal based on low levels of genetic diversity and vegetative compatibility types and the presence of one dominating mating type. Milgroom *et al.* (2008) proposed that these “clones have greater fitness than others” and that they are able to spread because they are well adapted to the new environmental conditions.

2.2.2 Multiple introductions

Not all introduced populations are characterized by reduced genetic diversity. Many population genetic studies have found substantial levels of variation in fungal populations outside their area of origin, suggesting multiple introduction events (Dlugosch & Parker 2008). Multiple introductions can increase the diversity of the population through the introduction of different alleles. This is especially apparent where the introduction events are from different geographic sources. This situation can result in invasive populations that are more genetically diverse than a single source population. In a study by Gobbin *et al.* (2006), high levels of genetic diversity due to large numbers of alleles were found within and amongst introduced *Plasmopara viticola* populations from Europe. They suggested that several genotypes had been introduced into Europe and that these introductions occurred multiple times. The source of these introductions was probably with grapevine propagation material

that was introduced into Europe multiple times since the early 1800's. Similarly, the European race of *Gremmeniella albietina* that causes scleroderris canker has been introduced into North America multiple times as indicated by RAPD marker analyses (Hamelin *et al.* 1998). Likewise, multiple introductions have also been suggested for *Phaeosphaeria nodorum* in North America (Stukenbrock *et al.* 2006) and *Diplodia pinea* in South Africa (Burgess *et al.* 2001b).

2.2.3 Population structure and reproductive mode

Introduced populations typically display changes in population structure and reproductive mode (Sakai *et al.* 2001, Desprez-Loustau *et al.* 2007). For example, where sexual reproduction is common in a fungus, random association amongst alleles is expected (Milgroom 1996). Newly colonized populations could show evidence of linkage disequilibrium (non-random associations between unlinked markers) due to influences such as small populations, genetic drift or the introduction of populations with different allele frequencies (Gladieux *et al.* 2008). Linkage disequilibrium created by drift can be reduced, over time, through continual migration of individuals amongst populations, gene flow and growth of the population (Slatkin 1987, Carlier *et al.* 1996, Dlugosch & Parker 2008, Gladieux *et al.* 2008). Shifts in the reproductive mode could, however, be hampered if only one mating type in a sexually reproducing pathogen is introduced, resulting in the establishment of asexual populations (Gladieux *et al.* 2008). The presence of only one mating type is, therefore, an obvious indication that a pathogen has been introduced (Goodwin *et al.* 1994, Engelbrecht *et al.* 2004).

2.3 Establishment

Many factors combined determine whether a pathogen population will become successfully established in a new environment. Thus, effective establishment is determined by various life-history characteristics of the pathogen, properties of the host such as susceptibility and genetic diversity, as well as by the environment (Sakai *et al.* 2001).

2.3.1 Competition

One of the main factors contributing to successful establishment of plants and animals lies in their ability to compete. Thus, invader species typically have enhanced capabilities to utilize the local resources as compared with native species (Sakai *et al.* 2001, Allendorf & Lundquist 2003). Although this characteristic has been observed in plant pathogens, its contribution to

invasion success is poorly understood and only a few examples have been documented. In Africa, Latin America and the Pacific Islands, the introduced fungal pathogen *Mycosphaerella fijiensis* dominates and has replaced populations of *Mycosphaerella musicola*, which causes Sigatoka disease of banana (Carlier *et al.* 1996). Likewise, it has been hypothesized that *Cryphonectria radicalis*, a local saprophytic species in Europe, is being replaced by the introduced pathogen *C. parasitica* (Hoegger *et al.* 2002). The classic example is that of the Dutch elm disease in Europe and North America where the more aggressive species, *Ophiostoma novo-ulmi* out-competes the non-aggressive strain *O. ulmi* (Houston 1985, Brasier 2001). This is due to the fitness advantage that *Ophiostoma novo-ulmi* has over *O. ulmi,* which includes traits such as competitive antagonism, enhanced ability to utilize host resources (pathogenicity) and better adaptation to the climate (Brasier 2001). Thus, a situation has been reached where *O. ulmi* is no longer present in Great Britain due to its complete replacement by *O. novo-ulmi* (Houston 1985).

2.3.2 Host specificity and range expansion

One of the main contributing factors to the successful establishment of plant pathogens as invasives lies in their capacity to survive and proliferate in a new environment. This typically depends on the host specificity of the pathogen as well as the availability of a susceptible host (Barrett *el al.* 2008). These factors could impact on the rate of range expansion and the boundary of the species (Antonovics 1976, Crawley 1986). Many obligate pathogens are usually host specific and occur in very specific environments (Lajeunesse & Forbes 2002). This is likely because they have often evolved in parallel with their hosts (Barrett *et al.* 2008).

Forest pathogens provide some good examples where host specificity is often high. These pathogens may have high levels of virulence, resulting in devastating mortality, but this effect is limited by the range and spread of the host species (Loo 2008). For example, tree pathogens in North America that are limited to either a single or low number of host species, include *Ophiostoma ulmi* and *O. novo-ulmi* (Dutch elm disease), *Ceratocystis fagacearum* (oak wilt), *Sirococcus clavigignenti-juglandacearum* (butternut canker), *Cronartium ribicola* (white pine blister rust) and *Cryphonectria parasitica* (chestnut blight) (Sinclair & Lyon 2005). Pathogens that have narrow host ranges, or infect single host species are more likely to undergo recurrent extinction and re-colonisation events, especially if host populations are small and fragmented (Thrall *et al.* 2001). These processes might then advance the loss of genetic diversity within the pathogen populations (Barrett *et al.* 2008).

In contrast to host specific pathogens, facultative pathogens, including many that infect agricultural crops, generally have multiple hosts and they possess the ability to live or survive saprophytically. They are thus less restricted by the presence of a specific host and could significantly increase their geographical distribution through infection of multiple hosts (Barrett *et al.* 2008). This is clearly evident in some *Phytophthora* species. *Phytophthora ramorum,* for example, infects over 100 native and non-native North American species of trees and shrubs, and over 30 in the UK and Europe, ranging in symptoms from leaf blights to shoot diebacks (Brasier 2008, Loo 2008). Pathogens with broad host ranges experience more stable environments and host population dynamics, and can, therefore, retain higher levels of within-population genetic variation (Barrett *et al.* 2008).

2.3.3 Host jumps

For some fungal pathogens, range expansion is not limited by host. Host jumps represent a common adaptive feature of fungi, and the switching from one host species to another has often lead to the emergence of diseases that have been particularly devastating (Rizzo *et al.* 2005, Slippers *et al.* 2005, Woolhouse *et al.* 2005). The distribution of the rust pathogen *Puccinia psidii* includes Central America, South America, the Caribbean, USA (Florida) (Coutinho *et al.* 1998, Glen *et al.* 2007) and recently, Hawaii (Uchida *et al.* 2006). This pathogen has been recorded to infect guava (*Psidium guajava*) and over 15 genera and 30 species in the family Myrtaceae, which are native to most of these areas. During the 1940's in Brazil, *P. psidii* underwent a host shift to infect exotic Eucalyptus where it was noticed causing severe infection, malformation and mortality in seedlings, young trees and coppice (Glen *et al.* 2007). *P. psidii* is now recognised as one of the most serious threats to *Eucalyptus* plantations grown world-wide (Coutinho *et al.* 1998, Booth *et al.* 2000, Glen *et al.* 2007). Australia is particularly at risk to this pathogen as 70 genera and 1646 species in Myrtaceae are native to Australia as well as over 850 species of Eucalyptus (Booth *et al.* 2000, Glen *et al.* 2007).

The canker pathogen *Chrysoporthe austroafricana*, currently known only from the African continent (Nakabonge *et al.* 2006), occurs on native *Syzygium cordatum* and *S. guineense* in the family Myrtaceae and provides another intriguing example of a distinct host shift in a relatively host specific tree pathogen (Heath *et al.* 2006). In South Africa, the pathogen has greatly increased its geographic range by jumping onto the exotic host *Eucalyptus grandis* (Myrtaceae) which is widely established as an important plantation species (Wingfield *et al.* 1989, Wingfield 2003). In addition, further host jumps onto *Tibouchina granulosa* that reside

in a different family (Melastomataceae), and planted as ornamental trees in South Africa, have been recorded (Myburg *et al.* 2002).

2.3.4 Hybridisation

Hybridisation and recombination are important driving forces in the evolution of invasiveness in fungal pathogens (Allendorf & Lundquist 2003, Brasier 2000). Interspecific hybridisation between two fungal species can produce a hybrid species that has the ability to infect novel hosts with enhanced pathogenicity leading to greater levels of invasiveness than either of its parent species (Brasier 2001). For example, the hybridisation of introduced poplar rust pathogens *Melampsora medusae* and *M. larici-populina* in New Zealand resulted in a hybrid with a broader host range than that of the parent species (Spiers & Hopcroft 1994, Burdon *et al.* 2006). Similarly, the hybridisation of *M. occidentalis* and *M. medusae medusae* that infect only *Populus trichocarpa* and *P. deltoides* respectively, have hybridised to form the species *Melampsora* x*columbiana.* This phenotypically different species is capable of infecting both hosts mentioned above and hybrid clones of these hosts (Newcombe *et al.* 2000). Another example of a fungal hybrid is *Puccinia graminis tritici* and *P. graminis secalis* that can infect lines of *Hordeum vulgare* which are resistant to the parent species (Watson & Luig 1958, Burdon & Thrall 2008). In the oomycetes, an allopolyploid recombination between hybrids *Phytophthora alni uniformis* and *Phytophthora alni multiformis* resulted in a new species, *Phytophthora alni* ssp. *alni* (Brasier *et al.* 2004). This persistent subspecies is part of a new taxon (*P. alni*), and is spreading throughout European riparian forests, killing alders (Ioos *et al.* 2006).

Not all hybrid species are sufficiently fit to establish and cause disease or to survive. Rather, there commonly needs to be some selection to produce a hybrid that is more fit than its parents. For example, although *Ophiostoma ulmi* and *O. novo-ulmi* cause devastating disease on elm trees, hybrids of these two species found in Europe are relatively unfit, rare and thus short-lived (Brasier *et al.* 1998). They are unable to compete with their parents and, therefore, do not contribute towards the disease epidemics (Brasier *et al.* 1998).

2.4 Spread

Successful invasion relies on the ability of a pathogen to spread to new locations. The number of spores (propagule pressure) (Lockwood *et al.* 2005), dispersal mode, reproductive mode and survival rates are critical factors regulating the spread of invasive species (Giraud *et al.*

2008). Spread is either through short-distance dispersal, which would include gradual range expansion of the pathogen in the present location, or long distance founder events (Gladieux *et al.* 2008). In fungi, large numbers of spores can be dispersed locally by means of rain-splash, via soil movement, or they can be water-borne as in the case of *Phytophthora* spp. (Lacey 1967). Some also have insect vectors as is true for *Ceratocystis* spp. and *Ophiostoma* spp. (Moller & DeVay 1968, Wingfield *et al.* 1993). These restricted or local methods of dispersal could result in reduced dispersal as distance from the source increases and could be a factor that would generally decrease effective population size (Barrett *et al.* 2008).

Long-distance dispersal of plant pathogens is typically associated with either human intervention in transporting infected plant material and seeds, or naturally via wind-dispersed spores (Brown & Hovmøller 2002, Stukenbrock *et al.* 2006). Pathogens that have high levels of dispersal (wind-blown spores) and thus high geneflow, will tend to display higher levels of population genetic diversity and isolation over distance, than those with more limited modes of dispersal (Barrès *et al.* 2008, Barrett *et al.* 2008). Some soil-borne pathogens such as *Armillaria* spp. spread by means of vegetative growth via rhizomorphs and can clonally expand their range by a few meters each year (Richardson *et al.* 2005, Prospero *et al.* 2008). Using microsatellite fingerprinting profiles, Coetzee *et al.* (2001) showed that various *Armillaria mellea* samples collected from the Company Gardens in Cape Town, South Africa, form part of one genet. This genet is estimated to be between 108 and 575 years old, which has spread to a diameter of about 345 m since its introduction into the country by early European settlers (Coetzee *et al.* 2001).

3.0 THE INVASION OF PINES AND ASSOCIATED PATHOGENS

Forest ecosystems provide interesting examples for studies that consider the dynamics within and among host-pathogen populations. They are also useful in efforts to determine the factors that shape the genetic structure of host and pathogen populations (Hamelin *et al.* 1998). This is particularly true because trees have long life cycles and diseases can be studied over sufficient periods of time to be able to understand patterns of host pathogen interaction. For the purpose of this review, a focus on *Pinus* spp. and their pathogens has been chosen to exemplify patterns of invasion.

3.1 Pines as invasives

Pines are considered to be the most successful invasive hardwood trees (Moran *et al.* 2000). Their invasive success has largely been facilitated by humans who have exploited the ability of these trees to grow well and to adapt to new environments, especially in nutrient-poor habitats (Poynton 1977, Le Maitre 1998). Thus, they have been taken from their native ranges in the Northern Hemisphere and widely planted in exotic locations, especially in the Southern Hemisphere, for ornamental and commercial plantation purposes (Richardson *et al.* 1994, Richardson *et al.* 2007). Pines produce large quantities of seed and pollen that disperse effectively and isolated individuals can also give rise to colonies by selfing (Poynton 1977). As a result, *Pinus* spp. species are able to expand rapidly in new environments, and populations have exploded to the point where they have become invasive in both their native and introduced environments (Richardson & Higgins 1998). Southern Hemisphere countries are particularly severely affected with exotic pine species dominating indigenous landscapes, displacing native plant species and causing huge ecological disturbances (Richardson *et al.* 1994, Richardson & Higgins 1998). Twenty three *Pinus* species have become naturalized in the Southern Hemisphere, eighteen of which are invasive in South Africa, South America, Australia and New Zealand (Richardson & Higgins 1998, Richardson & Petit 2006).

Although pines are considered noxious weeds in many countries, they have, along with *Eucalyptus* spp., become some of the world's best forest plantation species and are of great importance to local economies (Richardson & Petit 2006). Pines are not only highly valued for products such as solid wood, pulpwood and fuel wood, but the plantations provide employment and are a source of wealth creation (Toro & Gessel 1999, Moran *et al.* 2000, Richardson *et al.* 2007). In some situations, they have also been useful in stabilising eroded environments (Toro & Gessel 1999). One species in particular, *Pinus radiata*, native to a small belt along the coast of California and the offshore island of Guadeloupe (Poynton 1977, Rogers 2002), is an important commercial plantation species in the Southern Hemisphere. Over four million hectares of *P. radiata* have been established in South Africa, Chile, Argentina, Australia and New Zealand, with Chile and New Zealand having in excess of 1.5 million ha each (Toro & Gessel 1999, Rogers 2002).

3.2 Pine pathogens

Anthropogenic movement of pines has resulted in pine pathogens being moved over long distances through the movement of contaminated planting stock (Diekmann *et al.* 2002). For example, over 80 different *Pinus* species from various countries in Europe, Asia and North

America have been introduced into South Africa (RSA) since the European colonisation of the Cape more than 350 years ago (Poynton 1977), bringing with them over 115 exotic pests and diseases (Lundquist 1987). These introduced pathogens have in many cases become established and they have subsequently spread to cause serious disease problems (Harrington & Wingfield 1998, Wingfield 1999, Wingfield *et al.* 2001). The movement of pines and pathogens around the world, both in their native and non-native environments, presents a variety of examples illustrating the manner in which disease epidemics can occur. These patterns of invasion are discussed below.

3.3 Categories of invasion

3.3.1 Native host - native pathogen

In situations where both the host and pathogen are native, both would have co-evolved over long periods of time in their centres of origin (Gilbert 2002). Gene for gene resistance and other selective pressures would have maintained the environment in a state of equilibrium or general homeostasis (Parker & Gilbert 2004, Burdon *et al.* 2006). Epidemics are thus unlikely to occur due to these strong ecological pressures (Wingfield 1999, Burdon *et al.* 2006). In many cases, native pathogens are present without causing substantial disease, as is evident in natural forests, where the host trees are relatively resistant to cankers and foliage diseases resulting in these pathogens having only a minor impact (Harrington & Wingfield 1998).

If a native pathogen were to gain pathogenicity due to an evolutionary change that was maintained in the populations from events such as chromosome loss, somatic hybridisation, or recombination (Burdon *et al.* 2006), the state of equilibrium could be altered. In such cases, a native pathogen would gain the capacity to infect a native host, causing noticeable disease. Additionally, changes in the ecosystem could also alter the state of equilibrium. Stumps left open after the felling of pines in natural forests can, for example, create infection sites for root rot pathogens such as *Heterobasidion annosum* and *Armillaria* spp. (Hood *et al.* 1991, Harrington & Wingfield 1998, Sinclair & Lyon 2005). This could increase the pathogen inoculum to unnaturally high levels, which would escalate attacks on healthy trees (Wingfield 1999).

Climate change is another factor that can change the equilibrium state of an ecosystem where host-pathogen co-evolution is in effect (Ayres & Lombardero 2000, Burdon *et al.* 2006). This

is especially true for pathogens that are repressed from causing disease due to unfavourable historic climate conditions (Evans 1984). Increased temperatures might induce stresses on the host (e.g. drought) that would make it more susceptible to infection (Desprez-Loustau *et al.* 2006). In contrast, some pathogens are very sensitive to temperature in terms of infection potential and reproduction (Peterson 1973, Gadgil 1977). Increases in temperature could favour pathogen virulence, increasing opportunities for infection, and they could also lead to shortened life cycles leading to disease development (Anderson *et al.* 2004).

3.3.2 Native host - introduced pathogen

Where pathogens are introduced into new environments where there has been no host–pathogen co-adaptation (Parker & Gilbert 2004, Barrett *et al.* 2008), serious disease problems can emerge due to lack of appropriate resistance genes in the native hosts. These encounters are referred to as ‘new encounter’ or ‘novel interaction’ (Parker & Gilbert 2004) and they include some of the worlds most serious tree disease problems including Dutch elm disease, white pine blister rust and chestnut blight (Anagnostakis 1987, Brasier 2001, Kinloch 2003, Sinclair & Lyon 2005) .

White pine blister rust caused by *Cronartium ribicola* is probably the best example of this category of disease on pines caused by a fungal pathogen. The pathogen was first introduced into Europe from Asia and then, in the 1890’s, into North America (Fernando & Owen 2004, Diekmann *et al.* 2002). In its area of origin in Asia, *C. ribicola* has co-evolved with the native pines which developed a high level of resistance to the pathogen (Butin 1995, Loo 2008), with only low levels of disease ever seen. In contrast, white pine blister rust is one of the most destructive tree diseases of all native North American white pines and has caused catastrophic disease epidemics (Butin 1995, Kinloch 2003, Fernando & Owen 2004). By killing adult trees, natural forests have been opened up, transforming the landscapes. Changes in local environments as a result of the forests being destroyed (Burdon *et al.* 2006), has also resulted in significant ecological damage (Fernando & Owen 2004, Loo 2008). Both the Grizzly bear (*Ursus arctors*) and the Clark’s nutcracker (*Nucifraga columbiana*), for example, are highly dependent on the seeds produced by white bark pine, and the decline of white pines has had a concomitant negative effect on these animals (Loo 2008).

Although intercontinental spread of *C. ribicola* was through anthropogenic movement, population studies have shown that recombination and long-distance spore dispersal also appears to be an important mechanism for survival and spread for this pathogen (Et-Touil *et*

al. 1999, Hamelin *et al.* 2005). Furthermore, the ability of the pathogen to infect multiple host species in North America is most likely due to its outcrossing nature which would increase its evolutionary potential for survival (Richardson *et al.* 2005). A considerable amount of research has gone into breeding for resistance against this pathogen through selection and screening (Sniezko *et al.* 2001, Kinloch 2003). However, the possibility of new races of the pathogen being re-introduced from Asia, or new strains developing, is a constant threat (Sniezko *et al.* 2001).

In California, pitch canker disease caused by *Fusarium circinatum* (= *F. subglutinans*) represents another good example of an introduced pathogen affecting a native host (Aegerter *et al.* 2003, Gordon 2006), and it is considered one of the most important diseases of pine globally (Gordon 2006, Wingfield *et al.* 2008). The epidemic of this disease on native *Pinus radiata* in California has resulted in extensive mortality of the species. These losses are detrimental considering this area is the only existing source of genetic material of *P. radiata* used extensively for commercial forestry plantations (Rogers 2002).

Analyses of vegetative compatibility groups and RFLPs analyses of mitochondrial DNA have displayed low levels of diversity in the Californian populations of *F. circinatum*, consistent with recent introductions and clonal propagation (Correll *et al.* 1992, Gordon *et al.* 1996). These introductions are thought to have originated from South Eastern U.S.A where they shared common genotypes in high frequency (Wikler & Gordon 2000), and where the disease has been known since 1945 (Hepting & Roth 1946). Mexico is considered to be the origin of *F. circinatum* (Gordon 2006) due to high levels of genetic diversity that have been found for isolates of the pathogen collected in that country (Wikler & Gordon 2000). In Mexico, epidemics occur only in "off site" plantations, regardless of the pathogen being widespread there. This reinforces the notion that the fungus is in "host-pathogen equilibrium" with the native forests including many different pine species in that region (Wikler & Gordon 2000).

3.3.3 Introduced pine - native pathogen

When pines are moved out of their natural habitat, one of two distinct situations can arise. Either the exotic pine is infected by native pathogens or alternatively, they can be infected by non-native pathogens that have been co-introduced or introduced at a later stage (Parker & Gilbert 2004). The latter situation is most common in the Southern Hemisphere (Wingfield 1999, Wingfield *et al.* 2001). In both these situations, pathogens that are considered of minor

importance where the pines are native can have a devastating effect on pines planted outside their native range (Harrington & Wingfield 1998).

There are various examples of introduced pines being affected by native pathogens. A well recognised example is found with the introduced *Pinus contorta* in Northern Sweden, native to North America and the native pathogen *Gremmeniella abietina* (Richardson *et al.* 2005). The pathogen causes the disease known as Scleroderris canker and it results in large-scale infection and damage to these introduced trees (Karlman *et al.* 1994). In contrast, it has relatively little impact on the two native forests species *P. sylvestris* and *Picea abies* (Barklund & Rowe 1981, Hellgren & Barklund 1992).

There are various examples of native pathogens infecting introduced pine species in Southern Hemisphere pine plantations (Gibson 1979). Certainly the best example is found in the case of Armillaria root rot, which is found in most Southern Hemisphere countries where pines are grown as non-natives in plantations (Coetzee *et al.* 2000, Wingfield *et al.* 2001). Because they are native, the species of *Armillaria* spp. are different in these countries, but the disease problems and general symptoms are very similar (Hood *et al.* 1991, Shaw & Kile 1991). Armillaria spp. have very wide host ranges and non-native pines have been seriously damaged by them in most countries where they have been established in plantations.

3.3.4 Introduced pine - introduced pathogen

A good example of the co-introduction of pines and fungal pathogens is found in the case of *Diplodia pinea* (syn. *Sphaeropsis sapinea*) in Southern Hemisphere plantations. This pathogen is known to exist as an endophyte in healthy pine tissue (Smith *et al.* 1996, Stanosz *et al.* 1997) and has clearly been introduced into the Southern Hemisphere along with its hosts (Gilmour 1966, Gibson 1979, Burgess & Wingfield 2002). It is also, however, an opportunistic pathogen that infects pines when they are stressed (Wingfield 1999). The stress factors can either be as a result of new and unsuitable planting environments, severe wounding due to hail damage or when the trees suffer from drought (Swart & Wingfield 1991). *D. pinea* has been particularly destructive in plantations of the non-native and susceptible host, *P. radiata*, in RSA, New Zealand and Australia (Chou 1976, Currie & Toes 1978, Swart *et al.* 1987) causing shoot blight, stunting and deformation of trees and branch and trunk cankers (Gibson 1979, Swart & Wingfield 1991).

Diplodia pinea also provides a good example where molecular tools have been useful in determining the correct taxonomic position of the pathogen (Burgess *et al.* 2001a, de Wet *et al.* 2003). This has allowed for an improved understanding of how the pathogen has been introduced around the world (Burgess *et al.* 2001b, Burgess *et al.* 2004a). This has been particularly important as a foundation for population genetic studies.

Four morphotypes of *D. pinea* have been described and these have been provided with the designations A, B, C and I (Smith & Stanosz 1995, Hausner *et al.* 1999, de Wet *et al.* 2000). Multiple gene genealogies and microsatellite markers subsequently revealed that morphotype "B" represents a discrete taxon known as *Diplodia scrobiculata* (de Wet *et al.* 2003). This species is limited to North America and Europe where it is genetically highly diverse (Burgess *et al.* 2004b). Using simple sequence repeat (SSR) markers, Burgess *et al.* (2001a) further showed that morphotype "I" represents *Botryosphaeria obtusa*. Only two morphotypes (A and C) are thus responsible for the disease caused by *D. pinea.* These morphotypes differ in morphology, distribution, host specificity and virulence (de Wet *et al.* 2003).

In the case of *D. pinea,* SSR markers have revealed that the A morphotype has a global distribution but is relatively clonal, suggesting that only some genotypes are successful as endophytes (Burgess *et al.* 2004a). The low diversity of the pathogen could be advantageous in disease management strategies where trees are selected for tolerance or bred for resistance. In contrast, the more virulent C morphotype is known only to Indonesia (de Wet *et al.* 2002). The introduction of this morphotype into other countries should clearly be avoided by strict quarantine regulations.

4.0 DOTHISTROMA NEEDLE BLIGHT (DNB) AS A PINE PATHOGEN CASE STUDY

4.1 Introduction

Dothistroma needle blight, caused by a haploid ascomycete, *Dothistroma septosporum*, is considered to be one of the most important pathogens affecting exotic *Pinus* species, especially in the tropics and Southern Hemisphere (Gibson 1972, Ivory 1987, Harrington & Wingfield 1998, Bradshaw 2004). This fungal pathogen causes the well known disease commonly known as red band needle blight of pines or Dothistroma needle blight, and is

characterised by needle infections, defoliation, retarded growth and in severe cases, tree death (Gibson *et al.* 1964). The disease is identified by erumpent black conidiomata or fruiting bodies underneath the epidermis of the needles, which are normally surrounded by red or brown bands (Gibson 1972).

Dothistroma septosporum provides an excellent example to illustrate an introduced pathogen that has seriously damaged an introduced pine species. It further provides a good example of how the alteration of an environment by man has mediated both the introduction of a pathogen and the development of the disease. One of the key reasons why *Dothistroma* has become such an important pine disease problem globally, lies in the fact that extensive monocultures of a susceptible host have been established in areas where the environmental conditions are optimal for disease development.

The following section is not intended as a review of the literature pertaining to DNB as this topic has been treated extensively in previous reviews (Gibson 1972, Bradshaw 2004). Rather, this section serves as an introduction to the studies that make up this thesis, which includes investigations on the taxonomy and population genetics of *D. septosporum*. Here, I include the relevant characteristics of *Dothistroma*, especially in the Southern Hemisphere, in relation to the processes of invasion of plant pathogens as discussed above and presented in Figure 2. Furthermore, areas where research on the pathogen is lacking are highlighted.

4.2 Origin of the pathogen

Dothistroma septosporum has been known since the turn of the last century, where it was first described from *P. mugo* in Russia in 1911 (Doroguine 1911). Since then, the pathogen has been reported in over 45 countries in Eurasia, Africa, Oceania and the Americas (EPPO: http://www.eppo.org/QUARANTINE/fungi/Mycosphaerella_dearnessii/SCIRSP_ds.pdf, Ivory 1994) and infecting over 70 different species of pine (Bednářová *et al.* 2006). There have been no studies to ascertain the original, natural range of the species, although two hypotheses regarding its origin have been proposed. Evans (1984) suggested that the pathogen might be native to the high cloud forests of Central America. This was due to the presence of the pathogen on native pines, without causing epidemics, in secluded areas, far removed from anthropogenic activities that might have introduced the pathogen. Similarly, its occurrence in remote areas of the indigenous blue pine forests in the Himalayas prompted Ivory (1994) to suggest that it might also be native to these areas.

4.3 Introductions into new environments

There are no clear records detailing the introduction of Dothistroma into the Southern Hemisphere. However, as pines are not native to the Southern Hemisphere, it is reasonable to assume that *Dothistroma septosporum*, which is a pine specific pathogen, is also exotic in these regions. While pines were deliberately introduced into the Southern Hemisphere, the introduction of *Dothistroma* would have been accidental through infected pine seeds or plant material. It has been speculated that Dothistroma was introduced into New Zealand, for example, by forestry officials who visited East Africa in 1957 to observe the epidemics of the disease (Hirst 1997). The fungus was discovered five years after this visit, causing disease in central North Island forests (Gilmour 1967).

The increase in air traffic and development of pine plantations, especially after World War II, marks the period that Dothistroma was probably introduced into many different parts of the Southern Hemisphere (Table 3) (Gibson 1972). The first reports of *D. septosporum* in Central Africa were during the 1930's and 1940's (Barnes 1970, Gibson 1972), followed by Chile in 1957 (Gibson 1972), New Zealand in 1962 (Gilmour 1967) and Australia in 1975 (Edwards & Walker 1978). In each case, an epidemic of the disease was observed only years after the initial discovery. In all cases, the presence of Dothistroma was observed only after extensive plantations of the susceptible host, *P. radiata,* had been established. This was despite the fact that many other pine species were present in these countries for more than a century (Table 3). This could be attributed to a lag period which is sometimes required to allow for adaptive evolution to occur or, alternatively, the lag period could be attributed to ecological factors that are required for successful colonisation e.g. optimal temperature, correct inoculum pressure and host availability (Sakai *et al.* 2001).

4.4 Pathogen Colonisation

For the invasion process to be secured, conditions allowing successful colonisation, establishment and spread must be optimal for Dothistroma. In terms of colonisation, the infection process is important (Gadgil 1967); for establishment and spread, the availability and the susceptibility of the pine species is essential (Gibson 1972). In all cases, the environment is a critical factor (Gilmour 1981).

4.4.1 Infection process

Inoculum pressure is important for successful infection, and for *D. septosporum* on mature *P. radiata,* this has been estimated to be about 100 conidia/mm^2 (Hirst 1997). Conidia on the needle surfaces germinate and the germ tubes enter the host via stomata (Gadgil 1967, Peterson & Walla 1978), which is a process that can take three or more days (Gadgil 1967). Once infection has occurred, inter- and intracellular hyphal growth takes place within the necrotic mesophyll tissue. Tissue necrosis is a result of the production by the pathogen of the toxin dothistromin, which either kills the tissue directly or induces a host response resulting in cell death (Bradshaw 2004). This toxin is also responsible for the development of the typical red colour (Shain & Franich 1981) that is associated with the symptoms of this disease. Black conidiomata full of conidia develop within these red bands and further symptoms include necrosis of the entire needles causing premature defoliation (Gibson *et al.* 1964).

4.4.2 Environmental conditions

Temperature and moisture are the main environmental factors influencing the success of an infection in *D. septosporum*. Cool, wet conditions, such as several days of rain or overcast, humid weather, favour germination and spread of the pathogen (Gilmour 1981, Peterson 1973). Germination of conidia occurs only between 8-25 °C with an optimum at 18 °C (Ivory 1967b) and where there is high humidity (> 96 %) or extensive periods of needle wetness (Peterson 1973, Gadgil 1977, Gilmour 1981). Light does not affect conidial germination or growth on the needle surface, but it may affect the degree of infection and symptom expression (Ivory 1967b, Gibson *et al.* 1967) which may be related to dothistromin production (Bradshaw 2004). Gadgil & Holden (1976) noticed that shaded areas are less affected by disease than those exposed directly to the light and suggested it might be related to a host response. Soil nutrition is another factor that has an effect on host susceptibility. Soil deficient in available sulphur or high in nitrogen increases the host's vulnerability to infection by Dothistroma (Edwards & Walker 1978, Lambert 1986).

4.4.3 Life-cycle

Temperature has a significant effect on the life-cycle of *D. septosporum*. The period between germination and the appearance of symptoms has been reported in Kenya and Tanzania to range from between five weeks (in warmer climates) to four months. After the first symptoms have appeared, it can take a further 1- to- 2 weeks before conidia are produced, completing the life cycle (Gibson *et al.* 1964, Hocking & Etheridge 1967, Gibson 1972). If symptoms only appear in autumn, sporulation may be delayed until the following spring. In New

Zealand, the life-cycle of *D. septosporum* has been noted to be as short as two weeks in optimal environmental conditions (Gadgil 1974). *D. septosporum* in the Southern Hemisphere can thus have multiple generations in one growing season, increasing its infection and epidemic potential. In the colder climates of Europe, it can take a year or two before the *D. septosporum* life-cycle is complete. Infection usually occurs during the late spring or summer months and the pathogen over-winters in infected needles. Sporulation occurs only the following year during the spring and summer months (Butin 1985). Thus, in these colder climates, it can take years of continual infection before the effects can be seen because increment loss is proportional to the level of infection (Van der Pas 1981).

4.5 Establishment

4.5.1 Competition and niche opportunity

The ability of a species to out-compete other organisms that might potentially occupy the same niche could be advantageous to an invasive pathogen. *D. septosporum* is known as an ecologically obligate parasite, with the host providing a habitat that is non-competitive, rather than for a specific nutrient required by the pathogen. This is due to the slow growth, lack of specific nutrient requirements and inability of *D. septosporum* to survive for extended periods when competing with other saprophytes (Gibson 1972). Recently, Schwelm *et al.* (2008) showed that the toxin dothistromin, which is produced during early infection and colonisation, may have a role in competition. This would allow Dothistroma "to protect its niche from other micro-organisms" (Schwelm *et al.* 2008) and it could explain why Bradshaw *et al.* (2000) found higher levels of the toxin (5 to 500 fold more) in strains from the Bavarian Alps compared to isolates from New Zealand. In the Northern Hemisphere, where pines and this pathogen are native, *D. septosporum* would be under higher selection pressure to produce the toxin and protect its niche against many other pathogens and saprophytes. Non native pines would have a reduced number of pathogens and possibly endophytes, which would provide a greater number of niches for an invading pathogen to occupy. The loss of toxin production over time in New Zealand could, therefore, be due to a lack of selection pressure on the pathogen to retain increasing levels of virulence.

4.5.2 Species richness

Probably the single most important factor that has contributed to the success of *D. septosporum* as an invasive species in the Southern Hemisphere is the "niche opportunity" available for extensive establishment and spread. There has been a long history of different

pine species (over 80 in South Africa) being introduced into Southern Hemisphere countries (Table 3). Seeds and plant material have been obtained from various sources world-wide including seed merchants in Europe (Austria, France and Italy), America and Japan, along with other introductions from Holland, Portugal, Guatemala and Mexico (Poynton 1977). *D. septosporum* could have been introduced into the Southern Hemisphere any number of times from these Northern Hemisphere counties. It was, however, only after large areas of the susceptible host, *P. radiata* were planted, mainly as monocultures, that the pathogen established itself and spread, causing severe epidemics. The countries most affected are Chile, New Zealand and Australia, which combined, produce over 92.03 % of the total world production of *P. radiata* (Rogers 2002). It is interesting however, that *P. radiata* is not susceptible to the disease in its natural range in California, but that it does cause disease epidemics on non-native *P. radiata* plantations just a few kilometres up the North coast of California (Cobb *et al.* 1969).

4.5.3 Range expansion

Under optimal environmental conditions, *D. septosporum* is capable of extending its range within a relatively short period of time. Within seven years of the first report of it affecting the foliage of young *P. radiata* in the Usumbara Mountains of Tanzania, the disease was documented to have spread into all major plantations of *P. radiata* in Kenya, Malawi and Uganda (Gibson *et al.* 1964). Similarly, in New Zealand, range expansion of the epidemic was rapid with over 127 000 acres being infected within three to four years (Gilmour 1967).

Certain host factors could curb expansion of a DNB epidemic. The age of the host seems to be a critical factor for infection. *P. radiata* in particular shows a certain amount of resistance to pathogen infection once they reach maturity at an age of approximately 15 years or older (Gibson 1972, Ivory 1972). Seedlings and young trees are, therefore, most susceptible. Also, the establishment of large monocultures of pine does not necessarily mean that the plantation will be affected. For instance, no signs of *D. septosporum* have been observed on large plantations of the more resistant *P. patula* and *P. elliottii* in South Africa, despite the plantations being established for many years (Anonymous 2001).

4.5.4 Host jumps

Host jumps do not represent a mechanism whereby *D. septosporum* would increase its range as its host range is limited to pine species. Although the pathogen is known to infect over 70 different pine species (Bednářová *et al.* 2006), various levels of susceptibility are known.

Pinus radiata, P. nigra and *P. ponderosa* are the most susceptible while *P. patula* has been recorded as being one of the most resistant or immune species (Cobb & Libby 1968, Gibson 1979). *D. septosporum* has rarely been reported infecting other conifers including *Pseudotsuga menziesii* (Dubin & Walper 1967), *Larix decidua* (Bassett 1969), *Picea abies* (Lang 1987), *Picea sitchensis* (Gadgil 1984) and *Picea omorika* (Karadžić 1994). In all of these cases however, the trees were adjacent to highly infected sites with high inoculum pressures.

4.5.5 Hybridisation

As stated previously, hybridisation and recombination are important driving forces for the evolution of invasiveness amongst fungi (Allendorf & Lundquist 2003). Hybridisations have not been reported for *D. septosporum*, although three different varieties of the pathogen have been described based on differences in the average conidial length (Thyr & Shaw 1964, Ivory 1967a). There has, however, been considerable debate as to whether conidial size represents an appropriate character by which to distinguish among forms or varieties of *D. septosporum* (Funk & Parker 1966, Gadgil 1967, Sutton 1980). Evans (1984), studied a considerable collection of world-wide fungi, finding appreciable contrasts in both anamorph and teleomorph morphology. He contested the validity of varieties in *Dothistroma*, but acknowledged that morphotypes or ecotypes probably do exist. Bradshaw *et al.* (2000) also failed to support the distinction between the varieties based on morphology. They did find, however, in one of the first molecular analyses on this species, some ITS sequence differences between isolates from New Zealand, Europe, South America and parts of North America (Oregon and Canada) with those from North America (Minnesota and Nebraska). Because the numbers of isolates included in this study were low, no clear hypothesis could be formulated for the observation of sequence differences between isolates.

4.6 Spread

Once established, *D. septosporum* spreads via asexual conidia that are produced in the conidiomata on the needles. Short distance dispersal in the pathogen is very effective. When mature, the presence of water ruptures the epidermis and releases the spores (Peterson 1973), which are dispersed via run-off water from the needles or a splash take-off mechanism (Gibson *et al.* 1964, Ivory 1987). The spores can also become airborne in tiny mist droplets. Maximum conidial dispersal is thus found to occur under light rain or heavy mist conditions, where the spores can be carried over several distances. *D. septosporum* can be spread

effectively between trees in a plantation or nursery or even between plantations (Gibson *et al.* 1964, Ivory 1987), thus effectively expanding its range. Spores of *D. septosporum* can remain viable within dry plant material for many months depending on the environment. At 18 °C, spores can survive for up to a year, while at higher temperatures spores can survive between a few weeks up to five months (Gibson *et al.* 1964, Ivory 1967b). Spores will germinate again once exposed to water (Ivory 1987).

The introduction of *D. septosporum* into Australia is thought to have occurred via conidia present in mist clouds that blow over the Tasman Sea from New Zealand (Edwards & Walker 1978). This view is supported by that fact that stringent quarantine regulations in Australia would make it improbable that an introduction via plant material occurred (Edwards & Walker 1978, Bradshaw 2004). This hypothesis was substantiated when charred pieces of vegetation were found in New South Wales after a firestorm occurred during the burning of an infected plantation in New Zealand (Matheson 1985). The fungus in Australia is, therefore, hypothesised to be genetically the same as that in New Zealand.

Sexual ascospores are also produced by *D. septosporum* in ascostromata, but these generally occur later in the disease cycle and are produced primarily on dead needles or those that have been cast (Butin 1985). The sexual state of *D. septosporum*, which produces these sexual spores, known as *Mycosphaerella pini* (previously *Scirrhia pini*) (Funk & Parker 1966), has been observed only in certain countries in the Northern Hemisphere (Peterson & Graham 1974, Evans 1984, Kowalski & Jankowiak 1998). Thus, only the clonally produced asexual spores of *D. septosporum* have been found in the Southern Hemisphere.

4.7 Control

Three measures are currently utilized to inhibit spread and to control DNB. These include silvicultural practices to reduce inoculum, chemical spray applications and the development of DNB resistant *P. radiata* planting stock. Silvicultural practices include pruning lower branches to reduce the humidity within the trees, removing underbrush (weed control) and burning infected trees (Ahumada pers. communication, Bulman *et al.* 2004). Copper fungicides e.g. Bordeaux mixture (Cuprous oxide and copper oxychloride) applied aerially once or twice per year have been very effective (Peterson 1981, Gibson 1972) as they inhibit germination of spores (Franich 1988). Dothistroma Resistant (DR) *Pinus radiata,* bred in New Zealand, is available for commercial planting and is reported to have successfully

reduced mean infection levels by 12-15 % (Carson & Carson 1991, Dick 1989, Chou 1991, Bradshaw 2004). This DR breed might only be effective in New Zealand, however, as an extensive study on the *Dothistroma* populations in New Zealand showed them all to be clonal (Hirst 1997). Thus, resistance was bred against only one genotype. Based on the clonal reproduction of this pathogen, resistance in the host will be maintained unless a mutation which might confer greater pathogenicity is fixed during the frequent cycles of reproduction or a more virulent strain is introduced into the country.

4.8 Global epidemics

Most research on *D. septosporum* in the past focused on the epidemics that the pathogen caused in plantations of exotic pine species in the Southern Hemisphere (Ivory 1967a, Gibson 1972). Although the pathogen was known to be present in the Northern Hemisphere, there was no real cause for concern as the disease was not serious. This was partly due to natural selection pressures or temperatures and levels of humidity that were not optimal for disease development (Evans 1984). However, in the last two decades, an increase in the incidence and severity of DNB has been recorded in the Northern Hemisphere (Bradshaw 2004). Here, epidemic levels of the disease have been evident on susceptible, exotic plantations within affected countries: *P. nigra* subsp. *laricio* (Corsican pine) in the U.K. (Brown *et al.* 2003) and France (Aumonier 2002), *P. contorta* var. *latifolia* (lodgepole pine) in British Columbia (Woods 2003, Woods *et al.* 2005), and *P. nigra* (Austrian pine) in the Czech Republic (Jankovský *et al.* 2004) and Hungary (Koltay 2001) for example. Apart from these epidemics, new geographic areas and hosts have also been reported for this disease (Bradshaw 2004 and references therein, Bednářová *et al.* 2006). The concern in the Northern Hemisphere is that DNB may not be limited to pines grown in intensively managed plantations but that it may pose a serious threat to *Pinus* species in natural forests (Maschning & Pehl 1994, Aumonier 2002, Brown *et al.* 2003, Woods 2003, Woods *et al.* 2005). One of the explanations for this increase in epidemics, and the presence of *D. septosporum* on new hosts, is that global climate change has lead to previously unfavourable environments now being conducive to infection (Woods 2003). This has resulted in escalated international concern regarding this pathogen (Bradshaw 2004).

5.0 SUMMARY AND CONCLUSIONS

Biological invasions are caused by species that have been introduced into new areas and that threaten, or have the potential to cause harm in their new environment. In all cases, invasive species undergo a process that includes introduction into an area, colonisation, establishment and spread. These stages of invasions can either be studied in terms of the ecology of the species by studying life history traits and characteristics of the species or by studying the population genetics of the species.

Plant pathogens have not been as extensively studied as invasives to the degree that plants, animals and to some extent, insects have been treated. The more recent use of population genetics to study aspects related to invasions and the evolutionary potential of species has provided excellent opportunities to study the invasiveness of plant pathogens that otherwise might have been difficult.

In many cases for plant pathogens, every stage of the invasion process leaves a genetic 'imprint' in the genetic variation within and among populations (Gladieux *et al.* 2008). By studying the diversity and population structure of individuals within a population, information relating to origins of species, source and sink populations, recent population introductions etc, can be elucidated. All of these provide an improved understanding of the evolutionary, and thus invasion potential of these pathogens. Many examples have been provided in the text of this review.

The invasion process for plant pathogens can be categorised into four different situations. These include 1) Native host, native pathogen; 2) Native host, introduced pathogen; 3) Introduced host, native pathogen and 4) Introduced host, introduced pathogen. Using these different categories requires a clear understanding of the origin of the host and the origin of the pathogen. Pathogens in their native environments on native hosts generally do not cause disease, however, if the pathogen is then moved to a new environment it can either infect native or introduced plants to cause disease.

A central observation to have emerged from this review is that in many cases, it has been humans, either directly or indirectly, that have perpetuated the emergence of disease. This has either been through travel and trade, thereby creating an introduction pathway for pathogens to spread, or through the alteration of the environment, such as via agriculture or plantation

forestry, which can lead to the rapid emergence of disease epidemics. *Dothistroma septosporum*, as evident in the case study, provides a useful example of where both situations apply to the pathogen and the subsequent disease epidemics that have been associated with it. Thus, *D. septosporum* has become one of the most successful invasive pathogens, especially in the Southern Hemisphere on the widely grown exotic *P. radiata.*

The ecology of *D. septosporum* has been very well characterised on the basis of infection processes, life-cycle and mode of dispersal, in an attempt to better understand the pathogen and to develop control strategies to reduce its impact. However, there is a distinct lack of knowledge relating to the genetics aspect of the pathogen, its pattern of spread or the source of introductions. Many hypotheses have been raised regarding patterns of spread of *D. septosporum*, but most remain unconfirmed. The presence of a teleomorph in the Northern Hemisphere indicates that sexual reproduction is occurring in that area of the world. In contrast, in the Southern Hemisphere, only the asexual state is known. This suggests that while in its native range, *D. septosporum* could be evolving due to the possibility of recombination but that the pathogen in the Southern Hemisphere is reproducing clonally. This has already been established for the populations present in New Zealand where DR forms of *P. radiata* have been planted. The accidental introduction of more virulent genotypes in this, or other Southern Hemisphere countries, could result in new disease outbreaks. There is thus a critical need to study the populations of *D. septosporum* and to determine the genetic diversity and structure globally.

Accurate taxonomy is clearly a key factor in understanding the global distribution and management of *D. septosporum*. At the time of embarking on this study, only one species of Dothistroma was known to cause DNB. However, different varieties of the species had been described based on conidia morphology and the possibility that different morphotypes or ecotypes existed was a distinct possibility. Furthermore, the report of differences in ITS sequences between isolates from different countries made it important to further investigate the relationships that might exist between these isolates at a genetic level.

The main focus of the studies presented in this thesis will be to validate or substantiate some of the issues raised above. Because the morphology of different *D. septosporum* isolates has already been extensively studied, the work presented in this thesis will focus on expanding the studies started by Bradshaw *et al.* (2000) and Ganley & Bradshaw (2001). Molecular phylogenetic tools will thus be used to determine whether morphotypes or ecotypes exist by

considering the phylogenetic relationships of isolates from different countries. In addition, we will determine whether DNA sequence data validates the separation of *D. septosporum* into different varieties. Detailed studies on populations of *D. septosporum* from a world-wide collection are likely to yield valuable information pertaining to population diversities and structure of this pathogen. These data, to be obtained using microsatellite markers, will hopefully be valuable in determining aspects related to the genetics of the pathogen which could aid in the development of future management and quarantine strategies for *D. septosporum.*

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Table 1: Lists of some websites, databases, books and journals that are dedicated to the topic of biological invasions

Acronym / Name		URL / Editors / Authors / Publishers
Websites:		
APHIS	Animal and Plant Health Inspection Service General information	www.aphis.usda.gov/invasivespecies
2010 BIP	The 2010 Biodiversity Indicators Partnership	www.twentyten.net/
CABI		www.cabi.org/datapage.asp?iDocID=173
DAISIE	Delivering Alien Invasive Species Inventories for Europe	www.europe-aliens.org
GISP	Global Invasive Species Programme	www.gisp.org/
ISSG	Invasive Species Specialist Group	www.issg.org/
NOBANIS	North European and Baltic Network on Invasive Alien Species	www.nobanis.org/
	Alien Invader Plants	www.geocities.com/wessaaliens/index.htm
	Alien Invasive Species in China	www.chinabiodiversity.com/shwdyx/ruq/ruq-index-en.htm
	Japanese Knotweed Alliance	www.cabi.org/japaneseknotweedalliance/
Databases:		
AgNIC	Agriculture Newwork Information Center	laurel.nal.usda.gov:8080/agnic/
AKEPMP	Alaska Exotic Plant Mapping Project	agdc.usgs.gov/akepic/
APIRS		plants.ifas.ufl.edu/search80/NetAns2/
	Regulated Pest List	www.invasivespecies.org/NewInitiatives.html
	Alien Plant Invaders of Natural Areas in the U. S.	www.nps.gov/plants/alien/list/a.htm
	Alien Species in Hawaii	www.hear.org/
	Alien Species in Poland	www.iop.krakow.pl/ias/
	Lithuanian invasive species database	www.ku.lt/lisd/species_lists/fungi_all.html
Go to: http://42explore.com/invasive.htm "The Topic: Invasive species" for a list of over 70 additional websites.		
Books:		
Handbook of Alien Species in Europe (2008)		DAISIE 2008
Invasion Ecology (2008)		Lockwood JL, Hoopes MF, Marchetti MP (2008)
Invasive Alien Species: A Toolkit of Best Prevention and Management Practices (2001)		Edited by Wittenberg R & Cock MJ (2001)
Invasive species in a changing world (2000)		Edited by Mooney HA, Hobbs RJ
Biological Invasions - A global perspective. Scope 37 (1989)		Edited by Drake JA, Mooney HA, diCastri F, Groves RH, Kruger FJ, Rejmanek M, Williamson M.
The ecology of invasions by *Pinus* (Pinaceae) and *Hakea* (Proteaceae) species, with special emphasis on patterns, processes and consequences of invasion in mountain fynbos of the southwestern Cape Province, South Africa (1989)		Richardson DM
The ecology and management of biological invasions in southern Africa (1986)		Macdonald IAW, Kruger FJ, Ferrar AA
Journal:		
Biological Invasions		Editor-in-Chief: Daniel Simberloff / Springer Netherlands

Table 2: The ecological/life history traits and possible population genetic structure of plant pathogens during the different stages of invasion.

INVASION STAGES	PLANT PATHOGEN TRAITS	
	ECOLOGY / LIFE TRAITS	POPULATION GENETIC EVIDENCE
Origin / Source	Centres of diversity	areas of origin have high genetic diversity, large no of private alleles
	Host-pathogen interactions	Co-evolution between host and pathogen
		can determine historical source and sink patterns of migration
		source populations can be identified if introduced population shares identical genotypes
Introductions	Man-mediated via endophytes in plants, plant parts e.g. germplasms, seeds	
	Single introductions	low leves of diversity/clonality/one mating type
	Multiple introductions	high levels of genetic diversity/both mating types
Colonisation	Small number of individuals are introduced or spread into a new area - host infection takes place	founder effects or bottleneck = reduced genetic diversity
	Reproduction	
	asexual (spores, vegetative spread)	Gametic disequilibrium / linkage disequilibrium / clonal
	sexual	gametic equilibrium / random association amongst alleles
	homothallic	inbreeding- clonal or reduced genetic diversity
	heterothallic	outbreeding- large amount of genotypic diversity
Establishment	Host specificity	
	nonobligate pathogens (wide host range)	maintain higher levels of within population genetic variation
	obligate parasites (narrow host range or host specific)	experience frequent local extinction and re-colonisation events = loss of genetic diversity
	Range expansion	migration and geneflow = increases genetic diversity reducing effects of drift
	Host jumping	recombination events
	Hybrids	hybridisation events or recombinations
Spread	Long distance dispersal (air-borne spores)	low population sub-structure
	Short distance dispersal (rain-splash)	high population sub-structure

Table 3: Dates are provided for when pines in general and more specifically, *P. radiata*, were introduced into some Southern Hemisphere countries. Dates are also recorded for when the susceptible species *P. radiata* was extensively grown in plantations and when the first reports and epidemics of Dothistroma needle blight (DNB) occurred.

	Pines introduced	*P. radiata* introduced	Extensive plantations of *P. radiata*	First reports of DNB	Epidemics of DNB	References
Kenya			1945-1960 (1)	1960 on *P. radiata* (from Tanzania) (2)	1964 (3)	1) Lavery & Mead 1998; 2) Gibson 1972; 3) Gibson et al 1964
Tanzania				1957 -young *P. radiata* Tanganyika (Tanzania) (1)	1960 (1)	1) Gibson 1972
Malawi			1928-1934 resumed again in 1955 (1)	1940's on *P. radiata* (1)	1962 (1)	1) Gibson 1972; 2) Lavery & Mead 1998
Zimbabwe		1902 (3)	1928-1934 (2) resumed again in 1955 (1)	Middle 1930's on *P. radiata* (4)	1962 (1)	1) Gibson 1972; 2) Lavery & Mead 1998; 3) Poynton 1977 4) Barnes 1970
South Africa	late 17th century (1)	1850 (2)	1884 (2)	1965 on *P. canariensis* (3)	1984 - increased occurance (4, 5)	1) Poynton 1977; 2) Lavery & Mead 1998; 3) Gibson 1972; 4) Lundquist 1987; 5) Ivory 1994
Chile		1885 (1)	1940's (1)	1957 on *P. radiata* (2)	1964 - 1965 (2)	1) Toro & Gessel 1999; 2) Gibson 1972
New Zealand	Shortly before 1830 (1)	1859 (2)	1870's (3)	1962 on *P. attenuata* x *P. radiata* but probably present since late 1950's (4)	1966 (5)	1) Richardson & Higgins 1998; 2) Hirst et al 1999; 3) Lavery & Mead 1998; 4) Gilmour 1967; 5) Gibson 1972
Australia	1770's with British colonisation (1)	1857 (1)	1875 (2)	1975 on *P. radiata* (3)	1977 (3)	1) Richardson & Higgins 1998; 2) Lavery & Mead 1998; 3) Edwards & Walker 1978

Figure 1: Number of publications returned from a search of the ISI Web of Knowledge using the "topic" criteria and the search words "invas*" and "ecology*", and "biological" and "invas*" for the years 1968 to 2008. An increasing trend is observed from the year 1992 onwards.

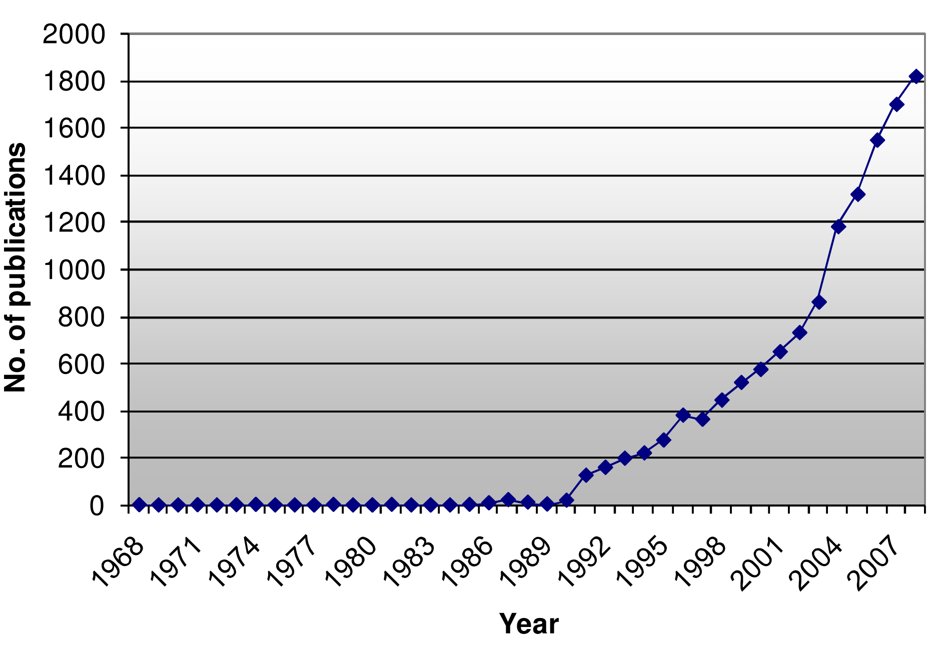

2000
1800
1600
1400
1200
1000
800
600
400
200
0
No. of publications
1968
1971
1974
1977
1980
1983
1986
1989
1992
1995
1998
2001
2004
2007
Year

Figure 2: The stages of invasion of a plant pathogen after it has been introduced onto a new area. Different scenarios of infecting native or exotic susceptible hosts, host-jumps or hybridisations are possible before the pathogen reproduces and spreads to new locations.

ORIGIN / SOURCE

INTRODUCTION

Native host

Exotic host

Infection

COLONISATION

Lag phase

ESTABLISHMENT

Host Jump

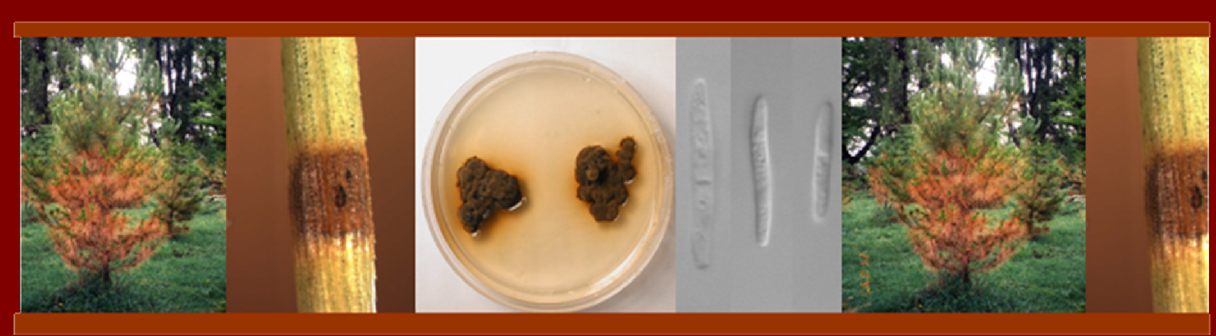
Hybridisation

Reproduction

SPREAD

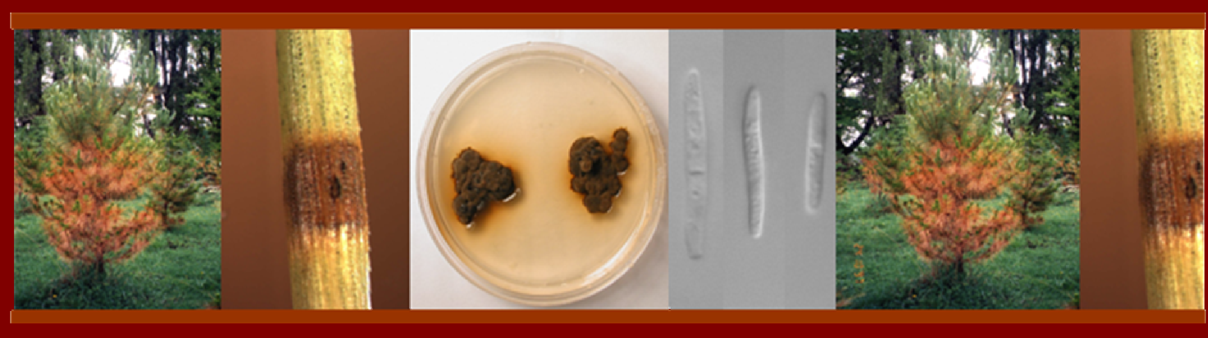
Long distance dispersal (LDD)

Short distance



Chapter 2

Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of Dothistroma, *D. septosporum* and *D. pini*



ABSTRACT

The red band needle blight fungus, *Dothistroma septosporum,* is a widely distributed pathogen of many pine species. Three morphological varieties of this pathogen have been described based on differences in conidial length. However, controversy exists as to whether spore size represents an adequate characteristic to distinguish between forms of *D. septosporum*. The aim of this investigation was to consider the phylogenetic relationships between *D. septosporum* isolates from different countries. An additional objective was to determine whether comparisons of DNA sequence data support the morphological varieties recognized for this species. DNA from portions of the nuclear ribosomal internal transcribed spacer (ITS), β-tubulin and elongation factor 1-α genes were sequenced and analysed for isolates from 13 different countries representing five continents. Results show that isolates of the pathogen encompass two divergent lineages representing distinct phylogenetic species. One phylogenetic species (Lineage I) is found worldwide, while the other (Lineage II), is restricted to the North-Central U.S.A. The names *D. pini* and *D. septosporum* are available for these species. The former name should apply to the phylogenetic species currently known only from the United States. The latter fungus has a worldwide distribution and is the causal agent of the serious disease known as red band needle blight that has damaged exotic plantations of *Pinus radiata* in the Southern Hemisphere. A PCR-restriction fragment length polymorphism (RFLP) diagnostic protocol is described that distinguishes between all the currently known *Dothistroma* species. The previous classification of *D. septosporum* isolates into different varieties based on morphology is inconsistent and not supported by our DNA analyses. We therefore reject further use of varietal names in *Dothistroma.*

Published as: Barnes I, Crous PW, Wingfield MJ, Wingfield BD (2004) Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of Dothistroma*, D. septosporum* and *D. pini. Studies in Mycology* **50:** 551–565.

INTRODUCTION

Dothistroma septosporum (Dorog.) M. Morelet, an ascomycetous pine needle pathogen, is the causal agent of the notorious red band needle blight disease. This fungus is known to infect over 60 different pine species (Ivory 1994). In situations where favourable conditions and high infection pressures exist, *D. septosporum* has also been reported infecting *Pseudotsuga menziesii* (Mirbel) Franco (Dubin & Walper 1967), *Larix decidua* P. Mill (Bassett 1969), *Picea abies* (L.) Karst. (Lang 1987), *Picea sitchensis* (Bong.) Carr. (Gadgil 1984) and *Picea omorika* (Pančić) Purkyne (Karadžić 1994), though no data exist to confirm that these incidents were caused by *D. septosporum*.

After the fungus infects via the stomata, initial symptoms appear as water-soaked lesions on the needles. Black conidiomata develop at these infection sites, which are characteristically surrounded by a red band, hence the common name of the fungus. Infected needles become necrotic and are cast (Figure 1). In severe cases, complete defoliation occurs, leading to growth retardation and tree death (Gibson *et al.* 1964). Red band needle blight is one of the most important diseases of pines, which has seriously damaged plantation forestry in many countries.

The red band needle blight pathogen has a cosmopolitan distribution, having been reported from more than 44 different countries in Eurasia, Africa, Oceania and the Americas (Data sheets on Quarantine pests: *Mycosphaerella dearnessi* and *Mycosphaerella pini* http://www.eppo.org/QUARANTINE/QP_fungi.htm, Ivory 1994). The severity of the disease appears to be related to a favourable climate in the Southern Hemisphere and to the exotic planting of susceptible host species such as *Pinus radiata* D. Don and *P. ponderosa* Laws. Thus, countries such as Chile, New Zealand and Kenya, where plantations are primarily monocultures of susceptible hosts, have experienced huge economic losses (Gibson 1974, van der Pas 1981). Control is limited to sanitary silvicultural practices, copper sprays and the planting of resistant tree species, families and clones (Carson & Carson 1989, Dick 1989, Chou 1991).

The taxonomic history of *D. septosporum* is beset with confusion. The species concept has two independent roots of origin: one stems from Europe and the other from the U.S.A. In Europe, Doroguine (1911) first described this fungus as *Cytosporina septosporum* Dorog. from Russia. *Cytosporina septosporum* was later transferred to the genus *Septoriella* Oudem. as *S. septosporum* (Dorog.) Sacc. (Trotter 1931).

In the U.S.A., the species became involved in taxonomic confusion stemming from a failure to distinguish between the red band fungus and the brown spot fungus, *Lecanosticta acicola* (Thüm.) Syd.. Initially, Saccardo (1920) described the red band fungus found on *P. ponderosa* in Idaho as *Actinothyrium marginatum* Sacc. Both Dearness (1928) and Hedgcock (1929) believed that the red band fungus was conspecific with *L. acicola* Syd., although Dearness referred to it as *Cryptosporium acicola* Thüm., and Hedgcock used the name *Septoria acicola* (Thüm.) Sacc. Sydow & Petrak (1942) later recognised that *A. marginatum* represented a *nomen confusum* and referred to the fungus as *L. acicola.* Independently, Hulbary (1941) described the red band fungus occurring on *Pinus nigra* Arn. var. *austriaca* Aschers. & Graebn, collected in Illinois, and erected the name *Dothistroma pini* Hulbary for it. Siggers (1944) discovered that the material previously referred to as *L. acicola*, *C. acicola*, *S. acicola* and *A. marginatum* on *P. nigra* var. *austriaca* was not conspecific with the type specimen of *L. acicola,* but rather with that of *Dothistroma pini*.

The connection between the American and European fungi was made when Gremmen (1968) and Morelet (1968) realized that the fungus described in Europe as *C. septosporum* was the same as *D. pini* causing red band needle disease in the U.S.A. Morelet (1968) synonymized all collections associated with red band needle blight and made a new combination in *Dothistroma* for the species epithet "*septosporum*" (as "*septospora*"), which is now widely accepted for the red band needle blight fungus.

Three different varieties of *D. septosporum* have been described based on differences in the average conidial length. *Dothistroma septosporum* var. *septosporum* (≡ *D. pini* var. *pini*) and *D. septosporum* var. *linearis* (≡ *D. pini* var. *linearis*), proposed by Thyr & Shaw (1964), are respectively the varieties with short (15.4–28 × 2.6–4 µm) and long (23–42 × 1.8–2.9 µm) conidia. *Dothistroma septosporum* var. *keniensis* (≡ *D. pini* var. *keniensis*), proposed by Ivory (1967), accommodates collections of the fungus with conidia of intermediate (15–47.5 × 1.5–3.5 µm) size. There has, however, been considerable debate as to whether conidial size represents an appropriate character by which to distinguish among forms or varieties of *D.*

septosporum (Gadgil 1967, Funk & Parker 1966, Sutton 1980). Evans (1984), studied a large number of collections of these fungi from many parts of the world and found considerable differences in both anamorph and teleomorph morphology. He contested the validity of varieties in *Dothistroma*, but acknowledged that morphotypes or ecotypes probably exist.

The aim of the present investigation was to consider the phylogenetic relationships of *D. septosporum* isolates from different countries, and further to determine whether morphotypes or ecotypes might exist for the fungus. An additional aim was to determine whether DNA sequence data reflect the separation of *D. septosporum* into different varieties.

MATERIALS AND METHODS

Isolates

A total of 32 isolates from various locations in 13 countries were chosen to represent a global distribution of *D. septosporum* (Table 1). They also included sufficient material to reflect the three varieties that have been described for the fungus. Further isolates, representing the species *Mycosphaerella dearnessii* M.E. Barr (the brown spot needle blight fungus, *L. acicola*), *D. rhabdoclinis* Butin and *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips were included in this study.

Isolates were obtained either directly from culture collections (Table 1), or from isolations made from infected needles. Infected needles collected from the field were first deposited in –70 ˚C freezers (minimum 1 h), in brown paper bags to kill possible contaminant insects or mites. Mature conidiomata from the needles were scraped from the needle surfaces and rolled across the surface of 2 % malt extract agar (MEA, Biolab, Midrand, Johannesburg) plates to release the conidia. Blocks of agar were cut from the plates in areas where there were many conidia but no contaminating debris. These blocks were then lifted and transferred to new MEA plates. Cultures were incubated at 20 ˚C until colonies formed. All cultures used in this study are stored in the culture collection (CMW), of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Duplicates of representative isolates have been deposited in the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands (Table 1).

DNA extraction, amplification and sequencing

Spores of representative cultures were spread onto 2 % MEA plates and incubated at 20 ˚C until colonies had formed (approx. 4 wks, 10-15 mm diam). Colonies were scraped from the plates, excess agar removed and placed directly into Eppendorf tubes. The colonies (constituting mycelium and spores) were freeze-dried and crushed with the aid of liquid nitrogen and a glass rod. Before DNA was extracted using the method described by Barnes *et al.* (2001), 800 µL of extraction buffer was added to the tubes, which were then incubated in a heating block for 15 min at 85 ˚C followed by another 1 h at 60 ˚C. DNA concentrations were measured with a NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, Delaware, USA). DNA from herbarium material was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). A single conidioma was scraped from a needle and excess plant material removed. The conidioma was then crushed between two slides before DNA extraction was continued. The success of this method, using one conidioma, was first tested on the Idaho material (CMW 15077) before attempting to extract DNA from the herbarium specimen.

Primers ITS1 and ITS4 (White *et al.* 1990), were used to amplify the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S gene of the ribosomal RNA operon. Parts of the β-tubulin gene were amplified using the primer pairs Bt2a/Bt2b and Bt1a/Bt1b (Glass & Donaldson 1995). The translation elongation factor (EF1-α) gene was amplified using the forward EF1-728F and reverse primer EF1-986R (Carbone & Kohn 1999).

PCR was performed in total volumes of 25 µL. The reaction mixtures consisted of ± 5 ng DNA template, 200 nM of the forward and reverse primers, 0.2 mM of each dNTP, 1U Taq DNA Polymerase with 10 × buffer (Roche Molecular Biochemicals, Mannheim, Germany) and 1.5 mM $MgCl_2$. The PCR cycling profile was as follows: 96 ˚C for 2 min, 10 cycles of 94 ˚C for 30 s, 55 ˚C for 30 s and 72 ˚C for 1 min. A further 30 cycles were included with the annealing time altered to 40 s and a 5 s extension after each cycle. Ten min at 72 ˚C completed the programme. PCR amplicons were visualized on 2 % agarose (Roche) gels stained with ethidium bromide under UV illumination. Amplicons were purified using Sephadex G-50 columns (SIGMA-Aldrich, Steinheim, Germany).

PCR amplicons were cycle-sequenced using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) following the manufacturer's protocol. The same primers used for the PCR reactions were used to sequence

the amplicons in both directions. Sequence reactions were run on an ABI PRISM™ 377 Autosequencer (Applied Biosystems) and sequence electropherograms were analyzed using Sequence Navigator version 1.0.1 (Applied BioSystems).

Phylogenetic analysis

Sequences were aligned using Clustal X (Thompson *et al.* 1997) and checked visually before analyses were run using PAUP v. 4.0 (Phylogenetic Analysis Using Parsimony) (Swofford 2002). Intron and exon positions were identified using the original sequences from which each primer set was designed. The *Neurospora crassa* sequence (GenBank M13630) was used for the β-tubulin gene regions and the *Puccinia graminis* sequence (GenBank X73529) for the EF1-α region. The random sequence (GenBank AJ544253) of *Saccharomyces cerevisiae* was used to identify the ITS1, 5.8S and ITS2 regions in our sequences.

The heuristic search option, based on parsimony, with random stepwise addition of 1000 replicates and tree bisection reconnection (TBR) as the swapping algorithm, was used to construct the phylogram. Gaps were treated as "new state" and, therefore, all characters were given equal weight. Confidence levels of the branching points were determined using 1000 bootstrap replicates. *Neofusicoccum ribis* (GenBank accession numbers AY236936, AY236878, AY236907) was used as the outgroup and was treated as a monophyletic sister group to the ingroup. A partition homogeneity test (PHT), was performed in PAUP with 100 replicates to determine the combinability of the four data sets. All sequences derived in this study have been deposited in the GenBank database with accession numbers AY808275–AY808308 (ITS), AY808170–AY808204 (β-tubulin 1), AY808205–Y808239 (β-tubulin 2) and AY808240–AY808274 (EF1-α). Sequence alignments and trees have been deposited in TreeBASE, accession number S1209, M2088-M2091. Percentage divergence within *D. septosporum* (other species were excluded) was calculated by dividing the number of variable positions in the aligned sequence by the total length of the consensus sequence.

Morphology

All cultures for growth rate studies were grown on 2 % MEA supplemented with 0.2 % yeast extract. Isolates CMW13004 from Poland, CMW11372 from South Africa and CMW10951 from the U.S.A. were used for growth rate studies at 5 ° intervals from 5–30 °C. The growth rates were determined by taking 2 mm plugs of actively growing cultures and placing a single plug at the centre of 35 mm, 2 % MEA Petri dishes. Three repeats of each culture were

incubated at the above temperature and the average colony diameter measured every seventh day for six weeks.

Descriptions and measurements of morphological characters were done directly from the fungal material obtained from the host tissue. Fungal structures were mounted in clear lactophenol or lactic acid, and observations were made using a Carl Zeiss (Carl Zeiss Ltd., Mannheim, West Germany) microscope. Spore lengths and widths from cultures and herbarium material were measured electronically using a Zeiss Axio Vision (Carl Zeiss) camera system.

PCR-restriction fragment length polymorphism (RFLP) diagnostic procedure

Potential restriction enzymes for species identification, i.e., enzymes interacting with three or fewer restriction sites on the ITS sequences, were identified using Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/). PCR-RFLP patterns were generated using the ITS PCR amplicons of CMW6841, CMW14822, CMW14820 and CMW12519. Amplicons (~10 µL) were digested with 5 units *Alu*I (Roche 10 U/µL) restriction enzyme in 20 µL reaction mixtures containing 2 µL 10× SuRE/Cut Buffer A and 7.5 µL water. CMW14822 was left undigested as a control. Reaction mixtures were incubated overnight at 37 °C followed by heat inactivation of the enzyme at 65 °C for 20 min. PCR-RFLP profiles were visualized on an ethidium bromide stained agarose gel (3 %), under UV illumination.

RESULTS

Isolates

The technique by which conidiomata are rolled across the surface of an agar plate was an effective means of easily obtaining pure cultures of *D. septosporum.* This method significantly reduces, and in some cases completely eliminates, contamination by the faster growing secondary pathogens that normally complicate isolation of this fungus.

DNA extraction, amplification and sequencing

Amplicons of the ITS region were ~520 bp long, the β-tubulin 1 region ~470 bp, the β-tubulin 2 region ~430 bp and the EF1-α region ~310 bp. Occasionally, for some isolates, an extra primer set of elongation factor primers EF1F – 5’TGCGGTGGTATCGACAAGCGT3’ and

EF1R- 5'AGCATGTTGTCGCCGTTGAAG3' (Jacobs *et al.* 2004) was used to generate sequences. Amplicons using this primer set were then ~760 bp in length.

The extraction of DNA using the DNeasy Plant Mini Kit, and subsequent PCR from one conidioma from the Idaho material (less than 1-yr-old) was successful, and was thus attempted on herbarium specimens ILLS 27093 and WSP 48361. PCR of the type of *D. septosporum* var. *linearis* (*Actinothyrium marginatum,* WSP 48361), although successful, gave faint bands and contained smears. Only the ITS sequence was recovered. Poor PCR could be the result of degraded DNA associated with the fact that the material was 47-yrs-old. PCR of the type of *Dothistroma pini* (ILLS 27093) from Illinois, which was 66-yrs-old, was not successful.

Phylogenetic analysis

Intron and exon positions were easily identified using the respective sequences of the gene regions from Genbank. Two introns were present in the ITS sequence and the aligned data set was 473 bp in length. None of the sequences of the β-tubulin-1 gene region contained introns and thus, no alignment was necessary. The amino acid alignment of the β-tubulin-2 gene region was somewhat different to that of *N. crassa.* Exon 3 and 6 were identified and intron C was absent. Only part of exon 4 was similar, but the rest of the sequence up to exon 6 was not comparable with the corresponding section of the *N. crassa* sequence. In total, the aligned sequences were 418 bp long. The EF1-α gene resulted in an aligned dataset of 346 bp in length and contained one intron.

Significant incongruence (P = 0.03) in the PHT was found among the four data sets of aligned sequences and thus they were not combinable. Phylograms for each gene region are thus represented individually (Figures 2–5). Only one most parsimonious tree is represented for data sets that produced multiple trees. Parsimony data and scores obtained from the heuristic search and analyses using PAUP are presented on each tree (Figures 2-5).

All four phylograms had very similar topology. The isolates of *D. septosporum* were resolved into two very distinct lineages, consistently supported with a 100 % bootstrap value (Figures 2–5). Lineage I included the majority of the isolates in this study, including isolates from all 13 countries represented in the data set.

The sequence obtained from the type material of *D. septosporum* var. *linearis* (WSP 48361), was also included in this clade (Figure 2). The ITS sequences in this lineage were identical while slight variation was observed randomly in the β-tubulin 1 (5 bp differences), β-tubulin 2 (1 bp difference), and EF-1α gene (2 bp differences) regions. Lineage II was limited to isolates originating from the North Central U.S.A. (Minnesota, Nebraska and Michigan). No variation among these isolates was evident for the four gene regions sequenced. From a total of 1508 bp of aligned sequences using only *D. septosporum* isolates, there were 147 bp polymorphisms distinguishing the two lineages. Most of the variation observed between the two lineages was in the conserved exon positions. Although the ITS had only 3 bp differences between the lineages, the β-tubulin-1 region contained 15 polymorphisms, the β-tubulin-2 showed 95 polymorphisms, and the EF-1α gene-regions had 34 polymorphisms. Percentage divergence between the two lineages was thus significant at 9.7 %, indicating the presence of a species boundary. Sufficient variation between the two lineages exists for the recognition of two separate taxa.

There was no evidence in the sequence data to justify recognizing the three varieties described based on morphological differences. Isolates from South Africa and Kenya, that might have been considered to represent the variety "*keniensis*", were identical in sequence to those from Idaho and France, representing the variety "*linearis*". These isolates could also not be distinguished from those from New Zealand and Chile that might have represented the variety "*pini*". All these isolates resided in Lineage I.

Morphology

In an attempt to find morphological differences between the two phylogenetic species distinguished within *D. septosporum sensu lato*, differences in growth rates, culture morphology and spore dimensions were investigated. Growth rates for the phylogenetic Lineage I represented by isolates CMW 13004 and CMW 13010 from Poland, and CMW 11372 from South Africa were 1, 3.2, 2.2, 1.9 and 1.4 mm per week at 25, 20, 15, 10 and 5 °C respectively. The growth rates for the Central U.S.A. isolates CMW 10930, CMW 10951 and CMW 14905, representing phylogenetic lineage II, were 0.9, 3.6, 2.7, 1.6 and 1.3 mm per week at 25, 20, 15, 10 and 5 °C. Optimum growth for isolates in both lineages was at 20 °C, while no isolate of either lineage grew at 30 °C.

Substantial variability in culture morphology was observed among isolates from different countries, isolates obtained within a single country and even subcultures of the same isolate

inoculated onto replica plates (Figure 6). In some cases, zones of red or blue pigment were observed in the agar surrounding the cultures. Pigment production was, however, not consistent within individual isolates and not observed at all in some isolates.

Dothistroma septosporum isolates chosen for spore measurements were selected 1) to represent isolates from all three varieties proposed in the literature (Table 1), and 2) from the two phylogenetic lineages revealed in this study (Figures 2–5).

Conidial length showed extreme variation, ranging from 12–50 µm in isolates belonging to Lineage I (Figure 7). Even spores from different conidiomata from the same tree differed in average measurement (data not shown). There was considerable overlap in size ranges for those isolates labeled as var. *linearis*, *keniensis* and *pini*, and no clear distinction between the isolates could be made. There was also no correlation between isolates from different continents, although conidia from the Southern Hemisphere tended to be shorter while those from the Northern Hemisphere were longer.

Although it was not immediately obvious, slight variation in morphology between isolates for the two lineages could be observed. The range of conidial dimension for isolates from Lineage II was smaller than that seen in Lineage I, and in general, there was a tendency for the isolates from the Central U.S.A. to have relatively short conidia, which were slightly wider than those produced by members of Lineage I (Table 2, Figure 8, 9). Conidial septation was also more clearly defined and obvious in Lineage II isolates than in Lineage I isolates (Figures 7, 8). The conidial dimensions of the type specimen of *Dothistroma pini* from Illinois (ILLS 27093) closely matched those of other collections from the North Central U.S.A., i.e. relatively short and wide conidia (Table 2).

Based on these observations we propose that isolates in the two phylogenetically distinct lineages be recognized as two discrete species. This separation is based on fixed nucleotide differences between isolates in the two lineages and variation in conidial dimensions. For conservation of names associated with the red band needle blight fungus, *Dothistroma septosporum* is retained for isolates belonging to Lineage I and *Dothistroma pini* is resurrected for isolates belonging to Lineage II.

Dothistroma pini Hulbary, Bull. Ill. St. nat. Hist. Surv. 21: 235. 1941. Figures 2–5, 8, 9, 10, 11, 12.

Conidiomata predominantly occurring in red bands on the upper and lower needle surfaces, separate to aggregated, sub-epidermal, becoming erumpent, and splitting the needle surface with one or two longitudinal slits; at maturity acervular, black, up to 1 mm in length, lined internally with pseudoparenchymatous cells giving rise to conidiophores; these cells brown, becoming paler at the point of conidiophore attachment. *Conidiophores* pale brown to hyaline, smooth, dense aggregated, subcylindrical to irregular, 1–4-septate, branched or simple, 15–27 × 2–3 µm. *Conidiogenous cells* integrated, hyaline, smooth, subcylindrical, tapering down to bluntly rounded apices, proliferating sympodially or percurrently near the apex, 7–12 × 2–3 µm. *Conidia* aggregated in cream to pale brown, slimy masses; smooth, thin-walled, hyaline, subcylindrical to narrowly obclavate or irregular, subobtuse at the apices, truncate to obconically subtruncate at the bases, (1–)3(–5) septate, (18–)25–35(–45) × 3–5 µm (av. 30 × 3.5 µm) *in vivo,* (11–)20–25(–27) × (2–)2.5–3(–3.5) µm (av. 22 × 3 µm) *in vitro*.

Notes: Amplification of the ITS/5.8S/ITS2 region using primers ITS1 and ITS4 elucidates three polymorphisms distinct from those seen in *D. septosporum sensu stricto* at positions 68, 115 and 318. The polymorphism at position 318 results in the addition of an *Alu*I restriction site in *D. pini* isolates. Upon digestion of the PCR product, this yields distinctive fragments of ~170 and ~350 base pairs in length.

Other specimens examined: **U.S.A.**, Michigan, Massaukee County, McBain, Riverside Township, isolated from *Pinus nigra*, Aug. 2001, G. Adams, herb. CBS 12203, culture CMW 14905 = CBS 116483; Michigan, Montcalm County, Stanton, Evergreen Township, from *Pinus nigra*, 2001, G. Adams, herb. CBS 12211, culture CMW 10951 = CBS 116486.

Dothistroma septosporum (G. Dorog.) M. Morelet (as "*septospora*"), Bull. Soc. Sci. nat. Archéol. Toulon Var 177: 9. 1968. Figures 2–5, 7, 9, 11, 12.

≡ *Cytosporina septospora* G. Dorog., Bull. Soc. Mycol. Fr. 27: 106. 1911.

≡ *Septoriella septospora* (G. Dorog.) Sacc. apud Trotter, Syll. Fung. 25: 480. 1931.

= *Actinothyrium marginatum* Sacc., Nuovo Giorn. Bot. Ital. 27: 83. 1920.

= *Dothistroma pini* var. *lineare* Thyr & C.G. Shaw, Mycologia 56: 107. 1964.

≡ *Dothistroma septosporum* var. *lineare* (Thyr & C.G. Shaw) B. Sutton, *The Coelomycetes. Fungi imperfecti with pycnidia acervuli and stromata (Kew)*: 173. 1980.

= *Dothistroma pini* var. *keniense* M.H. Ivory (as "*keniensis*"), Trans. Br. Mycol. Soc. 50: 294. 1967.

≡ *Dothistroma septosporum* var. *keniense* (M.H. Ivory) B. Sutton, *The Coelomycetes. Fungi imperfecti with pycnidia acervuli and stromata (Kew)*: 174. 1980.

Teleomorph: *Mycosphaerella pini* Rostr., Dansk bot. Ark. 17(1): 312. 1957.

≡ *Eruptio pini* (Rostr.) M.E. Barr, Mycotaxon 60: 438. 1996.

= *Scirrhia pini* A. Funk & A.K. Parker, Canad. J. Bot. 44: 1171. 1966.

≡ *Mycosphaerella pini* (A. Funk & A.K. Parker) Arx, Proc. K. Ned. Akad. Wet., Ser. C 86(1): 33 (1983) (homonym, Art. 53).

Conidiomata predominantly occurring in red bands on the upper and lower needle surface, separate to aggregated, sub-epidermal, becoming erumpent, and splitting the needle surface with one or two longitudinal slits; at maturity acervular, black, up to 1 mm in length, lined internally with pseudoparenchymatous cells giving rise to conidiophores; these cells brown, becoming paler at the point of conidiophore attachment. *Conidiophores* pale brown to hyaline, smooth, dense aggregated, subcylindrical to irregular, 0–4-septate, branched or simple, 7–25 × 2–3.5 µm. *Conidiogenous cells* integrated, hyaline, smooth, subcylindrical, tapering down to flattened apices, proliferating percurrently or rarely sympodially near the apex, 7–15 × 2–3 µm. *Conidia* aggregated in cream to pale brown, slimy masses; smooth, thin-walled, hyaline, subcylindrical to narrowly obclavate, long subobtuse at the apices, truncate to obconically subtruncate at the bases, (1–)3(–5)-septate, (18–)26–30(–40) × 2(–2.5) µm (av. 28 × 2 µm) *in vivo*, (15–)25–30(–40) × 1.5–2(–2.5) µm (av. 28 × 2 µm) *in vitro*.

Notes: Amplification of the ITS1/5.8S/ITS2 region using primers ITS1 and ITS4 results in three polymorphisms distinct from those seen in *D. pini* at positions 68, 115 and 318. The polymorphism at position 318 does not result in the addition of an *Alu*I restriction site, and thus, upon exposure of the PCR product to *Alu*I, the fragment retains its original length of ~520 base pairs.

Specimens examined: **Austria**, Thenneberg, from *Pinus nigra*, Apr. 2004, T. Kirisits, herb. CBS 12205. **Chile**, near Valdivia, from *Pinus radiata*, 2001, M.J. Wingfield, herb. CBS 12206. **Ecuador**, Lasso Highlands, *Pinus muricata* D. Don. 2001, M.J. Wingfield, herb. CBS 12207. **New Zealand**, Rotorua, FRI nursery, from *Pinus radiata*, 2001, M. Dick, herb. CBS 12208. **Poland**, Miechów Forest District, Goszcza Forest Unit, Compartment 71 h, approx. 20 km from Cracow, isolated from 19-yr-old *Pinus nigra* in a seed plantation, Jun. 2003,

Tadeusz Kowalski, herb. CBS 12209, culture CMW 13004 = CBS 116488, culture CMW 13010. **South Africa**, Tzaneen, from 6-yr-old *Pinus radiata*, 2002, M.J. Wingfield, herb. CBS 12210, culture CMW 11372. *Actinothyrium marginatum* Sacc., **U.S.A.,** Meadow Creek, Clearwater Ranger District, Idaho, isolated from *Pinus ponderosa*, Jun. 1957, Fred Matzner, WSP 48361; Idaho, Lochsa Historical Ranger Station, *Pinus ponderosa*, Jun. 2004, L.M. Carris, herb. CBS 12204. *Cytosporina septospora* Dorog, **Ukraine,** Kiev Guberniya, Smiela, *Pinus sylvestris* L., 25 Mar. 1914, L. Kaznowski, LE 116244, herb. CBS 11381.

PCR-RFLP diagnostic procedure

The ITS regions were selected for the construction of a simple diagnostic RFLP test to distinguish between *Dothistroma pini* and *D. septosporum s. str*. This gene region was chosen because it showed no variation within the two lineages. This lack of variation suggests that this method will remain robust even if other isolates from different countries are to be tested. At position 319 of the ITS GenBank sequences (GenBank sequences are shorter than observed PCR products due to the splicing off of sequence ends for alignment purposes), the transition from A to G creates an *Alu*I restriction site in *D. pini*, producing fragments of ~ 170 and ~350 base pairs in length. This restriction site is not present in *D. septosporum s. str*. The only other recognised *Dothistroma* species, *D. rhabdoclinis,* has a restriction site for *Alu*I at base pair position 371, giving it an RFLP profile distinguishable from those of the red band fungi (Figure 12).

DISCUSSION

Comparisons of DNA sequence data for four regions of the genome have shown clearly that the very serious pine disease known as red band needle blight, also referred to as Dothistroma needle blight, is caused by two distinct fungi. These fungi, *D. septosporum* and *D. pini*, make up two distinct phylogenetic lineages. *Dothistroma septosporum* has a worldwide distribution and it is the causal agent of the disease that has severely damaged plantations of *P. radiata*, grown as an exotic in the Southern Hemisphere. In contrast, *D. pini* is a serious pathogen of pines that currently appears to be restricted in distribution to the North Central United States.

DNA sequence comparisons provide no support for separating the red band needle blight fungus into three varieties based on conidial dimensions. Isolates from Idaho representing the variety "*linearis*" have the same DNA sequence as isolates from Africa representing the

variety "*keniensis,*" as do those from Chile and New Zealand thought to be of the variety "*pini*". We, therefore, support the views of Sutton (1980) and Evans (1984) rejecting the use of varietal names in *Dothistroma.* Although various morphotypes and ecotypes of *Dothistroma* have been suggested by Ivory (1967) and Evans (1984), no evidence of these was observed in the current study based on sequence data.

Species delimitations for a global collection of red band needle blight fungi were identified using multiple gene genealogies in this study. The 9.7 % divergence between these lineages, compiling polymorphisms in all four gene regions investigated, corresponds with what has been accepted as significantly different in previous species descriptions based on phylogenetic characters. For example, Couch & Kohn (2002) described a new species, *Magnaporthe oryzae*, based on a 9.7 % divergence observed within multilocus gene genealogies. Likewise, O'Donnell *et al.* (2004) recently presented formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade, based on fixed nucleotide characters observed in multiple gene phylogenies.

An important aspect of this study is that it incorporated a large number of isolates and sequences from four different gene regions. Bradshaw *et al.* (2000) compared several isolates of *D. septosporum* based on a small portion of the ITS region. Their results revealed only two nucleotide polymorphisms differing between North Central U.S.A. isolates and isolates from other parts of the world, and they therefore concluded that the fungi were conspecific. Goodwin *et al.* (2001), considered the phylogenetic relationships among *Mycosphaerella* species, and happened to include two *D. septosporum* sequences obtained from GenBank in their analyses. Although they were not aware of it, these two sequences coincidentally came from each of the distinct lineages recognised in the present study. The distinction between these isolates, and their differing placement in the larger *Mycosphaerella* group, can clearly be seen in the ITS ribosomal DNA phylogram in that paper. Although Goodwin *et al.* (2001) focussed on *Mycosphaerella* and did not discuss *Dothistroma*, their results support those presented here.

Recognition that two species cause the single disease known as red band needle blight has important consequences for disease control and quarantine. Our choice has been to retain the names that have been most closely associated with the red-band fungus and to amend the description of *D. septosporum* to exclude the genetically distinct isolates from Central U.S.A. We have consequently also restored the use of *D. pini* to represent this distinctly different

fungus that occurs in the North Central United States, including Illinois, where the type specimen of *D. pini* was collected. This specimen, described by Hulbary in 1941, could not be analysed based on sequence data but is morphologically consistent with isolates in phylogenetic Lineage II/*D. pini*. All other isolates associated with red band needle blight, including those from Western North America and Europe, are in Lineage I. They should be referred to as *D. septosporum* as proposed by Morelet (1968).

Dothistroma pini, as opposed to *D. septosporum*, has a limited host and geographical range. Within its range in Minnesota, Nebraska, Illinois, and Michigan, however, the exotic species, *P. nigra* is severely damaged by it, particularly in Christmas tree plantations (Peterson 1974). Our interpretation of the observations of Thyr & Shaw (1964) is that collections from Kansas and Kentucky assigned to the variety "*pini*" probably represent *D. pini*. If this were the case, then the host range of *D. pini* would be broadened to include the tree species considered in that study, *P. mugo* Turra (as *P. montana* Mill.).

The teleomorph *Mycosphaerella pini*, associated with the red band fungus, was not observed in the current study. So far, it has been reported only from Central America (Evans 1984), the western U.S.A. (Peterson 1974), western Canada (Funk & Parker 1966) and Europe (Kowalski & Jankowiak 1998). The original description of *M. pini* was from needles of *Pinus sylvestris* collected in Denmark. *Scirrhia pini*, a synonym (Evans 1984), was described from needles of *Pinus contorta* Douglas ex Loudon from British Columbia, Canada (Funk & Parker 1966), and has been linked taxonomically to the anamorph *D. septospora* var. *linearis* (Ivory 1967). This dictates that *M. pini* is connected to the fungus reflected by phylogenetic Lineage I with the anamorph *D. septosporum*. The separation of *M. pini* into a separate genus, *Eruptio* M.E. Barr (Barr 1996), was refuted by Crous *et al.* (2001), who showed that *Eruptio* is a synonym of *Mycosphaerella*.

In this study, we have been able to provide a simple and relatively rapid method to distinguish between *D. pini* and *D. septosporum*. This should be particularly useful because the fungi are similar in morphology and ecology, and cause similar symptoms on hosts in the genus *Pinus*. DNA sequencing facilities are not always available for comparison of fungi and the more accessible PCR-RFLP technique may facilitate correct identification.

The only other species of *Dothistroma* is *D. rhabdoclinis*. This fungus is associated with *Rhabdocline pseudotsugae* Syd. as a hyperparasite on *P. menziesii* (Butin *et al.* 2000).

Although *D. rhabdoclinis* is clearly distinguishable from *D. septosporum* and *D. pini* based on morphological and cultural as well as symptom and host differences (Butin *et al.* 2000), it can also be distinguished with this PCR-RFLP test and with sequence data.

Dothistroma or red band needle blight is one of the most important diseases of pines in the world. Some of the most serious damage caused by this disease has been seen in plantations of exotic species such as those of *P. radiata* in the Southern Hemisphere and plantations of native species, such as *P. ponderosa*, and exotics, such as *P. nigra,* in the United States. Recognition that two different fungi are associated with this disease has substantial implications for global tree health. Accidental introduction of *D. pini*, clearly a serious pathogen of *P. nigra*, could have very significant negative consequences in areas of Europe where this tree is native. Whether *P. radiata* and other species widely planted as exotics in the tropics and Southern Hemisphere are susceptible to *D. pini* is unknown but its accidental introduction into new areas could be catastrophic. Likewise, its introduction into temperate areas where as yet unelucidated, vulnerable hosts may grow, might have very severe consequences. The global distribution of *D. septosporum* implies that these fungi are easily moved into new environments, most probably with seeds. The potential threat of *D. pini* to pine forestry worldwide clearly deserves serious consideration.

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Table 1. Isolates of *Dothistroma* and related species examined in this study.

Fungus	Culture number [1]	Other culture numbers [1]	Country	Extra location information	Suggested variety [2]	Host	Collector	Date collected
Dothistroma septosporum	CMW 684	-	South Africa	Eastern Cape	var. *keniensis*	*Pinus radiata*	M.H. Ivory	1984
"	CMW 8658	-	South Africa	Hogsback, Eastern Cape	var. *keniensis*	*P. radiata*	J. Roux	2001
"	CMW 11372	CBS 116489	South Africa	Tzaneen, Limpopo	var. *keniensis*	*P. radiata*	I. Barnes	2002
"	CMW 10622	-	Kenya	Napkoi	var. *keniensis*	*P. radiata*	J. Roux	2001
"	CMW 10722	-	Kenya	Napkoi	var. *keniensis*	*P. radiata*	J. Roux	2002
"	CMW 9937	-	New Zealand	Karioi	var. *pini*	*P. contorta*	M. Dick	2001
"	CMW 9939	-	New Zealand	Rotorua	var. *pini*	*P. radiata*	M. Dick	2001
"	CMW 9943	-	New Zealand	Rotorua	var. *pini*	*P. radiata*	M. Dick	2002
"	CMW 6841	-	Australia	Canberra, Australian Capital Territory (A.C.T.)	var. *pini*	*Pinus* sp.	K. Old	2000
"	CMW 6845	-	Australia	Canberra, A.C.T	var. *pini*	*Pinus* sp.	K. Old	2000
"	CMW 6846	-	Australia	Canberra, A.C.T	var. *pini*	*Pinus* sp.	K. Old	2001
"	CMW 10247	-	Chile	Bio Bio, VIII Region	var. *pini*	*P. radiata*	M.J. Wingfield	2001
"	CMW 9304	-	Chile	Valdivia, X Region	var. *pini*	*P. radiata*	M.J. Wingfield	2001
"	CMW 8611	-	Chile	Valdivia, X Region	var. *pini*	*Pinus* sp.	M.J. Wingfield	2001
"	CMW 9920	-	Ecuador	Lasso Highlands, Cotopaxi	var. *pini*	*P. muricata*	M.J. Wingfield	2001
"	CMW 9992	CBS 383.74	France	Arboretum d' Amance, Amance, Meurthe et Moselle prefecture	var. *linearis*	*P. coulteri*	M. Morelet	-
"	CMW 13004	CBS 116488	Poland	Miechów Forest District, Cracow	-	*P. nigra*	T. Kowalski	2003
"	CMW 13007	-	Poland	Miechów Forest District, Cracow	-	*P. nigra*	T. Kowalski	2003
"	CMW 13010	-	Poland	Miechów Forest District, Cracow	-	*P. nigra*	T. Kowalski	2003
"	CMW 13123	ATCC MYA603	Slovakia	-	-	*P. sylvestris*	-	-
"	CMW 13122	ATCC MYA604	Germany	Bavarian Alps	var. *linearis*	*P. mugo*	-	-
"	CMW 14903	-	Austria	Vienna	-	*P. peuce*	T. Kirisits	2004
"	CMW 14904	-	Austria	Thenneberg	-	*P. nigra*	T. Kirisits	2004
"	CMW 14823	ATCC MYA-602	Canada	Goldstream River, British Columbia	var. *linearis*	*P. contorta* var. *latifolia*	-	1997

Table 1. Continued…

"	CMW15077	-	U.S.A.	Lochsa Historical Ranger Station, Idaho	var. *linearis*	*P. ponderosa*	L.M. Carris	2004
"	-	ILLS 27093 **T**	U.S.A.	DeKalb County, Illinois	*var. pini*	*P. nigra* var. *austriaca*	J.C. Carter	1938
Dothistroma pini	CMW 14822	ATCC MYA-610	U.S.A.	Bandon, Oregon	var. *linearis*	*P. ponderosa*	-	1983
"	CMW 10930	CBS 116485	U.S.A.	Crystal Lake, Crystal Township, Montcalm County Michigan	var. *pini*	*P. nigra*	G. Adams	2001
"	CMW 10951	CBS 116487	U.S.A.	Stanton, Evergreen Township, Montcalm County, Michigan	var. *pini*	*P. nigra*	G. Adams	2001
"	CMW 6400	-	U.S.A.	Stanton, Michigan	var. *pini*	*P. nigra*	G. Adams	-
"	CMW 14905	CBS 116483	U.S.A.	McBain, Riverside Township, Massaukee County, Michigan	var. *pini*	*P. nigra*	G. Adams	2001
"	CMW 14820	ATCC MYA-609	U.S.A.	Central, Minnesota	var. *pini*	*P. nigra*	-	1970
"	CMW 14821	ATCC MYA-606	U.S.A.	Lincoln, Nebraska	var. *pini*	*P. nigra*	-	1964
Actinothyrium marginatum (type of *D. septosporum* var. *linearis*)	-	WSP 48361	U.S.A.	Meadow Creek, Clearwater Ranger District, Idaho	*var. linearis*	*P. ponderosa*	F. Matzner	1957
Mycosphaerella dearnessii	CMW 9985	CBS 871.95	France	Le-Teich, Gironde prefecture (Aquitaine)		*P. radiata*	M. Morelet	1995
M. dearnessii	CMW 13119	ATCC 200602	China	Fujie		*P. elliottii*	Z.Y. Huang	-
D. rhabdoclinis	CMW 12519	CBS 102195	Germany	Wolfenbüttel		*Pseudotsuga menziessii*	H. Butin	1998
Neofusicoccum ribis	CMW 7773	-	U.S.A.	New York		*Ribes* sp.	G. Hudler	2000

[1]Abbreviations: ATCC, American Type Culture Collection; Virginia, U.S.A; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW, Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. ILLS, Illinois Natural History Survey, Illinois, U.S.A.; WSP, Washington State University, Washington, U.S.A.

[2] Varieties suggested are assigned based on conidial dimension and/or origin as defined by Thyr & Shaw (1964) and Ivory (1967). T = Type.

Table 2. Conidial dimensions of *Dothistroma* isolates residing in the two lineages, I and II, of the phylogenetic trees.

Origin of sample	Lineage	Length[1]	N[2]	Width[1]	N[2]
U.S.A. (Idaho, *A. marginatum*)	**1**	(21–)30–40(–50)	35	2(–3)	11
Austria	**1**	(22–)25–30(–45)	47	2(–3)	32
U.S.A. (Idaho)	**1**	(23–)26–36(–45)	32	(2–)2.5–3	11
Poland	**1**	(15–)25–30(–40)	52	1.5–2(–2.5)	13
U.S.A. (Michigan)	**2**	(16)19–26(–30)	125	2–3(–4)	44
South Africa	**1**	(14–)17–26(–33)	25	2–3	15
Chile	**1**	(17–)20–25(–30)	46	(1.5–)2(–3)	23
Ecuador	**1**	(14–)17–22(–24)	49	2(–3)	20
New Zealand	**1**	(12)18–23(–25)	34	(1.5–)2(–3)	33

[1]Conidial dimensions are presented as (min–)(mean – std. dev.) – (mean + std. dev.)(–max) in µm.

[2]Number of conidia counted.

Figure 1. Symptoms of *Dothistroma septosporum* infection on *Pinus* spp. A. 50–75 % infection on *P. radiata* in Chile. B. Tip die-back of infected *P. nigra* needles. C. Characteristically, needles from the lower branches show the first signs of disease. D. Severely infected needles showing complete necrosis and distinct red bands bearing mature conidiomata. E. Symptoms first appear as water soaked lesions followed by necrotic bands that turn reddish in colour. F. Mature conidiomata erupting through the epidermal tissue of pine needles.

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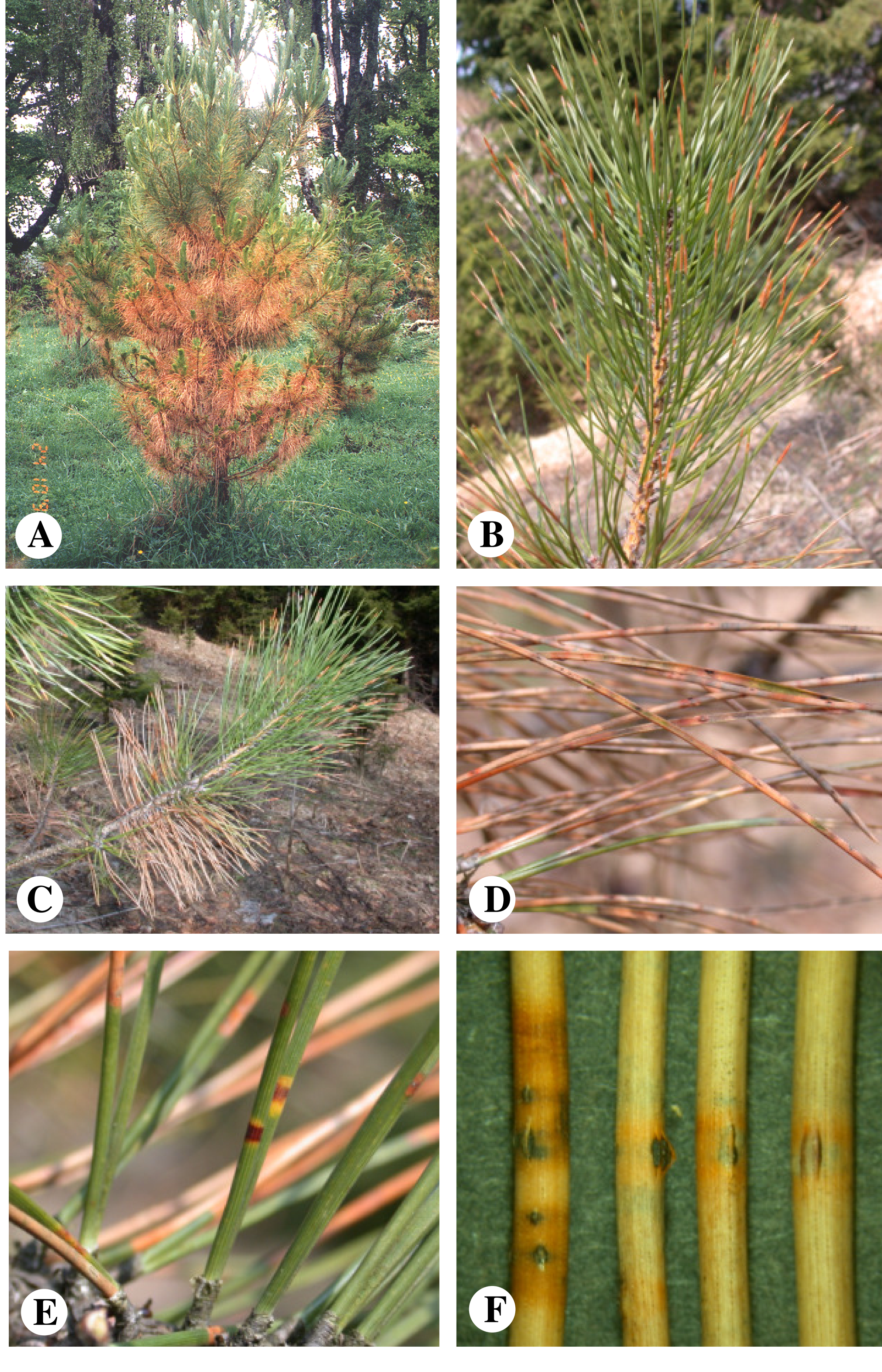


Figure 2. One of 9 most parsimonious trees inferred from nuclear ribosomal internal transcribed spacer (ITS)1, 5.8S and ITS2 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 473 characters, 90 variable characters were parsimony-uninformative and 57 were parsimony-informative. No variation within either lineage is observed. Bootstrap values are indicated below the branches while branch lengths are indicated above. *Neofusicoccum ribis* was used as the outgroup.

ITS1, 5.8S, ITS2
CI = 0.953
RI = 0.913
Tree Length = 191
g1 = -4.674

CMW15077 Idaho USA
CMW14822 Oregon USA
CMW14903 AUSTRIA
CMW14904 AUSTRIA
CMW14823 CANADA
CMW9992 FRANCE
CMW13004 POLAND
CMW13007 POLAND
CMW13010 POLAND
CMW13123 SLOVAKIA
CMW13122 GERMANY
CMW8658 RSA
CMW11372 RSA
CMW10622 KENYA
CMW10722 KENYA
CMW9937 NEW ZEALAND
CMW9939 NEW ZEALAND
CMW9943 NEW ZEALAND
CMW6841 AUSTRALIA
CMW6846 AUSTRALIA
CMW9304 CHILE
CMW9920 ECUADOR
CMW684 RSA
CMW8611 CHILE
CMW6845 AUSTRALIA
CMW10247 CHILE

LINEAGE I
D. septosporum

CMW10930 Michigan USA
CMW10951 Michigan USA
CMW14905 Michigan USA
CMW14820 Minnesota USA
CMW14821 Nebraska USA
CMW6400 Michigan USA

LINEAGE II
D. pini

21
100
12
51
93
52
27
CMW12519 *Dothistroma rhabdoclinis*
24
100
CMW9985 *Mycosphaerella dearnessii*
CMW13119 *Mycosphaerella dearnessii*
52
CMW7773 *Neofusicoccum ribis*

Figure 3. Phylogeny of the red band needle blight fungi based on the β-tubulin-1 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 367 characters, 28 variable characters were parsimony-uninformative and 45 were parsimony-informative. Within-species variation is observed for Lineage I. Bootstrap values are indicated below the branches while branch lengths are indicated above. *Neofusicoccum ribis* was used as the outgroup.

β-tubulin 1
CI = 0.919
RI = 0.955
Tree Length = 99
g1 = -1.254

CMW10247 CHILE
CMW9304 CHILE
CMW8611 CHILE
CMW9920 ECUADOR
CMW13123 SLOVAKIA
CMW13007 POLAND
CMW13004 POLAND
CMW9992 FRANCE
CMW6846 AUSTRALIA
CMW6845 AUSTRALIA
CMW6841 AUSTRALIA
CMW9943 NEW ZEALAND
CMW9939 NEW ZEALAND
CMW9937 NEW ZEALAND
CMW10722 KENYA
CMW8658 RSA
CMW684 RSA
CMW13010 POLAND
CMW11372 RSA
CMW10622 KENYA
CMW13122 GERMANY
CMW14903 AUSTRIA
CMW14904 AUSTRIA
CMW14823 CANADA
CMW14822 Oregon USA

LINEAGE I
D. septosporum

CMW10930 Michigan USA
CMW10951 Michigan USA
CMW14905 Michigan USA
CMW14820 Minnesota USA
CMW14821 Nebraska USA
CMW6400 Michigan USA

LINEAGE II
D. pini

CMW12519 *Dothistroma rhabdoclinis*
CMW9985 *Mycosphaerella dearnessii*
CMW13119 *Mycosphaerella dearnessii*
CMW7773 *Neofusicoccum ribis*

Branch labels: 1; 8 / 100; 1; 2 / 86; 1 / 61; 10 / 100; 7 / 100; 7 / 99; 17; 8 / 61; 13 / 100; 24

Figure 4. Phylogeny of the red band needle blight fungi based on the β-tubulin-2 sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Slight variation is observed within Lineage I while no variation is observed within Lineage II. Of 418 characters, 30 variable characters were parsimony-uninformative and 170 were parsimony-informative. Bootstrap values are indicated below the branches while branch lengths are indicated above. *Neofusicoccum ribis* was used as the outgroup.

β-tubulin 2
CI = 0.920
RI = 0.966
Tree Length = 337
g1 = -1.318

CMW14823 CANADA
CMW14822 Oregon USA
CMW9920 ECUADOR
CMW9304 CHILE
CMW8611 CHILE
CMW6846 AUSTRALIA
CMW10247 CHILE
CMW9937 NEW ZEALAND
CMW9939 NEW ZEALAND
CMW9943 NEW ZEALAND
CMW6841 AUSTRALIA
CMW6845 AUSTRALIA
CMW10722 KENYA
CMW10622 KENYA
CMW11372 RSA
CMW8658 RSA
CMW684 RSA
CMW15077 Idaho USA
CMW9992 FRANCE
CMW13004 POLAND
CMW13007 POLAND
CMW13010 POLAND
CMW13123 SLOVAKIA
CMW13122 GERMANY
CMW14903 AUSTRIA
CMW14904 AUSTRIA

LINEAGE I
D. septosporum

CMW10930 Michigan USA
CMW10951 Michigan USA
CMW14905 Michigan USA
CMW14820 Minnesota USA
CMW14821 Nebraska USA
CMW6400 Michigan USA

LINEAGE II
D. pini

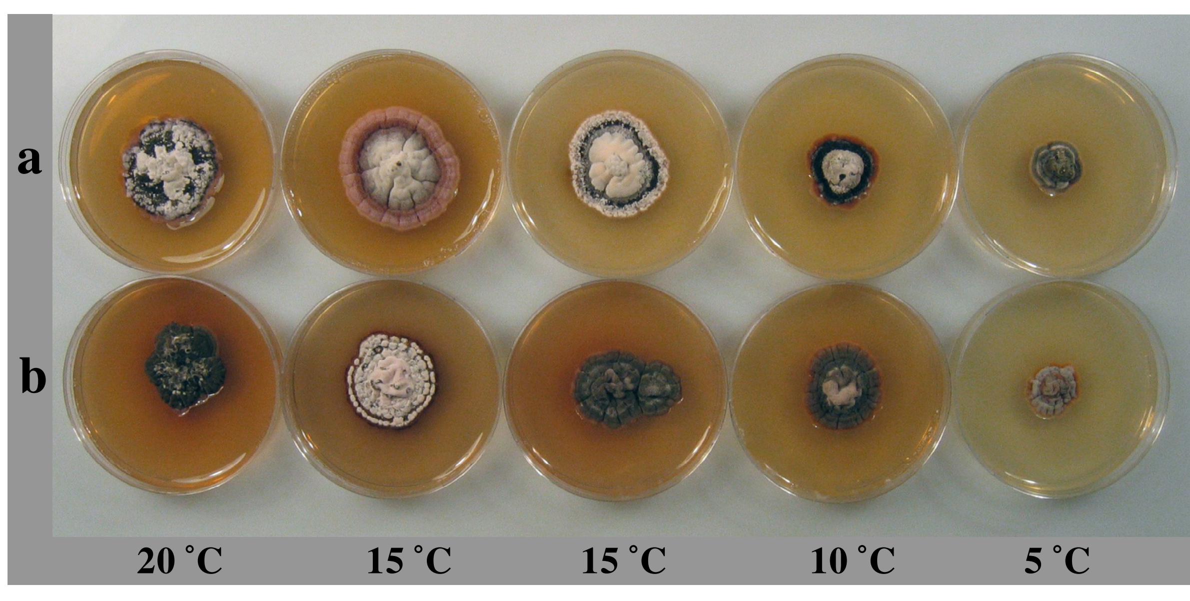
CMW9985 *Mycosphaerella dearnessii*
CMW13119 *Mycosphaerella dearnessii*
CMW12519 *Dothistroma rhabdoclinis*
CMW7773 *Neofusicoccum ribis*

42 / 100; 54 / 100; 32 / 100; 53 / 100; 57 / 100; 28 / 59; 43; 26; 1; 1

Figure 5. One of 12 most parsimonious trees inferred from the EF1-α sequences. The phylogram was obtained using the heuristic search option based on parsimony in PAUP. Of 346 characters, 87 variable characters were parsimony-uninformative and 130 were parsimony-informative. Bootstrap values are indicated below the branches while branch lengths are indicated above. *Neofusicoccum ribis* was used as the outgroup.

Elongation factor
CI = 0.906
RI = 0.917
Tree Length = 339
g1 = -2.036

Figure 6. Culture morphology of *Dothistroma* isolates from Lineages I (*D. septosporum s. str.*) and II (*D. pini*). Cultures, grown on 2 % malt extract agar (MEA), have approximately the same amount of growth at their respective temperatures after a six week period. Cultures vary considerably in morphology and colour within the same isolate at both the same (15 °C), and at different temperatures; a) Lineage I and b) Lineage II.


a
b
20 ˚C
15 ˚C
15 ˚C
10 ˚C
5 ˚C

gure 7. Variation in conidial dimensions found within isolates from Lineage I (*D. septosporum s. str.*). Conidia obtained directly from infected hosts. A–C. Austria. D, E. New Zealand. F, G. Ecuador. Scale bars = 5 µm.

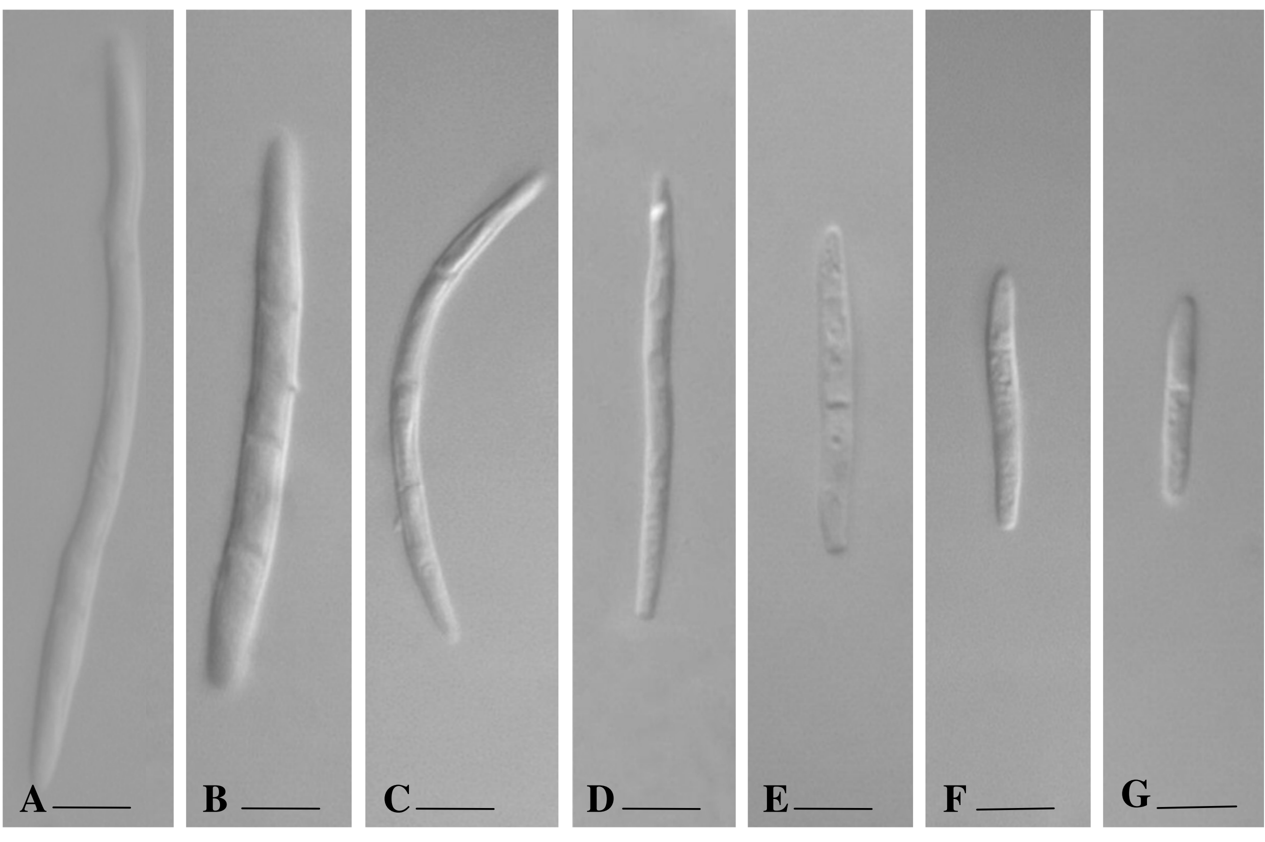

A
B
C
D
E
F
G

Figure 8. Variation observed in conidial dimensions and number of septa within isolate CMW 10930 (Michigan, U.S.A.), from Lineage II (*D. pini*). Scale bars = 5 µm.

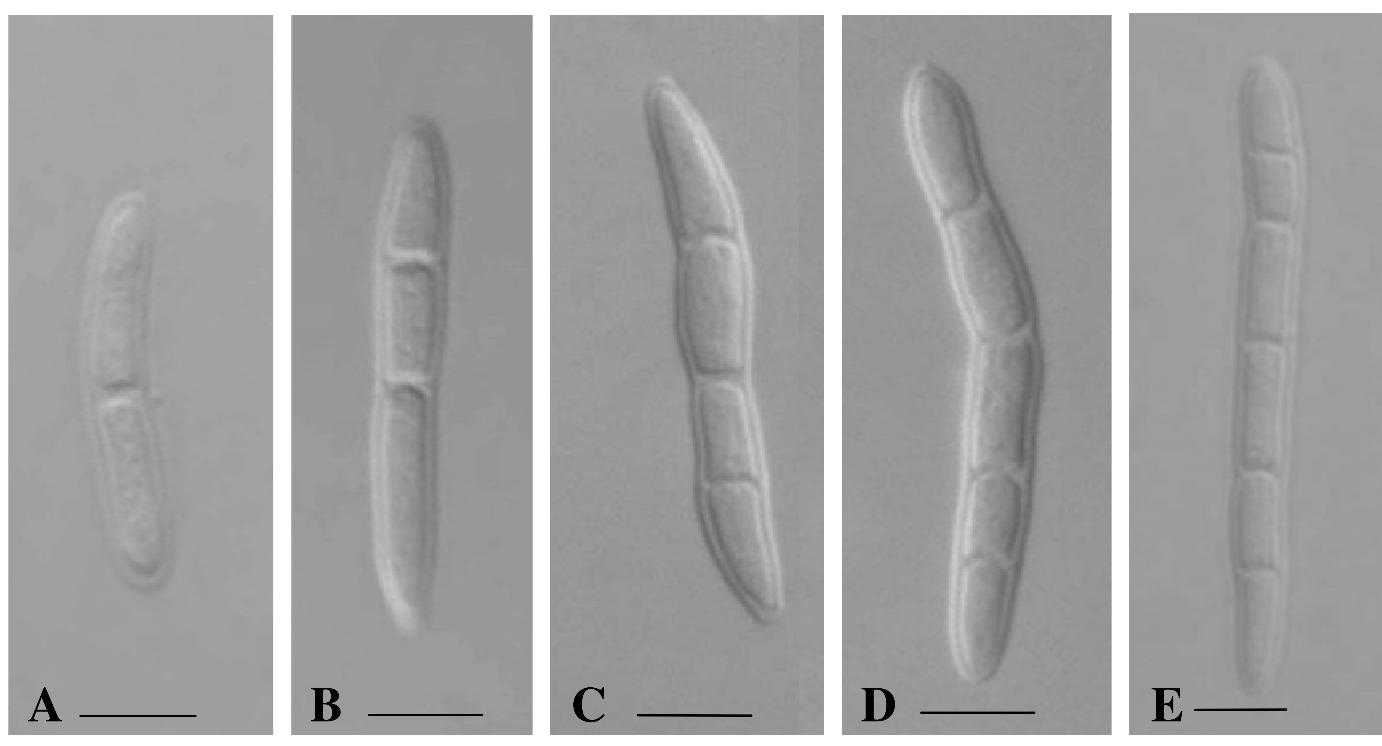

A
B
C
D
E

Figure 9. Variation in conidial morphology of *Dothistroma septosporum s. str.* on needles. A. Idaho, WSP 48361, type of *Actinothyrium marginatum*. B. Idaho, herb. CBS 12204. C. Chile, herb. CBS 12206. D. Austria, herb. CBS 12205. E. Ecuador, herb. CBS 12207. F. New Zealand, herb. CBS 12208. Scale bar = 10 µm.

A

B

C

D

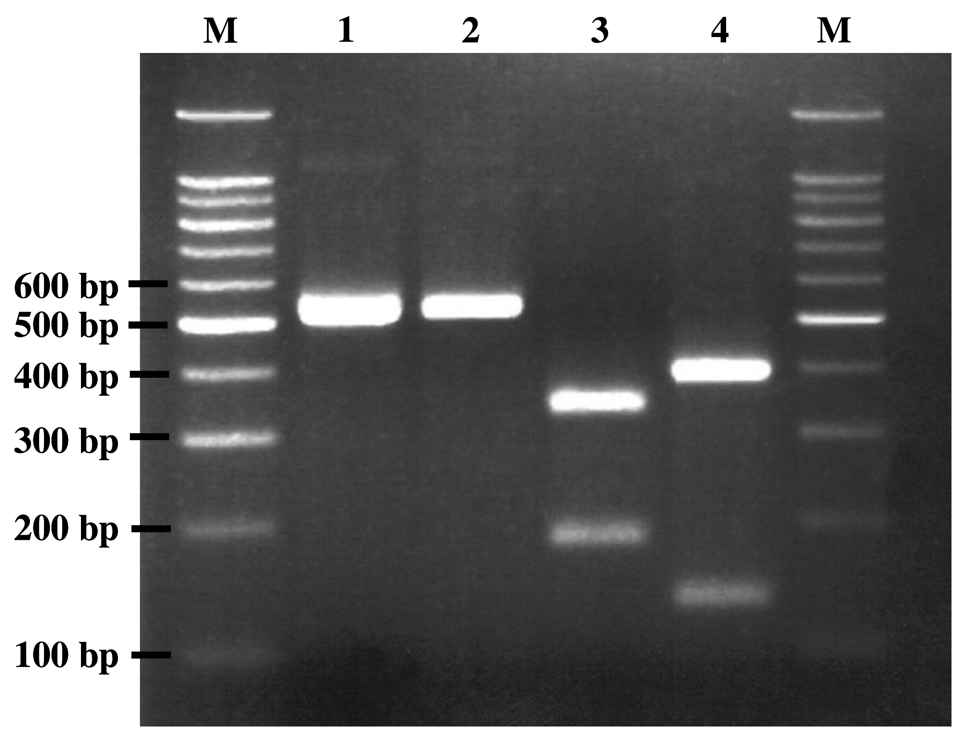
E

F

Figure 10. Conidia and conidiogenous cells of *Dothistroma pini* from Michigan on *Pinus nigra* (herb. CBS 12211) on needles (left), and on oatmeal agar (right). Scale bar = 10 µm.

Figure 11. Conidia and conidiogenous cells of *Dothistroma septosporum* from Poland on *P. nigra* (herb. CBS 12209). A. on needles. B. on oatmeal agar. Scale bar = 10 µm.

Figure 12. PCR-restriction fragment length polymorphism (RFLP) pattern of the three *Dothistroma* species digested with the restriction enzyme *Alu*I. A 100 bp marker (M) is on either side of the gel. Lane 1: uncut PCR amplicon (CMW 14822) used as a control; Lane 2: *D. septosporum* (CMW 6841) from Lineage I is not digested by *Alu*I, Lane 3: the digested product of *D. pini* (CMW 14820) from Lineage II producing 2 bands of ~170 and ~350 in length; Lane 4: digested product of *D. rhabdoclinis* (CMW 12519) producing 2 bands of ~120 and ~400 bp in length.


M
1
2
3
4
M
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp

Chapter 3

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

ABSTRACT

Dothistroma needle blight (DNB) is a serious disease of pines (*Pinus* spp.) with a world-wide distribution. It is caused by the ascomycete fungi *Dothistroma septosporum* (teleomorph: *Mycosphaerella pini*) and *D. pini* (teleomorph unknown). Recently, DNB was found on *Pinus peuce* in Austria, *P. pallasiana* in Ukraine and the European part of South-Western Russia, as well as on *P. radiata* and *P. wallichiana* in Bhutan. Based on DNA sequence comparisons of the ITS 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 *P. 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 U.S.A. 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 geographic areas during the last two decades, particularly in the Northern Hemisphere.

Published as: Barnes I, Kitisits T, Akulov A, Chhetri DB, Wingfield BD, Bulgakov TS, Wingfield MJ (2008) New host and country records of the Dothistroma needle blight pathogens from Europe and Asia. *Forestry Pathology* **38:** 178-195.

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 (Gadgil 1984, Gibson 1972), while on shade and ornamental trees, a loss of the aesthetic value resulting from defoliation can be a problem.

Until recently, the cause of DNB was attributed to the ascomycete pathogen, *Dothistroma septosporum* (Dorog.) Morelet (teleomorph: *Mycosphaerella pini* Rostr.), with the name *D. 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 recognised that *D. pini* is not a synonym of *D. septosporum*. The two *Dothistroma* species show slight differences in the morphology and dimensions of their conidia, 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), their original, natural range is still not precisely known.

To date, *D. pini* has only been conclusively identified from exotic *P. nigra* J. F. Arnold in certain areas of the North-Central U.S.A. (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 *P. 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* subsp. *laricio*) in the U.K. (Brown *et al.* 2003) and France (Aumonier 2002), lodgepole pine (*P. 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 geographic areas (Bradshaw 2004 & 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 & Pehl 1994, Aumonier 2002, Brown *et al.* 2003, Woods 2003, Kehr *et al.* 2004, Woods *et al.* 2005, Kirisits & 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 conclusively identify the causal agents of the pine needle blight disease on *Pinus peuce* Griseb. (Macedonian or Balkan pine) in Austria, on *P. mugo* Turra (Dwarf mountain pine) in Hungary, on *P. pallasiana* D. Don (Crimean pine) in Ukraine and the European part of South-Western Russia, as well as on *P. radiata* and *P. 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.

MATERIALS AND METHODS

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 placed at –70 ºC for a variable amount of time (2 hrs to 9 months) until processing.

In Austria, collections of needles were made in April 2004 from one, approx. 80 year-old, and four, approx. 25-30 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 U.S.A., 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 & A.J.L. Phillips were included in the DNA sequence comparisons (Table 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 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 on-line using MAFFT Version 5.8 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh *et al.* 2005) with the L-INS-I 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.

Morphology

For morphological characterisation, 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 to 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-sample 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 done 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 done separately for conidial measurements obtained from needles and from cultures.

RESULTS

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 & Wulf 2001, Brown *et al.* 2003, Barnes *et al.* 2004, Bradshaw 2004 & references therein, Kirisits & 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 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

the 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 due to 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.

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 to 3 days and reached colony diameters of approximately 7 mm after three weeks of incubation at 18 ºC. Isolates from *P. wallichiana* were the exception, however, as conidia only started germinating one to two weeks after being plated out and a further two weeks of growth was required, before colonies could be detected with the unaided eye.

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 Figure 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 (Figure 1). No variation between the isolates was observed in the ITS sequences. However, in the β-tubulin sequences, single base pair differences were found (Figure 1), which induced slight changes in the topology of the 4 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 (Figure 1). Slight variation between these isolates and other *D. pini* isolates from the U.S.A. were observed in the ITS sequence data (Figure 1). This was due to 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* (Figure 1).

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 regards to length, conidia of the *D. septosporum* 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 regards 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 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, 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 to the values from needles. Here, isolates of *D. pini* from Russia had the smallest conidia (means: 20.6 µm 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 to 3.5 µm) than the three *D. septosporum* isolates (range of means: 2.3 to 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 to 4.0 µm) than the two *D. septosporum* isolates measured (range of means: 2.3 to 3.0 µm), with statistically significant differences between these groups of isolates (Table 3).

Analyses of pooled data of *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).

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 U.S.A. *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 geographic

location. *Dothistroma septosporum* and *D. pini* do not show consistent differences in the length of their conidia (Tables 3 & 4). In contrast, *D. pini* has slightly wider conidia than *D. septosporum* (Tables 3 & 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 world-wide 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 *P. cembra* L.) native in this country and it has become relatively common in recent years (Brandstetter & Cech 2003, Kirisits & 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 & 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 to be 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 latter three 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 (Liesebach M, 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), due to 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.

DNB was not recorded during surveys of conifer tree diseases in Bhutan in the 1980s (Donaubauer 1986, Donaubauer 1987) or 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 & Singh 1968, Reddy *et al.* 1975, Ivory 1990, Ivory 1994, Zakaullah *et al.* 1987), *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 U.S.A. (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 sub-species of *P. nigra*: *Pinus nigra* subsp. *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 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. Due to its

drought tolerance, *P. pallasiana* has been extensively used in afforestation programs in the steppes of Southern Ukraine and South-Western Russia, outside its natural range (Dobrochaeva *et al.* 1987, Gorbok & 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 eight thousand hectares of forests (Usichenko & 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 & Akulov 2005, Usichenko & 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*, 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 & 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 & 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 (less than 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 due to 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 U.S.A. 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 U.S.A., 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 geographic 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 build-up 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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Table 1. Collection and isolation information as well as GenBank accession numbers of *Dothistroma* isolates examined in this study.

Locality	*Dothistroma* spp.	Host	Collectors	Culture no[1]	Additional culture and herbarium no.	Date collected	GenBank no. ITS	GenBank no. BT2	Isolation information
AUSTRIA									
Forest experimental garden,	*D. septosporum*	*Pinus peuce*	T. Kirisits, I. Barnes	CMW 23765	CBS 121010	April 2004	DQ926951	DQ926925	
"Knödelhütte", Institute of Silviculture,	*D. septosporum*	*P. peuce*	T. Kirisits, I. Barnes	CMW 23434		April 2004	DQ926952	DQ926926	isolates made from
BOKU, Vienna	*D. septosporum*	*P. peuce*	T. Kirisits, I. Barnes	CMW 23766		April 2004	DQ926953	DQ926927	different trees
	D. septosporum	*P. peuce*	T. Kirisits, I. Barnes	CMW 23433		April 2004	DQ926954	DQ926928	
HUNGARY									
Botanical garden of the University	*D. septosporum*	*P. mugo*	T. Kirisits, I. Barnes	CMW 23903		August 2005	DQ926956	DQ926931	isolates made from
of West Hungary, Sopron	*D. septosporum*	*P. mugo*	T. Kirisits, I. Barnes	CMW 23435		August 2005	DQ926957	DQ926932	different trees
	D. septosporum	*P. mugo*	T. Kirisits, I. Barnes	CMW 23906	CBS 121009	August 2005	DQ926960	DQ926935	
	D. septosporum	*P. mugo*	T. Kirisits, I. Barnes	CMW 23904		August 2005	DQ926958	DQ926933	same tree, different
	D. septosporum	*P. mugo*	T. Kirisits, I. Barnes	CMW 23905		August 2005	DQ926959	DQ926934	needles
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	same tree, different
	D. pini	*P. pallasiana*	A. C. Usichenko	CMW 23768		November 2004	DQ926965	DQ926940	needles
	D. pini	*P. pallasiana*	A. C. Usichenko	CMW 23769		November 2004	DQ926966	DQ926941	
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	isolates made from
	D. septosporum	*P. radiata*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23430		July 2005	DQ926962	DQ926937	same tree, different
	D. septosporum	*P. radiata*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23431		July 2005	DQ926963	DQ926938	needles
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	
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23896		July 2005	DQ926945	DQ926919	
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23897		July 2005	DQ926946	DQ926920	isolates made from
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23898	CBS 121007	July 2005	DQ926947	DQ926921	different trees
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23899		July 2005	DQ926948	DQ926922	
	D. septosporum	*P. wallichiana*	T. Kirisits, M. J. Wingfield, D. B. Chhetri, I. Barnes	CMW 23900		July 2005	DQ926949	DQ926923	
Lamey Goemba, Bumthang dzongkhag	*D. septosporum*	*P. wallichiana*	T. Kirisits, N. Gyeltshen, I. Barnes	CMW 23901	CBS 121008	July 2005	DQ926942	DQ926916	same tree, different
	D. septosporum	*P. wallichiana*	T. Kirisits, N. Gyeltshen, I. Barnes	CMW 23902		July 2005	DQ926943	DQ926917	needles

[1] Abbreviations: 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.

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

Fungus	Country	Host	Cultures[1]	Other culture no.[1]	GenBank Accession Numbers ITS	B - 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	U.S.A.	*P. nigra*	CMW 10951	-	AY808302	AY808232
D. pini	U.S.A.	*P. nigra*	CMW 14820	ATCC MYA-609	AY808304	AY808234
D. pini	U.S.A.	*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	U.S.A.	*Ribes* sp.	CMW 7773	-	AY236936	AY236907

[1]Abbreviations: CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; ATCC = American Type Culture Collection, Virginia, U.S.A; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

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).

Origin of sample	Host	Dothistroma spp.	Substrate	N[1]	Conidial length (µm)				Conidial width (µm)			
					Mean[2]	SD[3]	Range[4]	Sig.[5]	Mean[2]	SD[3]	Range[4]	Sig.[5]
AUSTRIA, Vienna	*Pinus peuce*	*D. septospoum*	Needle	93	21.4 a	3.2	14.2-31.5	***	3.0 c	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	*P. 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	*P. 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	*P. 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) (CMW 23898)	56	25.5 B	5.3	10.9-36.5		2.3 A	0.5	1.9-3.0	
UKRAINE, Tsjurupinsk area	*P. 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	*P. 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	*P. 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 ($p \leq 0.05$) 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 $p \leq 0.05$, ** = significant at $p \leq 0.01$, *** = significant at $p \leq 0.001$).

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

Substrate	Dothistroma spp.	N[1]	N[2]	Conidial length (µm)				Conidial width (µm)			
				Mean	SD[3]	Range[4]	Sig.[5]	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	

[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).

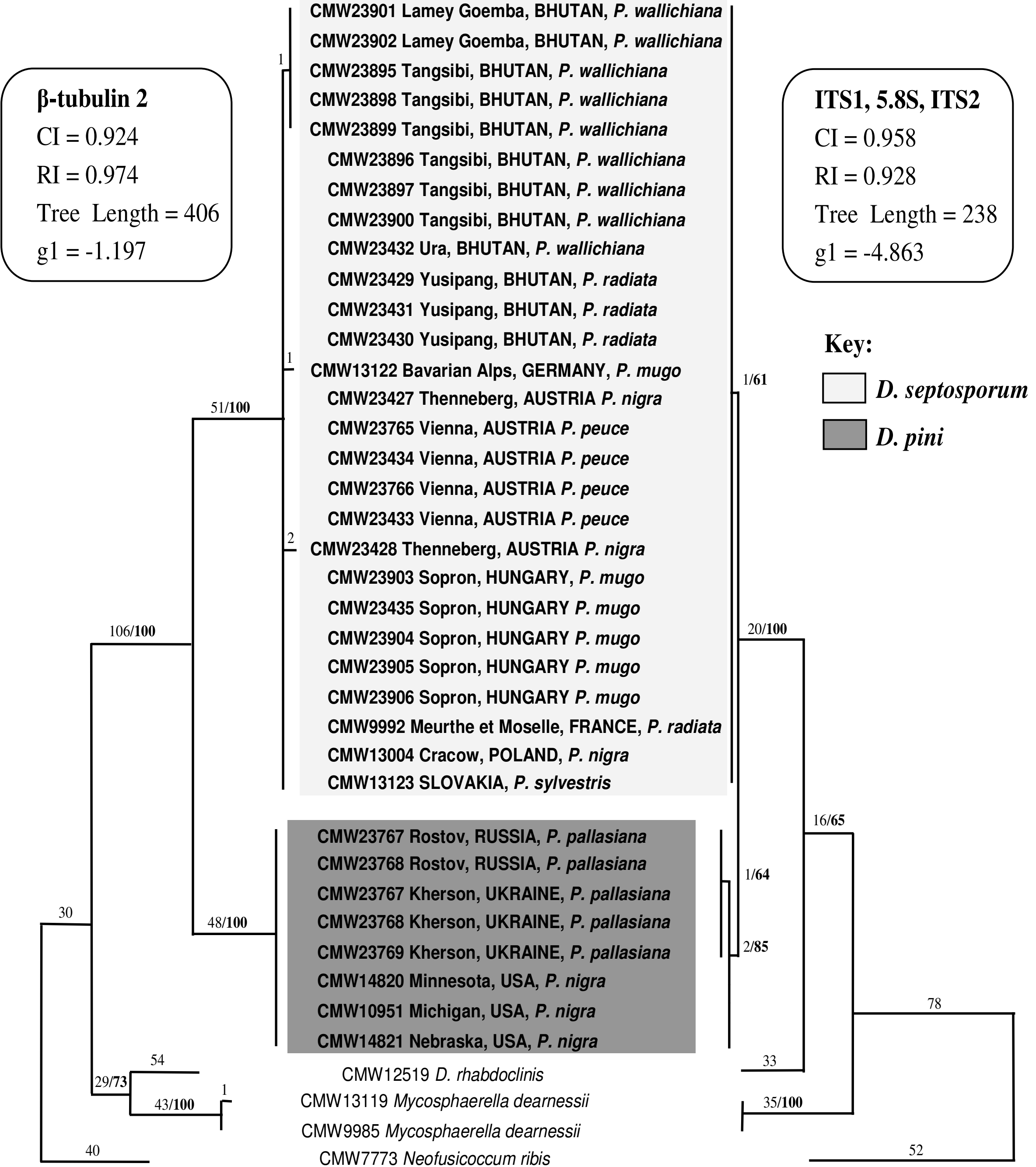
[2] Number of conidia measured.

[3] Standard deviation.

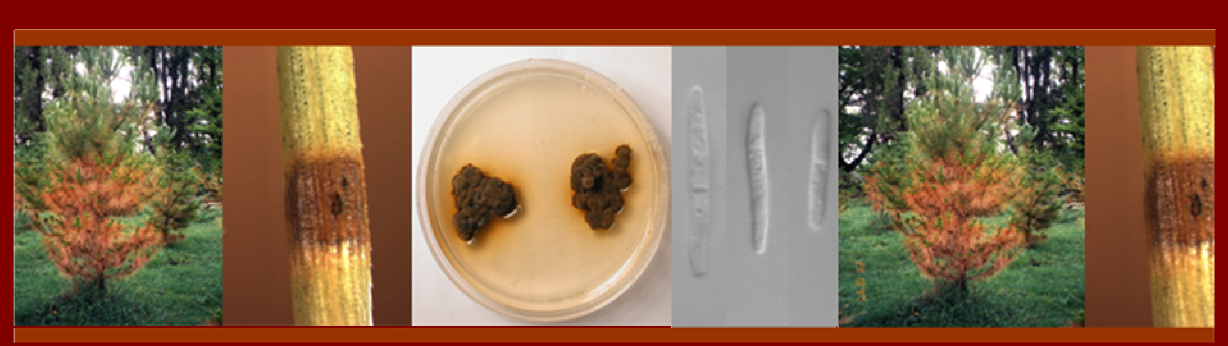
[4] Minimum-maximum.

[5] Mean values of condial length and width, within substrates "needle" and "culture", were significantly different between *D. septosporum* and *D. pini* (independent-samples t-test, *** = significant at $p \leq 0.001$). 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).

Figure 1. Phylogenetic trees derived from maximum parsimony analysis of β-tubulin and rDNA 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.

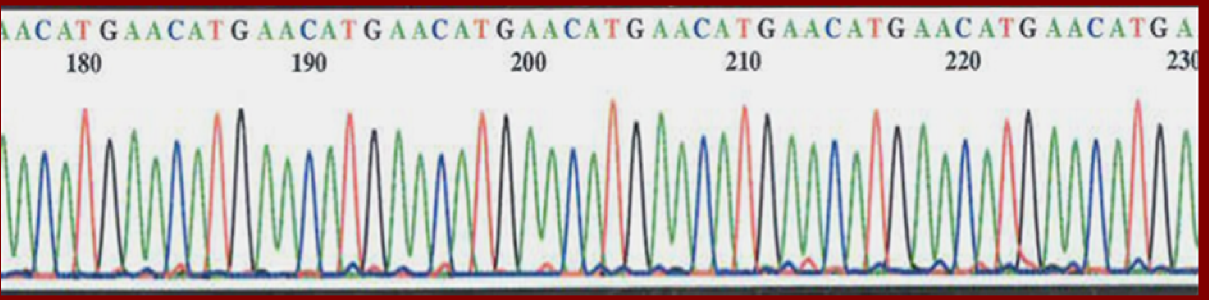


β-tubulin 2
CI = 0.924
RI = 0.974
Tree Length = 406
g1 = -1.197
ITS1, 5.8S, ITS2
CI = 0.958
RI = 0.928
Tree Length = 238
g1 = -4.863
Key:
D. septosporum
D. pini
CMW23901 Lamey Goemba, BHUTAN, P. wallichiana
CMW23902 Lamey Goemba, BHUTAN, P. wallichiana
CMW23895 Tangsibi, BHUTAN, P. wallichiana
CMW23898 Tangsibi, BHUTAN, P. wallichiana
CMW23899 Tangsibi, BHUTAN, P. wallichiana
CMW23896 Tangsibi, BHUTAN, P. wallichiana
CMW23897 Tangsibi, BHUTAN, P. wallichiana
CMW23900 Tangsibi, BHUTAN, P. wallichiana
CMW23432 Ura, BHUTAN, P. wallichiana
CMW23429 Yusipang, BHUTAN, P. radiata
CMW23431 Yusipang, BHUTAN, P. radiata
CMW23430 Yusipang, BHUTAN, P. radiata
CMW13122 Bavarian Alps, GERMANY, P. mugo
CMW23427 Thenneberg, AUSTRIA P. nigra
CMW23765 Vienna, AUSTRIA P. peuce
CMW23434 Vienna, AUSTRIA P. peuce
CMW23766 Vienna, AUSTRIA P. peuce
CMW23433 Vienna, AUSTRIA P. peuce
CMW23428 Thenneberg, AUSTRIA P. nigra
CMW23903 Sopron, HUNGARY, P. mugo
CMW23435 Sopron, HUNGARY P. mugo
CMW23904 Sopron, HUNGARY P. mugo
CMW23905 Sopron, HUNGARY P. mugo
CMW23906 Sopron, HUNGARY P. mugo
CMW9992 Meurthe et Moselle, FRANCE, P. radiata
CMW13004 Cracow, POLAND, P. nigra
CMW13123 SLOVAKIA, P. sylvestris
CMW23767 Rostov, RUSSIA, P. pallasiana
CMW23768 Rostov, RUSSIA, P. pallasiana
CMW23767 Kherson, UKRAINE, P. pallasiana
CMW23768 Kherson, UKRAINE, P. pallasiana
CMW23769 Kherson, UKRAINE, P. pallasiana
CMW14820 Minnesota, USA, P. nigra
CMW10951 Michigan, USA, P. nigra
CMW14821 Nebraska, USA, P. nigra
CMW12519 D. rhabdoclinis
CMW13119 Mycosphaerella dearnessii
CMW9985 Mycosphaerella dearnessii
CMW7773 Neofusicoccum ribis
1
1
2
51/100
106/100
48/100
30
54
29/73
43/100
1
40
1/61
20/100
16/65
1/64
2/85
78
33
35/100
52



Chapter 4

Microsatellite markers for the red band needle blight pathogen, *Dothistroma septosporum*



ABSTRACT

Twelve microsatellite markers were developed for population analyses of the fungal pathogen, *Dothistroma septosporum*. Intersimple sequence repeat polymerase chain reaction (ISSR-PCR) and an enrichment protocol (fast isolation by amplified fragment length polymorphism of sequences containing repeats [FIASCO]) were both used to identify 28 unique microsatellite regions in the genome. From 22 primer pairs designed, 12 were polymorphic. These markers, screened on two populations representing 42 isolates, produced 40 alleles across all loci with an allelic diversity of 0.09 - 0.76 per locus. Cross-species amplification showed variable success with *Dothistroma rhabdoclinis* and *Mycosphaerella dearnessii* and some sequence variation within isolates of *Dothistroma pini.* These markers will be used to further study the population structure and diversity of *Dothistroma septosporum*.

Published as: Barnes I, Cortinas MN, Wingfield MJ, Wingfield BD (2008) Microsatellite markers for the red band needle blight pathogen, *Dothistroma septosporum*. *Molecular Ecology Resources* **8:** 1026-1029.

INTRODUCTION

Dothistroma septosporum is one of the most important needle blight pathogens of *Pinus* spp. Infection is typified by distinct red bands on needles giving rise to the common name, red band needle blight. *Dothistroma septosporum* is thought to be native to the high cloud regions of Central America (Evans 1984). Its presence in remote areas in indigenous blue pine forests in the Himalayas prompted Ivory (1994) to suggest that it might also be native to these areas. The global spread of the pathogen is, however, attributed to the expanding pine trade in the early 1960's, particularly in the Southern Hemisphere, where *Pinus radiata* has been most severely affected (Gibson 1972).

The population biology of *D. septosporum* is poorly understood. In New Zealand, random amplified polymorphic DNA (RAPD) analyses have suggested that the pathogen population is clonal (Hirst *et al.* 1999). Studies of mating type distribution supported this view showing only one mating type present in New Zealand and Australia, but both occurring in other parts of Africa, Europe and the Americas (Groenewald *et al.* 2007). Understanding the population diversity and potentially, patterns of spread of *D. septosporum,* could help to reduce its impact on pine plantations and forests. Thus, the aim of this study was to develop polymorphic microsatellite markers that can be used effectively to differentiate between populations of this pathogen and assess levels of polymorphism and diversity within and between these populations. Cross-species amplification of these markers was also tested on the morphologically similar and closely related species *Dothistroma pini* (Barnes *et al.* 2004)*, Dothistroma rhabdoclinis* and *Mycosphaerella dearnessii.*

MATERIALS AND METHODS

Two techniques were used to screen for microsatellites: intersimple sequence repeat polymerase chain reaction (ISSR-PCR), which involves random amplification of

microsatellite regions using primers with repeat sequences (Barnes *et al.* 2001), and fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO), an enrichment protocol using biotinylated oligonucleotides and streptavidin-coated beads (Zane *et al.* 2002), with modifications (M-FIASCO) in Cortinas *et al.* (2006) (see supplementary material Figure 2).

Total DNA for all isolates was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For the ISSR-PCR's, 20 reactions generated with different combinations of the primers 5'DHB$(CGA)_5$, 5'DBD$(CAC)_5$, 5'HV$(GT)_5$G, 5'DDB$(CCA)_5$, 5'HVH$(GTG)_5$, 5'NDB$(CA)_7$C and 5'NDV$(CT)_8$ (see NC-IUB 1986, for sequence nomenclature), were carried out using DNA from a South African (CMW 8658) isolate of *D. septosporum*. Twelve reactions that showed clear banding patterns ranging in size from 200-2000 bp were purified using Sephadex G-50 (Sigma-Aldrich) in Centri-sep columns (Princeton Separations Inc.) and cloned using the pGEM®-T Easy Vector System (Promega). Approximately 200 clones were sequenced using the universal plasmid primer T7 (and SP6 for clones greater than 1000 bp). Sequencing reactions were prepared using Big Dye v3.1 (Applied Biosystems) and run on an ABI PRISM™ 3100 capillary autosequencer (Applied Biosystems). Genome "walking" was performed as described by Burgess *et al.* (2001) on sequences where the regions flanking the microsatellites were too short to design primers.

For M-FIASCO, approximately 1 µg of pooled DNA from isolates collected in Canada (CMW14823), Australia (CMW6846) and South Africa (CMW11372) was used in the combined digestion/ligation reaction. Enrichment of the amplified DNA was carried out using biotinylated probes $(CAC)_7$, $(AAG)_7$, $(TCC)_7$, $(CA)_{10}$ and $(CT)_{10}$. PCR amplicons of the enriched DNA were cloned using the TOPO TA Cloning® Kit for Sequencing (Invitrogen) and sequenced using the supplied M13 primers.

In total, 22 sets of primers were designed. Polymorphism for these primers was determined by sequencing the PCR amplicons obtained for the isolates of *D. septosporum* from Australia, Chile, South Africa and Poland (Table 1, Figure 1). PCR reactions were performed in 25 µL reaction volumes, consisting of 5-10 ng DNA template, 300 nM of the forward and reverse primer, 0.2 mM of each dNTP, 1U Expand High Fidelity Taq (Roche Molecular Biochemicals) and 1.2x Expand HF buffer containing 1.5 mM $MgCl_2$. The PCR conditions consisted of a 2 min denaturation step at 96 ºC followed by 10 cycles of 30 s at 94 ºC, 30 s at the specified annealing temperature for each primer and 45 s at 72 ºC. A further 25 cycles

were carried out with a 5 s extension after each cycle and the annealing time altered to 40 s. A final elongation step was carried out for 10 min at 72 ºC. One primer pair consistently yielded multiple bands and was discarded.

RESULTS

Nine sequences containing microsatellite regions were found using ISSR-PCR and 77 (from 469 clones) using M-FIASCO. After genome "walking" and removal of duplicate clones, three and 19 microsatellite regions were recovered, respectively, for each method. Of the 22 sets of primers that were designed, one primer pair consistently yielded multiple bands and was discarded. Of the remaining 21 primers, 12 were polymorphic and nine were monomorphic (Table 1).

Screening of the 12 polymorphic markers on populations from South Africa (N=24) and Poland (N=18) produced 40 alleles across all loci ranging from 2-6 alleles per locus (Table 2). Allelic diversity (Nei 1973) was between 0.09 and 0.76 per locus with an average heterozygosity of 0.46 (Table 2). The isolates from Poland showed higher gene diversity (H = 0.36) than those from South Africa (H = 0.2) and were also monomorphic for primer pair Doth_E as opposed to Doth_ I, O and DS1 for South Africa (Table 2). Pairwise linkage disequilibrium across loci was tested using MULTILOCUS 1.2 (Agapow & Burt 2001). Significant deviation ($P<0.05$) from equilibrium was observed in 46 (70 %) of the 66 pairwise comparisons. This departure from linkage disequilibrium is not, however, uncommon in haploid ascomycetes because of their predominantly asexual mode of reproduction.

In cross-species amplifications, 14 of the 21 markers amplified the corresponding microsatellite regions in *D. pini,* 10 in *D. rhabdoclinis* and 18 in *M. dearnessii* (Table 1). Considerable sequence variation and length polymorphism was observed between isolates of *D. septosporum* and *D. pini* in the polymorphic primer sets Doth_F and Doth_O as well as in the monomorphic primer sets Doth_A, Doth_D and Doth_P (Table 1). These markers could, therefore, be used in phylogenetic studies or species diagnosis. In the Genescan analyses locus Doth_A was monomorphic for allele 124 in *D. septosporum* and monomorphic for allele 114 in *D. pini* (data not shown). This primer would thus be useful as an internal diagnostic marker in genescan analyses, to screen for the presence of either of these two closely related species. The polymorphic markers developed in this study will provide a valuable tool for the future investigation of the global population diversity and structure of *Dothistroma septosporum.*

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Table 1. Polymorphisms, sequence variation and cross-species amplification of the primers[1] designed from the ISSR-PCR† and FIASCO methods for *Dothistroma septosporum.*

Primers	Repeat motif	*D. septosporum*				*D. pini*		*D. rhabdoclinis*	*M. dearnessii*
		CMW[2] 10247 Chile	CMW 6846 Australia	CMW 13010 Poland	CMW 11356 South Africa	CMW 6400 U.S.A.	CMW 23769 Ukraine	CMW 12519 Germany	CMW 9985 France
Doth_A	(CCA)	9 [3]	9	9	9	4*	4*	9	9
Doth_C	(GA)	5	5	5	5	BS	>500 bp, no microsats	5	5
Doth_D	(CAGC)	4	4	4	4	3 *	3*	-	4
Doth_E	(CATGAA)	13	13	6	9	13	-	13	13
Doth_F	(GA)	20	20	20	21	4	4*	20	20
Doth_G	(GA)	12	13	11	11	-	13	11	13
Doth_I	(GA)	10	9	11	10	10	12	11	9
Doth_J	(TG)	13	17	15	13	17	-	15	17
Doth_K	(GT)	-	20	9	21	6	6	-	-
Doth_L	(GT)	81	72	50	78	BS	BS	BS	BS
Doth_M	(CAGCACA)	5	5	6	6	7	10	6	7
Doth_O	(TGG)	10	10	10	8	5*	6*	-	10
Doth_P	(CGA)	5	5	5	5	0*	0*	-	5
Doth_S	(GT)	8	7	7	7	-	-	-	-
Doth_DS1†	(AC)	10	10	13	9	5	-	>600 bp, no microsats	10
Doth_DS2†	(CA)	13	13	15	14	BS	BS	-	13

[1] Primers that produced monomorphic alleles throughout all isolates are not shown.

[2] All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[3] The number of times the microsatellite motif is repeated as determined via sequencing.

– = multiple bands or no amplification during PCR reactions

BS (bad sequence) = positive PCR amplification although sequences were poor and thus unreadable

* = variation in sequence compared to those of *D. septosporum* (see GenBank no. EF591826/28/30/32/34 vs. EF591827/29/31/33/35)

Table 2. Primer details, core sequences, allelic properties and gene diversity (H, Nei 1973) of 12 polymorphic, PCR-based microsatellite markers developed for *Dothistroma septosporum.* Gene diversity was calculated separately for a population from Poland (N=18) and South Africa (N=24).

Locus name	Primer sequence (5?–3?)	Repeat motif	Size of cloned allele (bp)	GenBank Accession no.	T_a (ºC)	Individuals genotyped	Allele size range (bp)	No. of observed alleles	H
Doth_E	EF-(VIC): GAC ATG AAC GAG AAC TGC ATG C	$(CATGAA)_{13}$	254	EF591838	58	42	210 - 228	2	0.172
	ER: GCC AAA CTG CTC ACA AGT CTG								
Doth_F	FF-(PET): GAT ATG GAA TGA TGG AGG TGG C	$(GA)_9$ TT $(GA)_3$ AA $(GA)_6$: $(GA)_{20}$	174	EF591830	58	42	175 - 177	2	0.500
	FR: CGG AAC ATT TGT CAG CGA GGG								
Doth_G	GF-(PET): GAG TGG AAA GTA AGG GCT GAG G	$(GA)_4$ GG $(GA)_8$: $(GA)_{13}$	184	EF591839	58	42	183 - 186	2	0.245
	GR(2): GAA TTG CTG TAC TGG AAG ACC*								
Doth_I	IF-(VIC): GCA CTG CAA TTC GAC TGG GAC	$(GA)_{10}$	305	EF591841	58	42	303 - 307	3	0.582
	IR: CGC AGC AAG GCT TAG TGA ATC A								
Doth_J	JF-(NED): GAC TCC TCG GTC TGA TTC GTG	$(TG)_{17}$	193	EF591842	58	42	186 - 190	3	0.602
	JR: CAG CGA CGC CAT CAC GTA CTC								
Doth_K	KF-(6-FAM): GGT CTC AAG CTG ACG TGA TCG	$(GT)_5$ CT $(GT)_{14}$	357	EF591843	60	42	334 - 362	5	0.640
	KF(2): GCG AAG GAT GTC ACA GTC GAG*		271						
	KR: CGA GTC TGA GTT GGT CAC GAG								
Doth_L	LF-(NED): GTA AGG TCG CAG TCG GTG AAG	$(GT)_{72}$ with GC/AT/CT point mutations	393	EF591844	60	42	338 - 402	5	0.553
	LR: CCT AGA CTG TAA GCA CGC GTC								
Doth_M	MF-(PET): GAC TAA CAA CGC CTT CAA CAG T	$(CAGCACA)_6$	230	EF591845	58	42	214 - 228	2	0.337
	MR: GAA AGG TGG TAC ATA CGT CGG								
Doth_O	OF-(VIC): CGA GAA GCG ACG TGC ATC CTC	TGG TCG $(TGG)_3$ CGG $(TGG)_5$: $(TGG)_1$	204	EF591832	58	42	194 - 200	2	0.091
	OR: GCC ACG AGA GCG TCT TGT ACT								
Doth_S	SF: GTC GAT GTC ACG TTG AGA TGG	$(GT)_7$	331	EF591849	58	42	242 - 272	5	0.518
	SF(2): CGT ACA TGG TCA TCA GCG CTG*		168						
	SR-(6-FAM): GGT TAA TCC GAC CGT GAT GC								
Doth_DS1	DS1-F-(NED): GGA CAT TTG ACA GCT GTC CG	$(CA)_9$	144	EF591850	57	42	141 - 151	3	0.564
	DS1-R: GCA TGA GCG CGA GCT CAG AC								
Doth_DS2	DS2-F: GCC GCA ACC TCG GAT CAA GC	$(CA)_{13}$	380	EF591851	58	42	365 - 401	6	0.757
	DS2-F(2): GCT ACT GCC GGT GTA TAG CC*		189						
	DS2-R (VIC): CCC AAT GAC GTC TCA CCG TT								
No of isolates									42
Mean H									0.46

* Primers designed during genome walking

Figure 1. Microsatellite polymorphims and sequence variations observed within and between isolates of *D. septosporum* and *D. pini* using the microsatellite markers designed in this study. "*" Indicates that there is still sequence flanking the region of the locus which is represented. "-" Indicates gaps that are present in the aligned sequence.Primer sequences for the locus are indicated in bold.

D. septosporum	= (A)
D. pini	= (B)
CMW10247 Chile	= (1)
CMW6846 Australia	= (2)
CMW13010 Poland	= (3)
CMW11356 RSA	= (4)
CMW13122 Germany	= (5)
CMW6400 Michigan, USA	= (6)
CMW23769 Ukraine	= (7)
CMW14820 Minnesota, USA	= (8)

PRIMER A

Microsatellite polymorphism between *D. septosporum* and *D. pini*

```
(A)  *CCTCTTTGCCA\CAC\CAC\CAC\CAA\CAC\CAC\CAC\CGC\CGC\CTCCACTGCTTCTCTGTCACTCCTTGCTTCAACGTCACTCGCTCGC*
(B)  *CCTCGTTGCCA\CAC\CAC\CAC\---\---\---\---\---\CGC\CTCCACTGCTACTCTGTCACTCCTTGCTTCAACGTCACTCGCTCGC*
```

PRIMER D

Sequence variation between *D. septosporum* and *D. pini*

(A) **CGCAGCAGTTGTCATCGATTG**GACACATGACAATCGTCCCTAAAGCTACATACAGATCTCAGGACTCGAAGTTGATAGGCTCTCTTCTATAGCTGAA
(B) **CGCAGCAGTTGTCATCGATTG**GAGACGGTGTCGATCGTCTTCAAAGATTCATACAGATCTCGAAACTCGAAATTGAAAGGCTCTCTTCTATAGCTGA

(A) GTCCGTAGACTCCGGTAAATGGCTTCATTGATTCACTGTGTACGAAGGTCAGGAGCATGACATCCATTACATCTTGGGTAAGGAGGTAAGTCGGGGC
(B) AGTCCGTGGACTTCGGTAAATGGCTTCATTGATTTACTACGTACGAAGGTTAGGAGCATGACGTCCATCACATCTTGGGTAAGGAGGTAAGTCGGGG

(A) AACTTCCTGG-CAGCCAGCCAGCCAGCTCATTTATCCCTACTGATTAATCGCGTCTGCGTCCTGGCCGCCGAGCGGCTTCAATGGTTCGTTCTCGTC
(B) CAACTCCCTTGCAGCCAGCCAGCTCATTCATCCCTACTGAGCTGAGCAATCGCGTGTCCGTCCTGGCCGCCGAGCGGCTTCAATGGTCAATTTTCGT

(A) GTTTGCGTTTCGAGCTTTCTGCGAGTCTGTAGAAGTATGCGCGTCCAAGTCAATAGACCACTTACCAGTCTCCGGATTCCTTTTTCTGCTTTTTCTT
(B) TGTTTCCGATTCGAGCTTTCTGCAAGTTTGTGGAAGTATGCGCGTCCAAGTCAATAGACTACTTACCAGTCTCCGAATTGTTTTTCGGCTTTTTCCT

(A) TTGGTTCGACATTGTCCTCAAGGGTCTCTCATGACTAT**GACAATCTCATGCTGTGCCAC**
(B) TTCGTTCGACATTGTCCTCAAGGACCTCTCATGGCGAT**GACAATCTCATGCTGTGCCAC**

PRIMER E

(2) *TGAAGGCGAC\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\
(4) *TGAAGGCGAC\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\------\------\------\
(3) *TGAAGGCGAC\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\------\------\------\------\------\
(5) *TGAAGGCGAC\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\CATGAA\------\------\------\------\------\------\

(2) CATGAA\CCCTTTCTCTACTTCCCTTCTCAGCCGTGCCGTTCCGCTCACGACCATGAAACCGTCCTATTCTGAT*
(4) ------\CCCTTTCTCTACTTCCCTTCTCAGCCGTGCCGTTCCGCTCACGACCATCAAACCGTCCTATTCTGAT*
(3) ------\------CTCTACTTCCCTTCTCAGCCGTGCCGTTCCGCTCACGACCATCAAACCGTCCTATTCTGAT*
(5) ------\CCCTTTCTCTACTTCCCTTCTCAGCCGTGCCGTTCCGCTCACGACCATCAAACCGTCCTATTCTGAT*

PRIMER F

(4) *GCAGTGGAAA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\TT\GA\GA\TA\AA\GA\GA\GA\GA\GA\GA\--\GCGAAAGATGTGTCATCA*
(5) *GCAGTGGAAA\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\TT\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GCGAAAGATGTGTCATCA*
(2) *GCAGTGGAAA\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\TT\GA\GA\GA\AA\GA\GA\GA\GA\GA\GA\--\GCGAAAGATGTGTCATCA*

(A) **GATATGGAATGATGGAGGTGGC**GCGGAGGGAAGAGAGACGCAGTGGAAAGAGAGAGAGAGAGAGATTGAGAGAAAGAGAGAGAGAGAGCGAAAGA
(B) **GATATGGAATGATGGAGGTGGC**GGAGT-----GAGAGA----------GGTGAGAGAGAAAGAATCTAAGAGAGG---------------------

(A) TGTGTCATCAATTGTCAGCCTCAGTAGTCGTACAGTACATAGTTCTATAGTCCTGC**CCCTCGCTGACAAATGTTCCG**
(B) ---------------------CAGTAGTC-----------------------CTGA**CCCTCGCTGACAAATGTTCCG**

PRIMER G

(2) *GGAAGATGGATCGGTGAGG\GA\GA\GA\GA\GG\GA\GA\GA\GA\GA\GA\GA\GA\GGGTTTACAAGTTGATGTGGCGTATATACAACTTAG
(1) *GGAAGATGGATCGGTGAGG\GA\GA\GA\GA\GG\GA\GA\GA\GA\GA\GA\GA\--\GGGTTTACAAGTTGATGTGGCGTATATACAACTTAG
(4) *GGAAGATGGATCGGTGAGG\GA\GA\GA\GA\GG\GA\GA\GA\GA\GA\GA\--\--\GGGTTTACAAGTTGATGTGGCGTATATACAATTTAG
(3) *GGAAGATGGATCGGTGAGG\GA\GA\GA\GA\GG\GA\GA\GA\GA\GA\GA\--\--\GGGTTTACAAGTTGATGTGGCGTATATACAATTTAG

```
(2) AGTAAGGAGTGCGATCCTTTTGGTCTTCCAGTACAGCAATTCTGTACATTTACATTGGAGGTTTTTCATTTCCGCGTGACGAGGAGTGAAAAGCGC
(1) AGTAAGGAGTGCGATCCTTTTGGTCTTCCAGTACAGCAATTCTGTACATTTACATTGGAGGTTTTTCATTTCCGCGTGACGAGGAGTGAAAAGCGC
(4) AGTAAGGAGTGCGATCCTTTTGGTCTTCCAGTACAGCAATTCGGTCCATTTACATTGGAGGCTTTTCATTCCCGCGTGACGAGGAGTGAAAGCAGT
(3) AGTAAGGAGTGCGATCCTTTTGGTCTTCCAGTACAGCAATTCGGTACATTTACATTGGAGGCTTTTCATTCCCGCGTGACGAGGAGTGAAAGCAGT

(2) GTTTCGATCTCTCGTCTATCTTCGCCAGGGGATATGGG-----TAATGTCTCGCGCTTGATTTGACGGCTAAGTTGCGAGACTAG
(1) GTTTCGATCTCTCGTCTATCTTCGCCAGGGGATATGGG-----TAATGTCTCGCGCTTGATTTGACGGCTAAGTTGCGAGACTAG
(4) GTCTCGATCTCTCGTCTATCTTCGCCCGGGGATATGGG-----TAATGTCTCGCGCTTGATTTGACGGCTAAGTTGCGAGACTAG
(3) GTCTCGATCTCTCGTCTATCTTCGCCCGGGGATATGGGTTGGGTAATGTCTCGCGCTTGATTTGACGGCTAAGTTGCGAGACTAG
```

PRIMER I

```
(7) *CGGTGGGCTACATACTGGGGTTGAGATT\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GTCGTGGTTAGATATCTACAGCACCCACAA*
(3) *CGGTGGGCTACATACTGGGGTTGAGATT\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\GTCGTGGTTAGATATCTACAGCACCCACAA*
(4) *CGGTGGGCTACATACTGGGGTTGAGATT\GA\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\--\GTCGTGGTTAGATATCTACAGCACCCACAA*
(2) *CGGTGGGCTACATACTGGGGTTGAGATT\GA\GA\GA\GA\GA\GA\GA\GA\GA\--\--\--\GTCGTGGTTAGATATCTACAGCACCCACAA*
```

PRIMER J

```
(2) *GCTAGTCGAGCGCACGGCATGT\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\GGAAGAGAGCCATTTCAGCC*
(3) *GCTAGTCGAGCGCACGGCATGT\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\--\--\GGAAGTGAGCCATTTCAGCC*
(4) *GCTAGTCGAGCGCACGGCATGT\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\TG\--\--\--\--\GGAAGTGAGCCATTTCAGCC*
```

PRIMER K

```
(4) *AATGGCCATGGTC\GT\GT\GT\GT\GT\CT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\CCCTCTCTCTTCTCGTT*
(2) *AATGGCCATGGTC\GT\GT\GT\GT\GT\CT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\CT\--\CCCTCTCTCTTCTCGTT*
(3) *AATGGCCATGGTC\GT\GT\GC\GT\GT\GT\GT\GT\GT\--\--\--\--\--\--\--\--\--\--\--\CCCTCTCTCTTCTCGTT*
```

PRIMER L

```
(1) *CGGCGCACGGGTATGTGTAG\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT
(4) *CGGCGCACGGGTATGTGTAG\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT
(2) *CGGCGCAGGGGTATGTGTAG\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT
(3) *CGGCGCACGGGTATGTGTAG\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--\--

(1) \GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\AT\AT
(4) \GT\GT\GT\GT\GT\GT\--\--\--\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT
(2) \--\--\--\--\--\--\--\--\--\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\AT\AT
(3) \--\--\--\--\--\--\--\--\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GT\GC\GT\GT\GT\GT\GC\GT\GT\GT\GT\GT\GT\GT

(1) \GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\--\--\GT\GT\GT\GT\CT\GT\TGGAGAGGATGAGAGG*
(4) \GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\--\--\GT\GT\GT\GT\CT\GT\TGGAGAGGATGAGAGG*
(2) \GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\--\--\GT\GT\GT\GT\CT\GT\TGGAGAGGATGAGAGG*
(3) \GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\AT\AT\GT\GC\GT\GT\GT\GT\GT\GT\CT\GT\TGGAGAGGATGAGAGG*
```

PRIMER M

```
(7) *CGTTGG\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAACGCT*
(6) *CGTTGG\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\-------\-------\-------\CAACGCT*
(3) *CGTTGG\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\-------\-------\-------\-------\CAACGCT*
(2) *CGTTGG\CAGCACA\CAGCACA\CAGCACA\CAGCACA\CAGCACA\-------\-------\-------\-------\-------\CAACGCT*
```

PRIMER O

```
(2) *GCGGACAGGACTCGCCACAGCA\TGG\TCG\TGG\TGG\TGG\CGG\TGG\TGG\TGG\TGG\CGGCGAATACGACGAGAGTCGCGATGTTTTC*
(4) *GCGGACAGGACTCGCCACAGCA\TGG\TCG\TGG\TGG\TGG\CGG\TGG\TGG\TGG\---\---CGATTACGACGAGAGTCGCGATGTTTTC*
```

(A) CGAGAAGCGACGTGCATCCTCTGACGAATATGTCCAAAACGGAGGATTATGGATACAATTCGTATGAAGCTGGCGGACAGGACTCGCCACAGCA\T
(B) CGAGAAGCGACGTGCATCCTCTTGCGGATATGGCGAAGACTGAGGATTATGGATACAATTTGTATGAGGCTGGTGGACAAGACTCTCC------\-

```
(A) GG\TCG\TGG\TGG\TGG\CGG\TGG\TGG\TGG\TGG\TGG\CGGCGAATACGACGAGAGTCGCGATGTTTTCGGCCATGAGGAACATCACGATA
(B) --\---\---\---\---\GCG\TGG\TGG\TGG\TGG\TGG\---CGATTA---CGAGAGCCGCGACGTTTTCGGACATGAGGAACTTCACGATA
```

(A) TCA**AGTACAAGACGCTCTCGTGGC**
(B) TCA**AGTACAAGACGCTCTCGTGGC**

PRIMER P

Sequence variation between *D. septosporum* and *D. pini*

(A) **GGTCATCATTGACTCGTCGGG**TCCGCTTTCACACCCGGGATCTTGGCCCAGCATGACATCACACACTTTCCTCGATCGCTCTACCTCAAAACCGCAT
(B) **GGTCATCATTGACTCGTCGGG**TCCGCTTTCACACCCGGGATCTTGGCACAGCATGACATCACACACTTTCCTCGATCGCTCTTCCTCAAAACCGCAT

(A) CTTTCACAACAACACCGCTTCCCCTGTGCACCACATTTCACAGCAACGACGACGACGACGCCGCCGCAACACGATTTCGCGCTTTACGAATCTGCTA
(B) CTTTCACAACAACTCCACCACCTCCCCCATGCGCCACATAACACAGCAACAACGACAACGCCGCCGCAACCCGAATTTGCGCTTTACGAACCTGCCA

(A) TATATCAGCAAGCATTGACCCTGCTGCAGCATCCGAGCGGACCTGCCAACAGTGGCCAGTACAAGCCAACACCTGATCCTGACATTACTATTCACCA
(B) TATACCAGCAAGCATTGACCTTGCTGCAACACCCGAGCGGCCCTAACAACAGTGGCCAGCACAAGCCGACACCCGATCCTGACATTACTATTCACCA

(A) ACCGTCGCCACTCCGCAGTTACGCCAGCGCGAGG**CAAGACTCTGCCATCAGCGAC**
(B) ATCGTCGCCGCTCCGCAGTTACGCCAGCGCGAGG**CAAGACTCTGCCATCAGCGAC**

PRIMER S

(1) *GGTGAACGATGTCATACGAGGAATTCAGCTATCATTGCTAGGTCAGAG\GT\GT\GT\GT\GT\GT\GT\GT\GAGCATCACGGTCGGATTAACC
(3) *GGTGAACGATGTCATACGAGGAATTCAGCTATCATTGCTAGGTCAGAG\GT\GT\GT\GT\GT\GT\GT\--\GAGCATCACGGTCGGATTAACC

PRIMER DS1

(3) *CTGCTGTTGGGTATCATCTA\CA\CA\CA\CA\CA\CA\CA\CA\CA\CA\CA\CA\CA\AATCTCGGGCATCTCGGTATTATCACGCCGCATA*
(2) *CTGCTGTTGGGTATCATCTA\CA\CA\CA\CA\CA\CA\CA\CA\CA\CA\--\--\--\AATCTCGGGCATCTCGGTATTATCACGCCGCATA*
(4) *CTGCTGTTGGGTATCATCTA\CA\CA\CA\CA\CA\CA\CA\CA\CA\--\--\--\--\AATCTCGGGCATCTCGGTATTATCACGCCGCATA*
(8) *CGGCTGTTGGGTATCATCTA\CA\CA\CA\CA\AA\--\--\--\--\--\--\--\--\AATCTCGGGCATCTTGGTACTATCACGCTGCATA*

PRIMER DS2

(3) *CTGCCGGTGTATAGCCACAA\CA\CA\CA\CG\CA\CA\CA\CA\CG\CA\CA\CA\CA\CA\CA\TCAAAAACACTGGCGTCTATGCTAATGT*
(4) *CTGCCGGTGTATAGCCACAA\CA\CA\CA\CA\CA\CA\CG\CA\CA\CA\CA\CA\CA\CA\--\TCAAAAACACTGGCGTCTATGCTAATGT*
(2) *CTGCCGGTGTATAGCCACAA\CA\CA\CA\CA\CA\CA\CA\CG\CA\CA\CA\CA\CA\--\--\TCAAAAACACTGGCGTCTATGCTAATGT*

Figure 2. The M-FIASCO protocol utilized to enrich for microsatellite regions in *Dothistroma septosporum*. The protocol was prepared by María Noél Cortinas and incorporates a combination of pre-existing protocols given in the references section.

Additional publications using this method include:

Cortinas MN, **Barnes I,** Wingfield BD, Wingfield MJ (2006) Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis. Molecular Ecology Notes* **6**: 780-783.

Grobbelaar JW, **Barnes I,** Cortinas MN, Bloomer P, Wingfield MJ, Wingfield BD (2008) Development and characterization of polymorphic markers for the sap-stain fungus *Ophiostoma quercus. Molecular Ecology Resources* **9:** 399-401.

Procedure of Microsatellite capture in 5 Steps

	Steps	Why do you do this? What are the results?	Timetable min, hs, days	
1.	DNA Preparations			1 or 2 days
	1.1 Test digest with restriction enzyme	- To ensure your digestion was successfull (smear)	3h	
	1.2 Digestion-ligation of adaptors	- To be able to amplify DNA fragments	3h	
	1.3 PCRs of **D**igested **DNA** with **A**daptors (DDA)	- To increase amount of fragments for enrichment - To check adaptors were ligated successfully - To repair nicks - To verify if size selection is needed	3h 1h (gel) 3h 1h (gel)	
2.	Hybridizing DDA with µsat probes			1 or 2 days
	2.1 Incubation of DNA together with biotinylated probes	- To assemble the hyb-complexes (DDA/DNA-probe)	1h	
3.	Capture of microsatellites (enrichment)			
	3.1 Incubation with streptavidin-coated magnetic beads and competitor (tRNA)	- Formation of µsats capture complexes. - Non complementary DNA fragments remain in solution - tRNA reduces non-specific binding	30min	
	3.2 Washes - 3x non-stringent - 3x stringent	- Only ssDNA-probe complexes containing µsats remain captured with the beads during each wash (separation is accomplished with the aid of the magnetic rack)	2h	
	3.3 Elution	- To separate the complexes formed by ssDNA, probes and beads	15min	
	3.4 PCR post-capture - Might need to be repeated	- Back to double stranded DNA -To increase amount of DNA containing repeat sequences -To verify quality of enrichment. You are looking for a homogeneous representation of fragments as smears (between 200-700 bp)	3h 1h (gel)	
	3.5 Freeze enrichments: optional	- If not prepared for cloning or you want to make more enrichments before cloning.		
4.	Cloning: e.g. TOPO4 Kit			2 days
5.	Screening			2 days
	5.1 Colony PCR		3h, 1h (gel)	
	5.2 Sequencing PCR		3h, 1h (purif)	
	5.3 Seq. electrophoresis		O.N	6 to 8 days

M-FIASCO @ FABI

Fundamentally based on Hamilton *et al.* 1999 and Zane *et al.* 2002

		Volume (µl)		Temp. (ºC)	Time (Cycles or hours)	
1.	**DNA preparations**					
	Adaptor preparation			In thermocycler:		
	Fiasco1 A (10µM)[a]	100,0µl		96	2min	
	Fiasco2 B (10µM)[b]	100,0µl		94	1min	
	Total	200,0µl		Bench	until RT (room temp)	
1.1	**Genomic DNA digestion**					
	Test digestion					
	DNA (approx. 20-100ng)	10,0µl				
	Enzyme buffer (NEB 2)	2,0µl		37	O.N. (overnight)	
	BSA 100X	0,2µl				
	ddH_20	7,0µl				
	Enzyme (Mse*1*)	1,0µl				
	Total	20,0µl				
	Run 1% agarose gel					
1.2	**If test is OK, proceed to the full-scale digestion-ligation reaction**					
	DNA (approx. 1µg)	80,0µl				
	10x Enzyme buffer (NEB 2)	10,0µl	Enzymes are from New England Biolabs.			
	BSA 100X	1,0µl	NEB2 buffer is compatible with both ligase and Mse*1*			
	ddH_20	6,0µl				
	Enzyme (Mse*1*)	2,0µl				
	Ligase (2 000 000 U/µl)	1,0µl				
	ATP (1mM final)	10,0µl				
	Adaptor (10 µM)	10,0µl				
	Total	100,0µl				
	Incubation			37	O.N.	
	Inactivation			65	20min	
	Make PCR dilutions in ddH_2O	1:5 or 1:10				
1.3	**PCR post digestion-ligation**			**Cycling conditions**		
	DNA	5,0µl		94	2min	
	Buffer 10X with 15mM $MgCl_2$	2,5µl		94	30s	17, 20
	$MgCl_2$	2,0µl		53	1min	25 or 30
	Primer: Fiasco Mse*I*-N (4 bases)	3,0µl		72	1min	cycles
	dNTPs (10µM)	4,0µl		72	7min	
	ddH_2O	8,1µl		4	hold	
	Taq (FABI-taq or FastStart Roche)	0,4µl				
	Total	25,0µl				
	Run 1% agarose gel					
2.	**Hybridizing genomic DNA**					

2.1	**Probing reactions (adjusted to 100µl)**			**Hybridizing conditions**		
				(Can be done in a thermocycler)		
	in 0,5 ml eppi add:			96	10min	
				62	1h	
	DNA (from PCR step in 1.3)	10,0µl		or		
	Biotinylated probes (10µM)	6,0µl		96	10min	
	Hybridization solution[c]	82,0µl		40	1h	
	ddH_2O	2,0 µl		or		
	Total	100,0µl		96	10min	
				RT	1h	
	Optional: clean through Sephadex[d] before capturing					
3.	**Capture of microsatellites (enrichment)**					
3.1	**Incubation with the beads**					
	Use 1mg of beads per each hybridization mix (DYNAL, 1mg = 100 ul)					
	(e.g. hyb mix1 = dinucleotides, mix2 = trinucleotides from 2.1)					
	Wash together all the beads you will use: repeat wash 3 to 5 times					
	Add TEN100[e] to beads	100,0µl	for each 1mg of beads			
	Magnetize, remove supernatant		3 to 5 times			
	After the last wash resuspend in clean TEN100	40,0 or 50,0µl	for each 1mg of beads			
	Add to the resuspended beads:					
	tRNA (Sigma, R- 5636)	5-10µl	(10µg)			
	Mix well!!					
	and add:					
	hyb mixes (from step 1.2)	100,0µl				
	TEN 100	300,0µl				
	Incubate @		RT with agitation (150-200rpm)		30-60 min	
			or	33	3h	
3.2	**Enriching washes (mix gently)**					4-6h
	use magnetic rack					
	1 non Stringent TEN1000[f]	400µl			5min	
	2 non Stringent TEN1000	400µl			5min	
	3 non Stringent TEN1000	400µl			5min	
	4 Stringent Solution[g]	400µl			5min	
	5 Stringent Solution	400µl			5min	
	6 Stringent Solution	400µl		42	5min	
3.3	**Elution**					
	Add 150 µl TLE or ddH_2O			95	10min	
	After magnetizing, remove beads and collect supernatant in a clean tube					
	Precipitation					
	Add 1 volume isopropanol	150,0µl				
	NaOAc 3M	7,5µl				
	leave O.N. @ -20°C				O.N or 20 min	
	Centrifuge, remove supernatant				15-30 min	
	Wash with EtOH 70%					
	Centrifuge, vacuume dry				5-10 min	
	Resuspend in ddH_2O	30,0µl				
	Store at -20°C					

3.4	**PCR post-capture**					
				Cycling conditions		
	DNA (from steps 3.3)	2,0µl				
	Buffer 10X	2,5µl		94	2min	
	$MgCl_2$	2,0µl		94	30s	
	Primer Fiasco N (4 bases)	3,0µl		53	1min	30 cycles
	dNTPs (10µM)	4,0µl		72	1min	
	ddH_2O	11,1µl		72	7min	
	Taq (FABI-taq or FastStart)	0,4µl		4	hold	
	Total	25,0µl				
	Run 1% agarose gel					
	Clean PCR products with sephadex G-50					
	Taq 3' tailing					
	DNA (clean PCR product)	8,0µl				
	2mM dATP	4,0µl				
	Buffer 10X with 15mM $MgCl_2$	2,5µl		72	30min	
	Normal Taq polymerase	0,2µl (1U)				
	ddH_2O	10,3µl				
	Total	25µl				
	Clean PCR products with sephadex G-50					
4.	**Cloning**					
	Ligations	**PGEM**		**TOPO**		
	DNA	2,5µl	DNA	2,5µl		
	Ligase Buffer	5,0µl	Salt	1,0µl		
	Vector	0,5µl	Vector	1,0µl		
	ddH_20	0,5µl	ddH_2O	1,5µl		
	Ligase	1,5µl				
	Total	10,0µl		6,0µl		
				incubate for 30min		
	Follow the instructions of manufacturers for the transforming and growing of cells					
5.	**Screening**					
5.1	**Colony preparation**					
	Pick 20 colonies and grow in tubes in 2ml media with antibiotics (LB or terrific Broth)			37	grow O.N	
	alternative:					
	Grow in 96 well microtitre plates with 150 - 200 µl LB and antibiotic (add Glycerol for long term storage after colony PCR)			37	grow O.N	
	Dilute O.N cultures with ddH_2O					
	Cell suspension	5,0µl	It depends on concentrations of cells obtained in the O.N growth			
	ddH_2O	45,0µl				
	Total	50,0µl				
	Alternative: you can try growing the bacteria for only 3 h and make the colony PCR using the cell suspensions directly without dilutions					
	Denaturation in thermocycler (to open cells and liberate the DNA)			96	7- 10min	
				On ice until PCR		

			Cycling conditions		
5.2	**Colony PCR**				
	DNA	1,0µl			
	Buffer 10X	2,5µl			
	dNTPs (10µM)	2,5µl	96	5min	
	$MgCl_2$ (25mM)	2,0µl	94	30s	
	Primer M13 TopoF (10µM)[h]	1,0 µl	53	1min	30 cycles
	Primer M13 TopoR (10µM)[i]	1,0µl	72	1min	
	Taq (FABI-taq or FastStart)	0,12µl	72	7min	
	ddH_2O	14,88µl	4	hold	
	Total	25,0µl			

Cleaning PCR products before sequencing

Sephadex G-50 or Exo-Sap[j]

			Cycling conditions		
5.3	**Sequencing**				
	DNA	3,0µl			
	Big Dye v3.1	2,0µl			
	Buffer 5X	2,0µl	96	10s	
	Vector primer (10µM)	1,0µl	50	5s	25 cycles
	ddH_2O	2,0µl	60	4min	
	Total	10,0µl	4	hold	

References

Hamilton *et al.* 1999 protocol
Hamilton MB, Pincus EL, Di Fiore A, Fleischer C (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques* **27**, 500-507.

Zane *et al.* 2002 protocol
Zane L, Baegelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology* **11,** 1-16.

Appendix

[a]Fiasco1 A: TACTCAGGACTCAT
[b]Fiasco2 B: GACGATGAGTCCTGAG

Solutions as in Zane *et al.* 2002

[c]Hybridization solution	(SSC 4.2X, SDS 0.7%)
[e]TEN 100	(10mM Tris-HCl, 1mM EDTA, 100mM NaCl, pH 7.5)
[f]TEN 1000	(10mM Tris-HCl, 1mM EDTA, 1M NaCl, pH 7.5)
[g]Stringent solution	(SSC 0.2X, 0.1% SDS)

[d]Sephadex G-50 recipe to clean PCR and sequencing products

Dissolve 2g in 30ml ddH_2O
boil in microwave for 30 seconds
Use @ RT. Mix well before use
Store @ 4°C

Procedure:

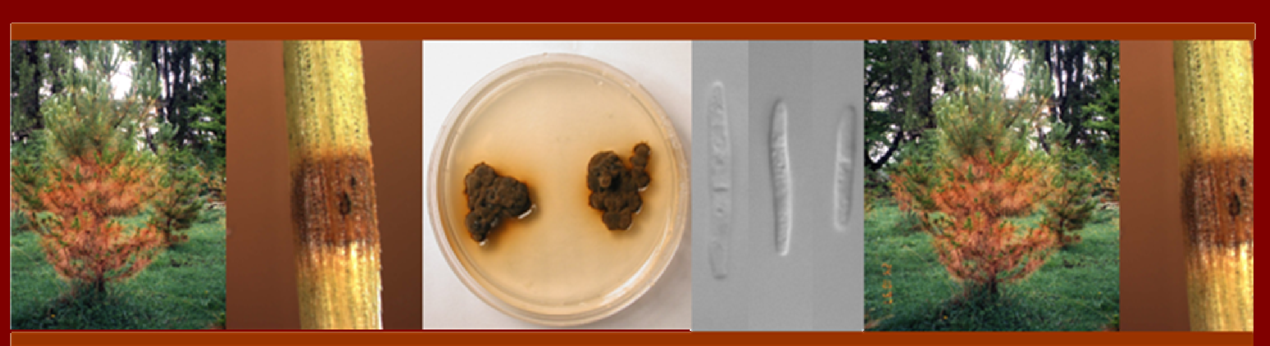
Fill CentriSep plastic columns with Sephadex G-50	650,0µl	
Centrifuge* with a collector tube and discard ddH_2O		2 min*
Add PCR or Seq products to the centre of the packed column	10,0µl - 60,0µl	
Centrifuge* and collect purif. DNA in a new clean tube		2 min*

* 0.7, 0.8 g = 2800 rpm in eppendorf 5415D

Additional for Sequencing....

Dry in a vacuum centrifuge	aprox.15 min

	Topo (M13) primers [h]5' GTA AAA CGA CGG CCA G [i]5' CAG GAA ACA GCT ATG AC	 16bp 17bp				
	[j]**Exo-SAP** Prepare a solution of 1:1 Exonuclease I and Shrimp Alkaline Phosphatase mixing the enzymes in ddH_2O. Store @ -20°C **Use 0.5-1 U of each enzyme for every 20 ul of PCR reaction product** incubate PCR product ready to use			 37 80	 15min 15min	
	Cleaning of sequencing reactions Sephadex G-50 or 96 well Ethanol precipitation (Ethanol/EDTA/Sodium Acetate precipitation protocol from ABI (Applied Biosystems, Protocol booklet 4337035 Rev. A, CA, USA)					



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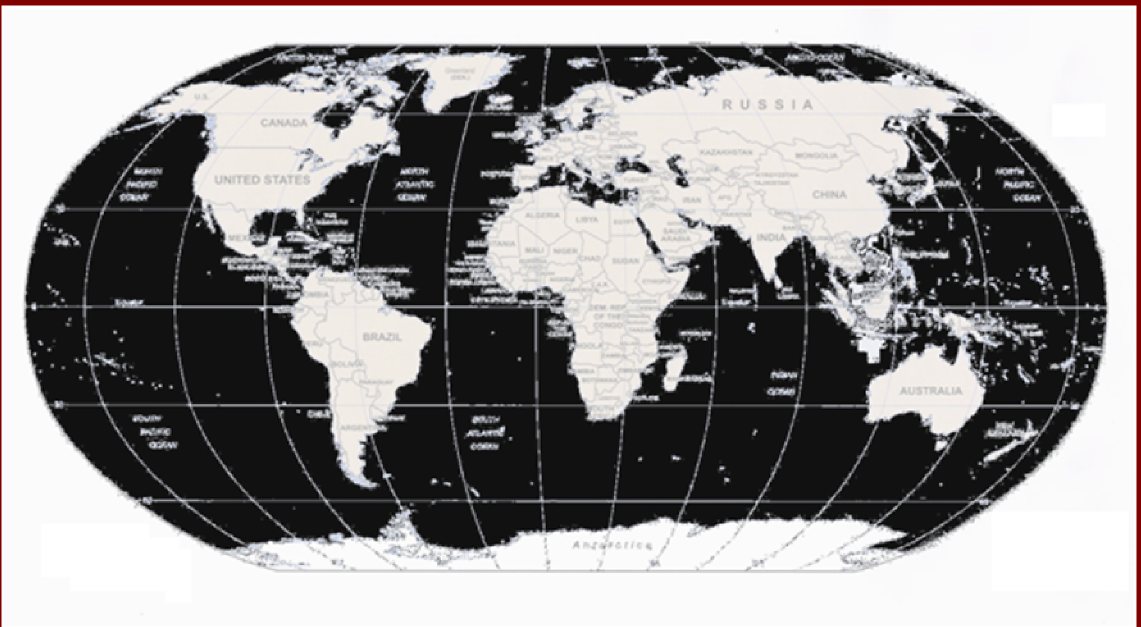

Global population structure and diversity of the red band needle blight pathogen, *Dothistroma septosporum*, reflects anthropogenic activity

ABSTRACT

Dothistroma septosporum is a haploid fungal pathogen that causes a serious needle blight disease of pines, particularly on *Pinus radiata* in the Southern Hemisphere. During the course of the last decade, the pathogen has also resulted in unexpected epidemics in the non-native plantations in the Northern Hemisphere. Unusually, *D. septosporum* is hypothesized to be endemic to the Himalayas as well as Central America, and there is clearly a distinct lack of knowledge regarding the population biology or genetic diversity of the pathogen. The aim of this study was to determine the population diversity and structure of a collection of *D. septosporum* isolates from 14 countries on six continents using microsatellite markers. In the Northern Hemisphere countries where pines are native and two mating type genes are present, high genetic diversity was found. Most of the populations from Europe were randomly mating showing little differentiation and high gene flow. Similar results were obtained for the North American (U.S.A) and Asian (Bhutan) populations. Isolates from most of the Southern Hemisphere countries had low genetic diversity and were represented by a single mating type. However, populations from Africa, which have had longer histories of pine introductions, had relatively high genetic diversity and were randomly mating. The results are consistent with the expected Northern Hemisphere origin of *D. septosporum,* and the patterns of diversity reflect the movement of germplasm and expansion of pine planting in the Southern Hemisphere.

INTRODUCTION

Biological invaders are considered to be any plants, animals, invertebrates or micro-organisms that have established in a new area and that threaten, or have a detrimental effect on, the biodiversity, ecology or safety of the new environment (Pimental *el al.* 2000, Sakai *et al.* 2001, Allendorf & Lundquist 2003, Anderson *et al.* 2004). Biological invasions by fungal plant pathogens have had huge impacts in forest ecosystems in both their native and non-native ranges and have been recognised for many years (Elton 1958, Desprez-Loustau *et al.* 2007). Important examples include Chestnut blight caused by *Cryphonectria parasitica* (Murr.) Barr and Dutch elm disease caused by *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier. The former pathogen was introduced into North America from Asia around 1900 and devastated American chestnuts throughout their native range (Anagnostakis 1987). Likewise, Dutch elm disease has caused disastrous pandemics on elm trees in their native ranges of Europe and North America (Brasier *et al.* 1998, Brasier 2001). The devastation caused by these diseases has been on native trees in natural forests, but plantation forestry, based on non-native trees particularly in the tropics and Southern Hemisphere, has also been seriously damaged by diseases (Wingfield *et al.* 2001, Wingfield 2003). Amongst these, *Dothistroma septosporum* (Dorog.) M. Morelet, that causes Dothistroma needle blight (DNB), is by far the most important invasive pathogen of non-native pine species (Gibson 1972, Ivory 1987, Bradshaw 2004, Barnes *et al.* 2008a). This disease is characterised by successive needle defoliation, retarded wood growth and in severe cases, tree death (Gibson *et al.* 1964).

Two haploid fungal species cause DNB (Barnes *et al.* 2004). These morphologically similar fungi can be distinguished from each other based on spore size, but more effectively using DNA-based identification techniques (Barnes *et al.* 2004, Chapter 6). *Dothistroma pini* has a limited host range and is known in the North-Central USA, Russia, Ukraine (Barnes *et al.* 2004, Barnes *et al.* 2008a) and has most recently been reported from Hungary (Chapter 6). In contrast, *D. septosporum* has been known since 1911 when it was first described from *P. mugo* in Russia (Doroguine 1911). Since then, the pathogen has been reported in more than 45 countries world-wide (Ivory 1994, Bradshaw 2004), infecting over 70 different species of pine (Bednářová *et al.* 2006). DNB owes its notoriety to this species and the epidemics it has caused in plantations of non-native *Pinus* spp. in the Southern Hemisphere (Gibson 1972, Ivory 1987).

Given its importance, it is surprising how little is known regarding the origin of *D. septosporum.* In this regard, there are two hypotheses (Evans 1984, Ivory 1994). Based on its presence on native pines in the high, minimally disturbed cloud forests of Central America in the absence of epidemics, one view is that *D. septosporum* is native in that region (Evans 1984). The fact that the pathogen has also been found on indigenous pine in remote forests in the Himalayas prompted Ivory (1994) to suggest that it might also be native to these areas. Given the fact that *D. pini* and *D. septosporum* are almost impossible to discriminate from each other in the absence of DNA based techniques, reports such as those listed above, and hypotheses regarding the origin of these pathogens, are spurious at best. Recently, the presence of *D. septosporum* has, however, been confirmed from native blue pine (*P. wallichiana*) trees in the Himalayas, in the absence of epidemics (Barnes *et al.* 2008a) and this area of origin seems probable.

One of the main factors attributed to the increase in biological invasions is the expansion of global travel and trade that has promoted the introductions of plant pathogens into new areas, mainly via infected plant material (Richard & Lonsdale 2001, Rossman 2001, Wingfield *et al.* 2001). Alternatively, introductions into new environments can be due to natural events such as long distance dispersal via wind-blown spores (Brown & Hovmøller 2002, Stukenbrock *et al.* 2006). *Dothistroma septosporum* provides a good example of how both forms of dispersal have increased its presence world-wide.

All pines are exotic to the Southern Hemisphere and pathogens such as *D. septosporum* in this region would have to have been accidentally introduced, probably on plant material. The increase in air traffic and the establishment of commercial pine plantations, especially after World War II, is how *Dothistroma* was most likely introduced into the Southern Hemisphere (Gibson 1972).

Within and between countries trade in plant material would have provided sources of new infections in the Northern Hemisphere. For example, in 1999 and 2000, planting stocks of *P. nigra* and *P. mugo* respectively, that were infected with *D. septosporum,* were intercepted when they were transported into the Czech Republic via Hungary (Jankovský *et al.* 2004). Intercontinental spread of the pathogen has most likely been due to trade in plant germplasm and human activity. *D. septosporum* in New Zealand is speculated to have been introduced by forestry officials who travelled to East Africa during 1957 to observe DNB (Hirst 1997). The pathogen was discovered five years later causing disease in central North Island forests of

New Zealand (Gilmour 1967). Natural long distance dispersal of *D. septosporum* into Australia, from New Zealand, via spores in mist clouds blown over the Tasman Sea has been suggested (Edwards & Walker 1978). This view is supported due to the strict quarantine regulations in Australia, which would make it unlikely that an introduction via plant material had occurred (Edwards & Walker 1978, Bradshaw 2004).

A characteristic of a successful invasive species lies in its ability to establish itself in a new environment and then to spread to new areas (Sakai *et al.* 2001). Short distance spread of the spores of *D. septosporum*, either as asexual conidia or sexual ascospores is very effective (Gibson 1972, Bradshaw 2004). The ability of the pathogen to expand its range can be observed from the chronological records of first reports from countries from both the Northern and the Southern Hemisphere.

In the Southern Hemisphere, the first report of *D. septosporum* was from Africa (Zimbabwe) in 1940 where young *P. radiata* were severely damaged (Barnes 1970, Gibson 1972). In 1957, the pathogen was then found in Tanzania and within seven years, the associated disease had spread to all major plantations of *P. radiata* in Kenya, Malawi and Uganda (Gibson 1972). Similarly in Chile and New Zealand, where 92 % of the worlds *P. radiata* is grown, (Toro & Gessel 1999, Rogers 2002), *D. septosporum* has caused disease epidemics since 1957 (Gibson 1972) and 1963 (Gilmour 1967) respectively. The pathogen was only found in Australia much later, in 1975 (Edwards & Walker 1978).

In Europe, *D. septosporum* has successfully invaded countries and established itself during the last 50 years. During this time, the range of the pathogen has expanded and disease epidemics have emerged. In Serbia (Yugoslavia), for example, the pathogen has been known since 1955 (Krstić 1958) but by 1988 the infections had reached epidemic proportions on both native and exotic hosts (Karadzić 1988). From there, the disease spread northwards, and by 1969 it had entered the southern part of Hungary (Marinković & Stajković 1969, Karadzić 1989). By 1995 (Szabó 1997), the disease had spread into all *P. nigra* monoculture plantations, most of which had been established during a pine afforestation programme in the 1960's (ÁESZ 2002). Approximately two years later, the disease was recorded in the Southern tip of Slovakia, close to the border of Hungary. Today, it is found throughout Slovakia on both native and non-native pine species. Plantations of *P. nigra* for Christmas trees are the most severely affected and pines growing in stressed situations in both Hungary and Slovakia show signs of tree mortality.

Dothistroma septosporum has been present in many European countries where there has been no real cause for concern because damage was minimal and epidemics were never seen (Evans 1984). In the last two decades, however, *D. septosporum* has emerged as a threat to native, and particularly exotic, pine species planted within Europe and other Northern Hemisphere countries. The disease is prominent, for example, in epidemic proportions on susceptible, exotic plantations of *P. nigra* subsp. *laricio* (Corsican pine) in the U.K. (Brown *et al.* 2003) and France (Aumonier 2002). Likewise, in British Colombia, although native pine species are infected, non-native lodgepole (*P. contorta* var. *latifolia)* pine plantations have experienced severe epidemics with 60 – 100 % mortality (Parker & Collis 1966, Woods 2003, Woods *et al.* 2005). These new epidemics have largely been attributed to global climate change (Woods 2003) and as a result, the pathogen is now of international concern.

Although the ecology of *D. septosporum* has been well documented, little is known regarding the genetics of the pathogen, its mode of spread or the sources of new introductions. In a preliminary study of the mating type distribution in *D. septosporum,* Groenewald *et al.* (2007) revealed that only one mating type was present in New Zealand and Australia. This was consistent with the study conducted by Hirst (1997) in which several populations collected in New Zealand and screened with RAPD markers, showed that these populations were clonal. Both mating types were, however, observed in other parts of Africa, Europe and the Americas (Groenewald *et al.* 2007) indicating the possibility of sexual reproduction in these areas.

The aim of this study was to consider the diversity and genetic structure of *D. septosporum* populations from a world wide collection of isolates from 14 countries over six continents using 12 polymorphic microsatellite markers. More specifically, we considered whether the patterns in genetic diversity and structure of *D. septosporum* populations reflected the movement of its host from the Northern Hemisphere to the Southern Hemisphere and whether epidemic populations on non-native pines in both the Northern and Southern Hemisphere reflect recent introduction events. In addition we considered the degree of genetic differentiation and variation that exists between populations and groups of populations and estimated geneflow between geographic locations. The frequency and distribution of mating types in the countries was also investigated.

MATERIALS AND METHODS

Sampling, fungal isolation and DNA extraction

In this study, isolates of *D. septosporum* were obtained from a variety of *Pinus* spp. representing 14 different countries (referred to as populations) from six regions. These included Africa (South Africa and Kenya), Europe (Austria, Czech Republic, Hungary, Poland, Romania, Slovakia), Asia (Bhutan), North America (U.S.A.), South America (Chile and Ecuador) and Australasia (Australia and New Zealand) (Table 1). The main pine species from which collections were made included *P. nigra* from the Northern Hemisphere and *P. radiata* from the Southern Hemisphere. The sampling strategy from plantations included collecing a handful of diseased needles from every alternate tree randomly chosen along two or more transects. Each alternate tree was sampled to reduce the potential of obtaining clones. Samples from native trees, or those not in plantations, were randomly collected from infected trees. Samples were collected from one or several locations in a country (Table 1).

Isolations were made as described in Barnes *et al.* 2004, from a single conidiomata on a needle, per tree. Cultures were grown on 2 % malt extract agar (MEA, Biolab, Midrand, Johannesburg) at 20 °C. All cultures have been deposited and are maintained at the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Genomic DNA was extracted from each isolate using freeze-dried, ground mycelium, from 2- to 3-month-old cultures with the aid of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Haplotype identification

The genotypes of isolates were determined at 12 microsatellite loci including Doth_E, Doth_F, Doth_G, Doth_I, Doth_J, Doth_K, Doth_L, Doth_M, Doth_O, Doth_DS1, Doth_DS2 described by Barnes *et al.* (2008b) and DCB2 of Ganley & Bradshaw (2001) (Figure 1). An internal diagnostic marker, Doth_A, was used to confirm the identity of the isolates as *D. septosporum* during allele scoring based on the fact that *D. septosporum* is monomorphic for the allele 124 while *D. pini* is monomorphic for the allele 114 at locus Doth_A (Barnes *et al.* 2008b).

Polymerase chain reactions (PCR) were carried out in 96-well plates. A total volume of 12.5 µl per well contained 5-10 ng DNA template, 0.06 U FastStart Taq DNA Polymerase (5 U/µl)

(Roche Diagnostics GmnH, Mannheim, Germany), 1x PCR buffer containing 2 mM $MgCl_2$, 0.25 mM of each dNTP, 100 nM of the forward and reverse primer, one of which was fluorescently labelled and 1 mM additional $MgCl_2$. The PCR conditions consisted of an initial denaturation step of 10 min followed by 10 cycles at 94 °C for 30 s, specified annealing temperature for 45 s (as described in Barnes *et al.* 2008b) and 72 °C for 1 min. A further 30 cycles were carried out using the same conditions above except that a 0.5 s increment was added to the elongation time. A final elongation time of 30 min at 60 °C was added to avoid the +A effect (Clark 1988, Magnuson *et al.* 1996) during genescan analyses. PCR amplicons were separated, with a 100 bp marker, on 2 % low electroendomosis agarose gels (Roche Diagnostics) stained with ethidium bromide and visually analysed under UV light (Vilber Lourmat, Omni-Science). PCR amplicons were not purified.

To facilitate multiplexing, PCR amplicons (for each individual) were combined according to the approximate size of the amplicons and type of fluorescent label attached to the primer. Samples were electrophorised on an ABI 3100 sequencer. Allele assignments were determined using ABI-Prism ® GENEMAPPER™ software version 3.0 (Applied Biosystems, Foster City, USA). Multilocus genotypes were obtained by combining the alleles present at all twelve loci for each isolate. All isolates having the same multilocus haplotype in a population were considered clones. Only one representative of each haplotype (clone-corrected) was retained for the analyses of each population. Clone-corrected datasets were thus used in all analyses unless otherwise stated.

Genetic data analyses

Genetic diversity

Allele frequencies were estimated for each SSR locus using the program POPGENE version 1.32 (http://www.ualberta.ca/fyeh/index.htm). Total number of alleles, unique alleles and expected heterozygosity, calculated as Nei's (1973) measure of gene diversity, were calculated for each population and region across all 12 loci.

In addition to calculating gene diversity, estimates of allelic richness were computed for each population and region in the program FSTAT for windows, version 2.9.3.2 (http://www.unil.ch/izea/softwares/fstat.html) (Goudet 2001). Unequal sample sizes were standardized, by rarefaction, to a uniform sample size of the smallest population (USA, $n = 7$) and region (Australasia, $n = 6$) as described by El Mousadik & Petit (1996). The clonal

fraction was calculated for each population by dividing the number of genotypes observed in the population by the total population size and subtracting this from one.

Measures of genotypic diversity (D) were quantified using non clone-corrected datasets in MULTILOCUS version 1.3 (Agapow & Burt 2001) as $(n/n\text{-}1)(1\text{-} \sum_i pi^2)$ where *pi* is the frequency of the *i*th genotype and *n* is the number of individuals sampled. Here, the multilocus genotype of all possible pairs of individuals is compared and the proportion of pairs that are different is calculated. Populations that are completely clonal will score a value of 0 while those where all individuals have different multilocus genotypes will score 1.

The random associations of alleles among different loci (consistent with sexual reproduction), were tested by calculating the index of association (I_A) in the program MULTILOCUS version 1.3 (Agapow & Burt 2001). I_A, which is a measure of multilocus linkage disequilibrium (Maynard Smith *et al.* 1993), was calculated for all isolates in a population using clone-corrected data sets. The I_A was only calculated for those populations in which both mating types were present.

The hierarchical partitioning of molecular variation within and among populations and among regions was assessed with an AMOVA-test implemented GENALEX version 6.1 (Peakall & Smouse 2006) using the complete dataset. The significance was tested by 1000 permutations of the dataset. Only regions with more than two populations were included in analyses.

Population differentiation

The level of pair-wise genetic differentiation between populations was tested using Weir's θ (Weir 1996) for haploids, which is an estimate of Wright's F_{ST}, using the program MULTILOCUS. The observed θ was calculated and compared to values of 1000 artificial randomized datasets to test for the null hypothesis of no population differentiation ($\theta = 0$). If $\theta = 1$, no alleles are shared between the populations. Corresponding estimates of the number of migrants per generation was calculated as $M=[(1/\theta) - 1]/2$ (Cockerham & Weir 1993).

Population structure

The program STRUCTURE version 2.2 (Pritchard *et al.* 2000, Falush *et al.* 2003) was used to determine the optimal number of populations (K) and to assign individuals to these distinct populations based on their genotype data and not by their geographic location. The program uses a Bayesian Monte Carlo Markov Chain (MCMC) clustering algorithm and the

simulations assumed a model of mixed ancestry and correlation of allele frequencies within clusters. Individuals are assigned to clusters to minimize Hardy-Weinberg disequilibrium and linkage disequilibrium between the loci within each cluster. The MCMC scheme was run for 100 000 iterations after a burn-in period of 10 000 and twenty simulations were performed for K ranging from one to 14 to verify the convergence of the Log likelihood values for each value of K. Delta K (ΔK), a statistic based on the rate of change in the log probability of data with respect to the number of clusters, was used to help interpret the real number of clusters (Evanno *et al.* 2005). After the optimal K was determined, a final parameter of 1 million MCMC replicates and a burn-in period of 100 000 was run for the assignment of individuals into K populations.

Mating type distribution

The mating genotypes (MAT1-1 and MAT1-2) were assayed using a set of primers defined by Groenewald *et al.* (2007) to amplify the mating-type idiomorphes of *D. septosporum*. Degenerate primers (Dot Mat1r 5'- TTGCCTGACCGGCTGCTGGTG-3' and Dot Mat2r 5'-CTGGTCGTGAAGTCCATCGTC-3') and species-specific primers (*D. septo* Mat2f 5'-GTGAGTGAACGCCGCACATGG-3' and *D. septo* Mat1f 5'-CGCAGTAAGTGATGCCCTGAC-3') were multiplexed in a single reaction. PCR reactions were carried out in 96-well micro titre plates with a total volume of 12.5 µl in each well consisting of: 10 ng - 20 ng DNA, 1x PCR buffer containing 2 mM $MgCl_2$, 0.5 mM $MgCl_2$, 0.12 mM of each dNTP, 200 nM of each of the four primers and 0.032 U FastStart Taq DNA Polymerase (5 U/µl) (Roche Diagnostics GmnH, Mannheim, Germany). Cycling conditions consisted of an initial denaturation of 5 min at 94 ^{o}C followed by 40 cycles at 94 ^{o}C for 20 sec, 65 ^{o}C for 20 sec, 72 ^{o}C for 40 sec and a final elongation of 7 min at 72 ^{o}C. To determine whether populations were deviating from the null hypothesis of a 1:1 ratio of mating types, a χ^2-test was applied to the frequencies of the two mating types.

RESULTS

Haplotype identification

A total of 471 (Table 1) isolates were successfully recovered from the infected pine needles. All isolates screened with the internal diagnostic marker, Doth_A, produced an allele size of 124, consistent with *D. septosporum,* except for 13 isolates from Hungary that produced an allele size of 119 (See Chapter 6). These isolates were excluded from further analyses. After clone correction, 240 unique haplotypes were obtained.

Genetic data analyses

Genetic diversity

From 12 microsatellite loci, a total of 130 alleles were produced ranging from three alleles at Locus_O to 24 alleles at Locus_L. The allele frequencies at each locus are recorded for all populations in Table 2. Other indices of variation are reported in Table 3 for each population (country) and region (continent).

Isolates from the USA had the highest percentage of unique alleles at 25 % followed by Austria (11.76 %) while RSA, Chile, Ecuador and New Zealand contained no unique alleles. Only Austria and Hungary showed 100 % polymorphism for the 12 loci. Expected heterozygosity ranged from 0.04 in New Zealand to 0.60 in Austria. Similarly, allelic richness, based on rarefaction to the smallest sample size, ranged from 4.32 to 1.78. On the continental scale, Europe contained the highest percentage of unique alleles at 42 % followed by North America at 25 % and South America at 15 %. Expected heterozygosity and allelic richness was highest in Europe (H = 0.58, A = 4.0) and lowest in South America (H = 0.24, A = 1.9) and Australasia (H = 0.12, A = 1.33).

Overall, the clonal fraction for the Northern Hemisphere countries was low (ave. 17 %) compared to the Southern Hemisphere (ave. 62 %). The Czech Republic and isolates from Bhutan contained no duplicate haplotypes while in New Zealand, the clonal fraction was 91 %. Genotypic diversities were higher in the Northern Hemisphere countries ranging from 0.833 to one. The genotypic diversity for isolates from the Southern Hemisphere ranged from 0.964 (Kenya) to 0.091 (New Zealand). Three haplotypes were shared between Chile and Ecuador and one between New Zealand and Australia.

AMOVA analyses showed that on a global scale, with the continents representing regions, most of the variation observed was distributed within populations (61 %), but a significant proportion of the variation was also attributed to differences among regions (32 %) (Table 5). Only 8 % of the variation was partitioned among populations within regions. AMOVA analyses on individual regions showed that for both the South American and Australasian groups, a high percentage of the variation was within populations (99 % and 96 % respectively) with no genetic differences observed among these populations. A large proportion of the variation within Africa was observed between Kenya and South Africa (33 %, Table 5) and, within Europe, although variation among populations was low (Φ-PT = 0.088), it was still significant for population structure.

Population differentiation

Theta values ranged from 0.004 (between Czech and Austria) to 0.704 (between USA and Australia) and were significant for 25 of the 91 population pairs indicating an absence of genetic differentiation between 12 of the populations sampled (Table 6). All populations paired with RSA and Kenya, from Africa, had high and significant pairwise theta values ($P < 0.05$) ranging from 0.283 (between Austria and Kenya) and 0.616 (between Australia and Kenya). The numbers of migrants per generation were substantial within the European populations (113 migrants between Austria and Czech). For the Southern Hemisphere countries, geneflow was indicated between Ecuador and Chile (76 migrants) and between New Zealand and Australia (12 migrants).

Population structure

STRUCTURE analyses revealed the highest posterior probability for five populations among all the isolates (Figure 2). Assignments of the individuals into these clusters closely resembled that of their geographic distribution (Figure 3). The Southern Hemisphere isolates were partitioned into two clusters; the majority of isolates from Africa (Kenya and RSA) formed one cluster and, validating the AMOVA results, the remaining isolates from Chile, Ecuador, New Zealand and Australia formed a second cluster. For the Northern Hemisphere, three additional clusters were observed. The isolates from USA and Bhutan were assigned to the same cluster while in Europe, a fair amount of the isolates from Romania, Hungary, Slovakia, Czech and Austria clustered together. Isolates from Poland displayed differences in allele frequencies when compared with those from the rest of Europe and were partitioned into their own cluster (Figure 4). Baysian estimates also indicated a slight potential for K=2 populations (Figure 2). When the partitioning of K=2 was further investigated (Figure 2, Figure 3), the separation was mainly between the populations in the Northern Hemisphere versus those in the Southern Hemisphere, except for Bhutan which grouped together with the Southern Hemisphere (Figure 3).

Mating type distribution

Two products were obtained using the Doth Mat primers including a 823 bp product of the MAT1-1 idiomorph and a 480 bp product pertaining to the MAT1-2 idiomorph. Both mating types were present in all the Northern Hemisphere populations. Only Poland deviated from the null hypothesis of a 1:1 ratio of random mating favouring the MAT1-2 idiomorph (Table 4). Isolates from Kenya and RSA in Africa in the Southern Hemisphere, contained both mating types while Ecuador, Chile, Australia and New Zealand isolates had only the MAT1-2

idiomorph (Table 4). The hypothesis of random mating was rejected in five out of the nine populations that were analysed using the I_A statistic at $P<0.01$ (Table 4). Those that appeared to have random mating were populations from the Czech Republic, Hungary, Bhutan and Kenya. In Austria, when the analyses were refined to 27 isolates from *P. nigra* in one plantation, results indicated random mating with $I_A = 0.27$ and $P = 0.037$.

DISCUSSION

The epidemics caused by *Dothistroma septosporum* in many parts of the world have been exacerbated by planting of susceptible hosts, particularly in monocultures and under environmental conditions conducive to disease development (Gibson 1972, Ivory 1987, Brown *et al.* 2003, Woods *et al.* 2005). Clustering methods, based on multilocus microsatellite markers applied to a worldwide collection of isolates of *D. septosporum,* in this study, partitioned the isolates roughly corresponding to geographic origin. This clustering made it possible to show how the patterns of genetic diversity, population structure and distribution of mating types in the fungus reflect the anthropogenic nature of its worldwide distribution in the Southern Hemisphere. They also provide an interesting insight into the establishment, reproduction and range expansion of *D. septosporum* on non-native trees in the Northern Hemisphere, particularly in Europe.

Other than those for Poland, the European populations of *D. septosporum* were characterised by high levels of genetic diversity, high levels of geneflow, low population differentiation, and the presence of two mating types. Essentially these populations from Austria, Hungary, the Czech Republic and Romania constitute one panmictic population, where most of the genetic variation (91 %) distributed in populations is reflected by the large numbers of unique alleles present in these individual populations. These populations, mainly on exotic *P. nigra,* were genetically very similar, despite their occurrence in different countries. This is explained by the fact that these countries border on each other and thus have no barriers to geneflow.

The absence of physical barriers to geneflow suggests that migration of *D. septosporum* individuals, which was particularly evident between Austria and the Czech Republic, would occur between the European countries. Migration or range expansion of *D. septosporum* during the last 50 years in Europe, due to effective dispersal of conidia and extensive movement of pine material, has evidently resulted in the homogenisation of allele frequencies

in isolates from this region. This has, in turn, resulted in an even distribution of genetic structure of the pathogen in these countries. The range expansion of an apparently native pathogen is not unusual and is well known for many other plant pathogens (Banke & McDonald 2005, Stukenbrock *et al.* 2006, Gladieux *et al.* 2008)

The equal proportions of mating types found in the populations of *D. septosporum* from Europe and the high genetic diversity observed in these isolates, suggests that recombination between individuals is occurring. This is most likely due to sexual reproduction as is common for outcrossing populations of fungi (Milgroom 1996). This interpretation is supported by the fact that in most cases, the populations consisted of unique haplotypes and by the fact that the sexual state of the fungus has been recorded from many European countries (Bradshaw 2004) including those considered in his study. Apparently, regular sexual recombination and population growth over time has gradually dissipated most of the evidence of introduced populations in Europe, which would be revealed by genetic bottlenecks and multilocus linkage disequilibrium (Dlugosch & Parker 2008).

Isolates from Poland represented a clear example of an introduction into an area where a genetic bottleneck (Dlugosch & Parker 2008) has apparently shifted the allele frequency of a population. As a result, the Poland population was partitioned in its own cluster, which would account for the majority of the 9 % among-population variation observed in Europe. Dothistroma needle blight in Poland was first observed in a strip of trees near the edge of an old forest nursery in 1990 (Kowalski & Jankowiak 1998) which could be the source of the introduction into that country, probably via infected nursery stock. Seven years later, *D. septosporum* was found over 500 km away, seriously infecting a 12-year-old seed plantation (Kowalski & Jankowiak 1998), which was also the collection site for the samples in this study. The Poland population is also the only one from Europe analysed in this study that was not in Hardy-Weinberg equilibrium, with the MAT 2 phenotype predominating (73 %) in the samples collected. This however, has not affected the diversity of the pathogen in the region, which is still comparatively high compared to those from the rest of Europe. The Poland population also included many unique alleles that might have resulted from multiple introductions into the country. These introductions would most likely have originated from a source different to other populations considered in this study.

The isolates from Bhutan and USA included in this study were very small in number but they were included in order to determine whether any of the genotypes in those countries might be

present in samples from other countries. This would have made it possible to determine migration patterns for the pathogens. The USA isolates were very distinct from those originating elsewhere in the world and had a large number of unique alleles, despite the small sample size. These populations were genetically isolated with high levels of population differentiation. In contrast, while the Bhutan isolates did not cluster with those from Europe, they shared many of the same alleles. This is particularly interesting because the Himalayas have been suggested as a possible area of origin for *D. septosporum* (Ivory 1990, Ivory 1994). In addition, the isolates were from trees that did not show signs of disease (Barnes *et al.* 2008a), which is a common feature for pathogens present in their native ranges and under selection pressure (Parker & Gilbert 2004, Burdon *et al.* 2006). Human-mediated movement of the pathogen out of an area such as the Himalayas, followed by large natural range expansion could have aided the efficient spread of *D. septosporum* throughout Europe. This would have been strongly supported by planting of susceptible, often non-native hosts for wood production and the spread would have most likely continued gradually from east to west. Recent reports of *D. septosporum* from Finland and Sweden (Hantula, pers. communication) provide an example of such local range expansion of the pathogen.

Patterns of genetic diversity and the clustering of isolates of *D. septosporum* from countries of the Southern Hemisphere sampled, reflect the anthropogenic movement of pines into these regions. In the African cluster, the high levels of genetic variability in the Kenyan and South African populations, and the presence of both mating types, indicate that multiple introduction events of *D. septosporum* have occurred. This is consistent with the long history of pine being moved into these countries (Poynton 1977, Richardson *et al.* 2007) that would have provided many pathways and opportunities for introduction of the pathogen (Lundquist 1987, Diekmann *et al.* 2002). A similar situation has emerged for pine pathogen *Diplodia pinea* (Desm.) J. Kickx. Fil. on *P. radiata* in the Southern Hemisphere (Burgess *et al.* 2001), *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni populations in Europe (Gobbin *et al.* 2006) and the European race of *Gremmeniella albietina* (Lagerberg) Morelet var. *abietina* in North America (Hamelin *et al.* 1998).

The Kenyan population of *D. septosporum* was the most diverse sample in the Southern Hemisphere as reflected by gene and genotypic diversity as well as the number of unique alleles. It is also the country considered in this study which has had the longest history of commercial *P. radiata* plantations and *D. septosporum* infections (Gibson 1972, Lavery & Mead 1998). The presence of both mating types in a 1:1 ratio in the African countries and the

lack of linkage disequilibrium for the Kenyan isolates suggest that sexual reproduction could be important in the life cycle of the pathogen in Africa. This could account for the high genotypic diversity observed in the populations due to recombination and re-assortment of alleles during sexual reproduction (Milgroom 1996). It is unusual, therefore, that the sexual state of *D. septosporum* has not been reported from Africa (Groenewald *et al.* 2007), although this might simply be due to time and intensity of sampling.

Founder effects were evident in *D. septosporum* populations from Ecuador, Chile, New Zealand and Australia. The low levels of diversity and clonal structure that were observed for isolates from these countries suggests that these populations have lost alleles in association with their movement, arrival, and establishment outside their native range. This is common for introduced pathogens. For example, *Fusarium circinatum* Nirenberg & O'Donnell, which causes pitch canker of pine, has reduced genetic variation in its areas of introduction in comparison to those in its native geographic range (Wikler & Gordon 2000). Linkage disequilibrium and shifts in the reproductive modes of populations are additional indicators of pathogens that have been moved out of their natural range (Taylor *et al.* 1999). Both these indicators are apparent in the populations of *D. septosporum* from South America and Australasia in this study where only the MAT 2 phenotype was observed. These studies using a larger sample size, confirmed the same results obtained by Groenewald *et al.* (2007).

Low genetic diversity and the presence of a small number of clonal lineages in the South American and Australasian cluster observed in this study has clearly not been a limiting factor for the successful establishment and spread of *D. septosporum*. Populations that have gone through a bottleneck resulting in lower levels of genetic diversity, are thought to be at a disadvantage due to the possibility of alleles conferring adaptive abilities to survive in their new environment being deleted (Sakai *et al.* 2001, Allendorf & Lundquist 2003). *D. septosporum* disease epidemics have, however, been most devastating in the Southern Hemisphere. In this case, a small number of virulent genotypes have likely adapted to the environmental conditions and susceptible host tissue and these would have spread, expanding their range via asexual reproduction. This would be similar to the situation for many other introduced plant pathogens including *Phytophthora infestans* (Mont.) de Bary (Goodwin *et al.* 1994), *Diplodia pinea* (Desm.) J. Kickx. Fil. (Burgess *et al.* 2001) and *Ceratocystis fimbriata* (Ellis & Halsted) f. *platani* Walter (Engelbrecht *et al.* 2004).

The results of this study suggest that there has been direct movement of *D. septosporum* between Ecuador and Chile. This was evident from the fact that both countries shared common genotypes, all the alleles were identical and there was a lack of population structure (1 % among population variation) between these countries. Pine plantation forestry based on *P. radiata* has been practiced in Chile since the 1940's (Toro & Gessel 1999) and to a lesser degree in Ecuador since 1925 (Miller 1974). Dothistroma needle blight has been known in Chile since 1957 (Gibson 1972) but was only reported from Ecuador in 1983 (Evans & Oleas 1983). Given the genetic similarity of the *D. septosporum* populations in these countries, it is likely that the pathogen was accidentally moved from Chile that has shared *P. radiata* germ plasm with Ecuador (F. Montenegro, pers. communication).

It has been speculated that *D. septosporum* moved from New Zealand to Australia naturally across the Tasman Sea (Edwards & Walker 1978, Matheson 1985). Results of this study showing a single haplotype present in New Zealand and at two locations sampled in Australia supports this view. However, the Australian samples included additional genotypes and unique alleles not found in New Zealand. This suggests that, despite rigorous quarantine regulations, other sources of introductions have occurred into Australia. These would most likely have been human-mediated due to the geographically isolated position of the continent and they would most probably have occurred through the importation of pine germplasm such as seed.

Results of this study clearly show that the *D. septosporum* populations in New Zealand are clonal. This is consistent with previous studies (Hirst 1997) where only one haplotype was found in samples covering a relatively large area. In that study, collections made in the 1960's were identical to those made 30 years later. It is evident that the same clone continues to persist almost 50 years later. New Zealand is well known to have maintained very strict quarantine regulations for many years (www.maf.govt.nz/quarantine) and evidently, new introductions of *D. septosporum* have not occurred. There was, however, a single isolate in the New Zealand collection that had a mutation at one locus in a microsatellite region resulting in a unique genotype. The observed mutation in a single isolate suggests that *D. septosporum* has the potential to evolve, and thus to overcome the extensive resistance barrier that has been bred into *P. radiata* in that country.

The number of isolates available for this study from Kenya was limited. However, there were no shared genotypes between isolates from New Zealand and Kenya and only a small

proportion (22 %) of the alleles were common to the fungus in these areas. It was thus not possible to validate the hypothesis of Hirst (1997) that *D. septosporum* in New Zealand originated in Kenya. Yet the data suggests that this is probably not the case.

The worldwide distribution of *D. septosporum* has provided an outstanding example of human intervention, albeit unintentional, facilitating both the movement and the establishment of this pathogen in many areas of the world where pines are planted as non-natives. Although available cultures from countries considered in this study varied in number, intriguing patterns of local range expansion and global spread emerged. There remains substantial opportunity to expand knowledge gained in this study with larger numbers of isolates from areas that could not be optimally sampled. Larger numbers of isolates from North America, Central America and the Himalayas are particularly likely to provide evidence for the centre of origin of *D. septosporum*.

In the future, optimal climatic conditions, influenced dramatically by climate change (Woods *et al.* 2005), and the planting of susceptible trees in monocultures are likely to increase the potential for *D. septosporum* to infect, establish, and expand its range. This is apparently already occurring in areas such as British Columbia and parts of Europe. *D. septosporum* provides an outstanding model to study biological invasions due to the fact that it has become established both in the Northern and Southern Hemisphere where it can be viewed alternatively as an alien invasive or a native pathogen, influenced by planting practices, other human activities and climate change. Whereas populations of the pathogen in the Northern Hemisphere are genetically homogenous and sexually recombining, those in the Southern Hemisphere, with the exception of Africa, have low levels of diversity and a single mating type gene. It is thus important to monitor and prevent the further spread of different, possibly more virulent genotypes and opposite mating type genes, into these predominantly monoculture plantations of susceptible hosts.

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Table 1: Isolates of *Dothistroma septosporum* from different countries and hosts used in this study

Country	Locality	Host	Number of isolates	Date collected	Collectors
SOUTH AFRICA	Hogsback, Eastern Cape	*P. radiata*	73	Aug 2001	J. Roux
	Haenertsburg area, Tzaneen, Limpopo	*P. radiata*	27	Sep 2002	I. Barnes
KENYA	Napkoi	*P. radiata*	11	Jan 2001	J. Roux
ECUADOR	Lasso Highlands, Cotopaxi	*P. muricata*	17	2001	M.J. Wingfield
CHILE	Canteras plantation, Bio Bio, VIII Region	*P. radiata*	27	2001	M.J. Wingfield
	Canteras plantation, Bio Bio, VIII Region	*P. radiata*	1	2007	R. Ahumada
	Dollinco, Valdivia, X Region	*P. radiata*	23	2001	M.J. Wingfield
	Naguilan, Valdivia, X Region	*P. radiata*	8	2001	M.J. Wingfield
NEW ZEALAND	Karioi	*P. ponderosa*	1	Aug 2001	M. Dick
	Karioi	*P. contorta*	1	Aug 2001	M. Dick
	FRI Nursery, Rotorua	*P. radiata*	3	Aug 2001	M. Dick
	Hokonui Forest	*P. ponderosa*	1	Aug 2001	M.J. Wingfield
	Lake Okareka, Rotorua	*P. radiata*	1	Aug 2001	M.J. Wingfield
	Kaingora Forest	*P. radiata*	15	Feb 2003	M.J. Wingfield
AUSTRALIA	Canberra (A.C.T)	*P. radiata*	6	2000	K. Old
	State Forests of New South Wales in Tumut	*P. radiata*	35	June 2003	A.J. Carnegie
BHUTAN	Yusipang, Thimphu dzongkhag	*P. radiata*	3	July 2005	T. Kirisits, M. J. Wingfield
	Ura, Bumthang dzongkhag	*P. wallichiana*	1	May 2005	H. Konrad
	Tangsibi, Bumthang dzongkhag	*P. wallichiana*	6	July 2005	T. Kirisits, M. J. Wingfield
	Lamey Goemba, Bumthang dzongkhag	*P. wallichiana*	2	July 2005	T. Kirisits, N. Gyeltshen
HUNGARY	Botanical garden of the University of West Hungary, Sopron	*P. mugo*	5	August 2005	T. Kirisits
	Near Diszel	*P. nigra*	45	May 2007	I. Barnes
AUSTRIA	Hollenstein/Ybbs, Lower Austria	*P. sylvestris*	5	July 2004	T. Kirisits
		P. mugo	5	July 2004	T. Kirisits
		P. nigra	2	July 2004	T. Kirisits
		P. cembra	1	July 2004	T. Kirisits
	Forest experimental garden, “Knödelhütte”, BOKU, Vienna	*P. peuce*	4	April 2004	T. Kirisits
	Thenneberg, province Lower Austria	*P. nigra*	27	June 2004	T. Kirisits
	Near Wr. Neustadt, Lower Austria	*P. sylvestris*	1	June 2006	T. Kirisits
	Raumberg, Styria	*P. mugo*	2	June 2006	T. Kirisits
	National park “Gesäuse”, Styria	*P. sylvestris*	8	June 2006	T. Kirisits
		P. mugo	2	June 2006	T. Kirisits
POLAND	Miechów Forest District, Goszcza Forest Unit, Near Cracow	*P. nigra*	34	June 2003	T. Kowalski
CZECH REPUBLIC	Tisnov, Riegrova road	*P. nigra*	1	July 2006	L. Jankovsky
	South Moravia, Lanzhot, Forest district Lanzhot, Loc:	*P. nigra*	12	July 2006	L. Jankovsky
SLOVAKIA	Strazovske vrchy, valley between Zliechov and Kosecke Podhradie	*P. nigra*	24	July 2006	L. Jankovsky
ROMANIA	Valea Putnei (near fishing pond), Suceava	*P. nigra*	4	July 2007	G. Hoch
U.S.A.	Lochsa Historical Ranger Station, Idaho	*P. ponderosa*	1	2004	L.M. Carris
	Missoula Lola National Forest, Montana	*P. contorta v. latifolia*	9	May 2006	D. Six

Table 2: Allele frequencies of *D. septosporum* isolates from different countries based on 12 microsatellite markers

		EUROPE						ASIA	NORTH AMERICA	AFRICA		SOUTH AMERICA		AUSTRAL-ASIA	
Locus	**Allele**	**Austria**	**Czech**	**Hungary**	**Poland**	**Romania**	**Slovakia**	**Bhutan**	**USA**	**Kenya**	**RSA**	**Chile**	**Ecuador**	**Australia**	**New Zealand**
DS1	Allele A	0.02	-	-	-	-	0.04	0.08	-	-	0.74	-	-	-	-
	Allele B	0.02	-	-	-	-	-	0.92	-	-	-	1	1	1	1
	Allele C	**0.02**[1]	**0.23**	-	-	-	-	-	-	-	-	-	-	-	-
	Allele D	0.30	0.15	0.25	0.07	0.67	0.35	-	0.86	0.22	0.03	-	-	-	-
	Allele E	0.11	-	0.11	0.33	-	0.17	-	-	0.78	-	-	-	-	-
	Allele F	0.20	0.08	0.25	0.22	-	-	-	-	-	0.23	-	-	-	-
	Allele G	0.18	0.23	0.25	-	0.33	0.35	-	0.14	-	-	-	-	-	-
	Allele H	**0.05**	**0.08**	-	-	-	-	-	-	-	-	-	-	-	-
	Allele I	**0.07**	**0.15**	**0.06**	**0.37**	-	**0.09**	-	-	-	-	-	-	-	-
	Allele J	**0.02**	**0.08**	**0.08**	-	-	-	-	-	-	-	-	-	-	-
DCB2	Allele A	0.56	0.38	0.58	1	0.67	0.83	-	1	0.67	1	0.25	0.5	0.5	1
	Allele B	0.44	0.62	0.42	-	0.33	0.17	1	-	0.33	-	0.75	0.5	0.5	-
F	Allele A	**0.02**	-	**0.03**	-	-	-	-	-	-	-	-	-	-	-
	Allele B	0.64	0.62	0.33	0.74	-	0.70	1	-	0.78	0.23	1	1	1	1
	Allele C	0.33	0.38	0.64	0.26	1	0.30	-	-	0.22	0.77	-	-	-	-
	Allele D	-	-	-	-	-	-	-	**0.57**	-	-	-	-	-	-
	Allele E	-	-	-	-	-	-	-	**0.43**	-	-	-	-	-	-
E	Allele A	-	-	**0.03**	-	-	-	-	-	-	-	-	-	-	-
	Allele B	**0.02**	-	-	-	-	-	-	-	-	-	-	-	-	-
	Allele C	0.13	0.38	0.19	0.22	-	0.39	-	-	-	0.03	-	-	-	-
	Allele D	0.73	0.62	0.69	0.78	1	0.43	0.83	0.14	0.44	0.57	-	0.08	-	-
	Allele E	0.09	-	0.08	-	-	0.09	-	-	-	-	-	0.08	-	-
	Allele F	0.02	-	-	-	-	0.09	-	-	0.56	0.40	-	-	-	-
	Allele G	-	-	-	-	-	-	0.08	0.14	-	-	-	-	-	-
	Allele H	-	-	-	-	-	-	-	**0.71**	-	-	-	-	-	-
	Allele I	-	-	-	-	-	-	0.08	-	-	-	0.08	0.08	-	-
	Allele J	-	-	-	-	-	-	-	-	-	-	-	0.08	1	0.50
	Allele K	-	-	-	-	-	-	-	-	-	-	0.33	0.25	-	0.50
	Allele L	-	-	-	-	-	-	-	-	-	-	**0.50**	**0.33**	-	-
	Allele M	-	-	-	-	-	-	-	-	-	-	**0.08**	**0.08**	-	-
G	Allele A	-	-	-	-	-	-	-	**0.71**	-	-	-	-	-	-
	Allele B	**0.02**	-	-	-	-	-	-	-	-	-	-	-	-	-
	Allele C	0.09	0.08	0.08	0.04	-	0.04	1	0.14	0.44	0.60	-	-	-	-
	Allele D	0.78	0.92	0.92	0.96	1	0.96	-	0.14	0.56	0.40	0.75	0.75	-	-
	Allele E	0.09	-	-	-	-	-	-	-	-	-	0.25	0.25	1	1
	Allele F	**0.02**	-	-	-	-	-	-	-	-	-	-	-	-	-
J	Allele A	0.02	-	0.06	-	-	-	0.17	0.14	-	-	-	-	-	-
	Allele B	-	-	-	-	-	-	-	**0.86**	-	-	-	-	-	-
	Allele C	0.13	-	0.03	0.19	0.33	-	0.08	-	-	0.40	1	1	-	-
	Allele D	0.09	0.23	0.19	0.19	-	0.04	0.17	-	1	0.54	-	-	-	-
	Allele E	0.44	0.54	0.53	0.48	-	0.78	0.33	-	-	0.06	-	-	-	-
	Allele F	0.27	-	0.17	0.07	0.67	0.04	0.08	-	-	-	-	-	-	-
	Allele G	0.02	0.15	-	0.07	-	-	0.08	-	-	-	-	-	0.75	1
	Allele H	-	0.08	0.03	-	-	-	-	-	-	-	-	-	0.25	-
	Allele I	-	-	-	-	-	**0.13**	-	-	-	-	-	-	-	-
	Allele J	-	-	-	-	-	-	**0.08**	-	-	-	-	-	-	-
	Allele K	**0.02**	-	-	-	-	-	-	-	-	-	-	-	-	-
O	Allele A	0.11	-	0.06	0.19	0.33	-	-	-	0.33	0.31	-	-	-	-
	Allele B	0.87	1	0.94	0.81	0.67	1	1	-	0.67	0.69	1	1	1	1
	Allele C	0.02	-	-	-	-	-	-	1	-	-	-	-	-	-
M	Allele A	**0.02**	-	-	-	-	-	-	-	-	-	-	-	-	-
	Allele B	0.07	0.08	0.06	0.37	-	0.09	0.08	-	-	0.60	-	-	-	-
	Allele C	-	-	0.06	-	-	-	-	-	-	-	-	-	1	1
	Allele D	0.36	0.46	0.56	0.33	0.33	0.48	0.08	0.86	0.33	0.40	-	-	-	-
	Allele E	-	-	0.08	-	-	-	-	0.14	-	-	-	-	-	-
	Allele F	0.11	0.15	0.06	-	0.33	-	0.25	-	-	-	-	-	-	-
	Allele G	0.09	-	-	-	-	-	0.08	-	-	-	1	1	-	-
	Allele H	**0.18**	-	**0.06**	**0.07**	-	**0.04**	-	-	-	-	-	-	-	-
	Allele I	0.09	-	-	0.11	0.33	0.09	0.25	-	-	-	-	-	-	-
	Allele J	**0.02**	**0.23**	**0.03**	**0.04**	-	-	-	-	-	-	-	-	-	-
	Allele K	-	0.08	-	-	-	0.04	0.17	-	-	-	-	-	-	-
	Allele L	0.02	-	0.08	-	-	-	0.08	-	-	-	-	-	-	-
	Allele M	**0.02**	-	-	-	-	**0.22**	-	-	-	-	-	-	-	-
	Allele N	-	-	-	-	-	0.04	-	-	0.11	-	-	-	-	-
	Allele O	**0.02**	-	-	-	-	-	-	-	-	-	-	-	-	-
	Allele P	-	-	**0.03**	**0.07**	-	-	-	-	-	-	-	-	-	-

Table 2: Continued…

		EUROPE						ASIA	NORTH AMERICA	AFRICA		SOUTH AMERICA		AUSTRAL-ASIA	
Locus	Allele	Austria	Czech	Hungary	Poland	Romania	Slovakia	Bhutan	USA	Kenya	RSA	Chile	Ecuador	Australia	New Zealand
I	Allele A	0.02	-	-	-	-	0.30	-	0.86	-	-	-	-	-	-
	Allele B	0.58	0.54	0.97	0.59	0.67	0.57	0.75	-	0.22	0.03	0.25	0.25	1	1
	Allele C	0.13	-	-	-	-	-	0.17	-	0.78	0.86	0.58	0.75	-	-
	Allele D	0.02	-	-	0.30	-	0.04	-	0.14	-	0.11	0.17	-	-	-
	Allele E	0.18	0.38	0.03	0.11	0.33	0.09	0.08	-	-	-	-	-	-	-
	Allele F	**0.07**	**0.08**	-	-	-	-	-	-	-	-	-	-	-	-
L	Allele A	0.04	0.08	0.06	-	-	-	-	0.14	-	-	-	-	-	-
	Allele B	**0.04**	-	-	-	-	-	-	-	-	-	-	-	-	-
	Allele C	0.56	0.62	0.50	0.19	0.33	0.61	0.17	-	-	-	-	-	-	-
	Allele D	0.04	0.08	0.06	-	-	0.09	0.58	0.14	-	-	-	-	-	-
	Allele E	-	-	-	**0.78**	-	-	-	-	-	-	-	-	-	-
	Allele F	-	-	**0.03**	-	-	**0.04**	-	-	-	-	-	-	-	-
	Allele G	-	0.08	-	-	-	-	0.17	-	-	-	-	-	-	-
	Allele H	0.04	-	0.11	-	-	0.04	-	-	1	-	-	-	-	-
	Allele I	**0.02**	-	-	-	-	-	-	-	-	-	-	-	-	-
	Allele J	**0.02**	-	**0.19**	-	-	**0.04**	-	-	-	-	-	-	-	-
	Allele K	-	-	-	**0.04**	-	**0.04**	-	-	-	-	-	-	-	-
	Allele L	-	-	-	-	**0.33**	-	-	-	-	-	-	-	-	-
	Allele M	-	**0.08**	-	-	-	-	-	-	-	-	-	-	-	-
	Allele N	**0.11**	**0.08**	**0.06**	-	-	-	-	-	-	-	-	-	-	-
	Allele O	0.04	-	-	-	-	-	-	-	-	-	-	0.25	0.75	1
	Allele P	-	-	-	-	-	-	-	-	-	-	-	-	**0.25**	-
	Allele Q	0.02	-	-	-	-	-	-	0.14	-	-	-	-	-	-
	Allele R	0.02	-	-	-	-	0.04	0.08	-	-	0.80	-	-	-	-
	Allele S	-	-	-	-	**0.33**	-	-	-	-	-	-	-	-	-
	Allele T	-	-	-	-	-	-	-	-	-	-	**1**	**0.75**	-	-
	Allele U	-	-	-	-	-	0.09	-	-	-	0.20	-	-	-	-
	Allele V	-	-	-	-	-	-	-	**0.43**	-	-	-	-	-	-
	Allele W	**0.02**	-	-	-	-	-	-	-	-	-	-	-	-	-
	Allele X	-	-	-	-	-	-	-	**0.14**	-	-	-	-	-	-
K	Allele A	**0.47**	**0.54**	**0.61**	**0.30**	**0.33**	**0.04**	-	-	-	-	-	-	-	-
	Allele B	-	-	-	-	-	-	**0.17**	-	-	-	-	-	-	-
	Allele C	-	**0.08**	-	-	-	-	-	-	-	-	-	-	-	-
	Allele D	-	-	-	-	-	0.04	-	-	-	-	0.17	-	-	-
	Allele E	0.18	0.08	0.08	0.04	0.33	0.48	0.58	0.14	1	0.40	0.42	0.25	1	1
	Allele F	0.13	0.08	0.06	0.37	-	0.04	0.08	0.57	-	0.29	0.08	-	-	-
	Allele G	0.07	0.15	0.08	0.30	-	0.30	-	0.14	-	0.14	-	0.08	-	-
	Allele H	0.04	-	-	-	-	-	0.17	-	-	0.17	-	-	-	-
	Allele I	**0.07**	-	**0.03**	-	**0.33**	**0.09**	-	-	-	-	-	-	-	-
	Allele J	**0.02**	**0.08**	**0.14**	-	-	-	-	-	-	-	-	-	-	-
	Allele K	0.02	-	-	-	-	-	-	0.14	-	-	-	-	-	-
	Allele L	-	-	-	-	-	-	-	-	-	-	**0.33**	**0.67**	-	-
DS2	Allele A	**0.07**	-	**0.03**	**0.04**	-	**0.04**	-	-	-	-	-	-	-	-
	Allele B	**0.02**	-	**0.03**	-	-	-	-	-	-	-	-	-	-	-
	Allele C	**0.09**	**0.15**	**0.09**	**0.04**	**0.33**	**0.09**	-	-	-	-	-	-	-	-
	Allele D	-	-	**0.06**	-	-	-	-	-	-	-	-	-	-	-
	Allele E	0.40	0.31	0.23	0.33	-	0.09	-	-	0.11	-	-	-	-	-
	Allele F	0.09	0.08	0.20	-	0.33	0.39	-	-	-	-	1	1	1	1
	Allele G	0.07	-	0.09	-	-	-	-	-	-	0.34	-	-	-	-
	Allele H	0.07	-	0.03	0.44	-	-	-	1	-	-	-	-	-	-
	Allele I	-	**0.15**	-	-	-	-	-	-	-	-	-	-	-	-
	Allele J	**0.02**	**0.15**	-	-	-	-	-	-	-	-	-	-	-	-
	Allele K	-	-	0.03	-	-	-	-	-	0.89	0.40	-	-	-	-
	Allele L	0.02	-	-	-	-	-	0.17	-	-	-	-	-	-	-
	Allele M	-	-	-	-	-	**0.22**	-	-	-	-	-	-	-	-
	Allele N	-	-	-	-	-	-	0.83	-	-	0.26	-	-	-	-
	Allele O	-	-	**0.03**	-	-	**0.04**	-	-	-	-	-	-	-	-
	Allele P	**0.04**	**0.08**	-	-	-	-	-	-	-	-	-	-	-	-
	Allele Q	-	-	-	**0.15**	-	-	-	-	-	-	-	-	-	-
	Allele R	**0.04**	-	**0.14**	-	-	**0.04**	-	-	-	-	-	-	-	-
	Allele S	**0.04**	-	-	-	-	-	-	-	-	-	-	-	-	-
	Allele T	**0.02**	**0.08**	**0.06**	-	**0.33**	**0.09**	-	-	-	-	-	-	-	-

[1]Allele frequencies in bold represent alleles that are unique to the continent

Table 3: Summary diversity statistics of *Dothistroma septosporum* isolates within populations and regions

	CONTINENT	COUNTRY	N[1]	No. of haplotypes[2]	Total no. of alleles	Unique alleles	% alleles unique	% loci polymorphic	H[3]	Allelic richness	D[4]
Northern Hemisphere		AUSTRIA	61	45	85	10	11.76	100	0.60	4.32	0.979
		CZECH	13	13	47	3	6.38	92	0.53	3.55	1
		HUNGARY	50	36	61	2	3.28	100	0.50	3.63	0.977
		POLAND	34	27	39	2	5.13	92	0.46	2.81	0.980
		ROMANIA	4	3	25	2	8	75	0.41	N/A	0.833
		SLOVAKIA	24	23	53	2	3.77	92	0.49	3.42	0.996
	EUROPE	**Total**	**186**	**147**	**109**	**46**	**42.20**	**100**	**0.58**	**4.00**	**0.995**
	ASIA (Bhutan)	**Total**	**12**	**12**	**36**	**2**	**5.55**	**67**	**0.33**	**2.68**	**1**
	NORTH AMERICA (USA)	**Total**	**10**	**7**	**28**	**7**	**25**	**75**	**0.31**	**2.32**	**0.911**
Southern Hemisphere		KENYA	11	9	23	2	8.70	75.00	0.31	2.28	0.964
		RSA	100	35	30	0	0	92	0.43	1.91	0.857
	AFRICA	**Total**	**111**	**44**	**37**	**2**	**5.41**	**100**	**0.49**	**2.57**	**0.884**
		CHILE	59	12	22	0	0	42	0.22	1.79	0.835
		ECUADOR	17	12	24	0	0	50	0.24	1.92	0.934
	SOUTH AMERICA	**Total**	**76**	**20**	**27**	**4**	**14.81**	**50**	**0.24**	**1.90**	**0.873**
		AUSTRALIA	41	4	15	1	6.67	25	0.10	N/A	0.618
		NEW ZEALAND	22	2	13	0	0	8	0.04	N/A	0.091
	AUSTRALASIA	**Total**	**63**	**5**	**16**	**1**	**6.25**	**33**	**0.12**	**1.33**	**0.528**

[1] N = Total number of isolates

[2] Equivalent to samples that have been clone corrected

[3] H = Nei's (1973) gene diversity

[4] Genotypic diversity

Table 4: Linkage disequilibrium tests and the distribution and frequency of mating types within world-wide populations of *Dothistroma septosporum*

Countries	Clone-corrected sample size	Clonal fraction	Index of association (I_A)	*P*-vlaue[1] of I_A	Frequencies		χ^2 values
					MAT1-1	MAT1-2	
AUSTRIA	45 (27)[2]	0.26	0.406 (0.27)	< 0.001* (0.037)	0.44	0.56	0.56
CZECH	13	0.00	0.222	0.093	0.46	0.54	0.08
HUNGARY	35	0.30	0.11	0.146	0.56	0.44	0.50
POLAND	27	0.21	0.704	< 0.001*	0.27	0.73	5.54 [3]
ROMANIA	3	0.25	-	-	1	0	-
SLOVAKIA	23	0.04	0.433	0.002*	0.45	0.55	0.18
BHUTAN	12	0.00	-0.155	0.718	0.42	0.58	0.33
USA	7	0.30	2.850	< 0.001*	0.86	0.14	3.57
KENYA	9	0.18	0.730	0.011	0.67	0.33	1.00
SOUTH AFRICA	35	0.65	0.702	< 0.001*	0.46	0.54	0.26
CHILE	12	0.80	-	-	0	1	-
ECUADOR	12	0.29	-	-	0	1	-
AUSTRALIA	4	0.90	-	-	0	1	-
NEW ZEALAND	2	0.91	-	-	0	1	-

[1] *The null hypothesis of random mating is rejected at P<0.01

[2] Values in parenthesis indicate the results obtained from the refined analyses of I_A for 27 isolates from *P. nigra* in one plantation in Austria.

[3] Mating type frequencies depart from the null hypothesis of a 1:1 ratio at P < 0.05 with 1 degree of freedom.

Table 5: Analyses of molecular variance within populations, among populations and between regions of *Dothistroma septosporum*

Dataset	Number of populations	Number of regions	% Variation						ϕPT-statistics	*P*- value[1]
			Among Regions	df	Among Pops	df	Within Pops	df		
Africa	2	1	-	-	33%	1	67%	42	0.333	0.001*
South America	2	1	-	-	1%	1	99%	22	0.006	0.319
Australasia	2	1	-	-	4%	1	96%	4	0.040	0.537
Europe	6	1	-	-	9%	5	91%	141	0.088	0.001*
North America	1	-	-	-	-	-	-	-	-	-
Asia	1	-	-	-	-	-	-	-	-	-
All Regions	14	6	32%	5	8%	8	61%	226	0.395	0.001*
Northern vs Southern Hemisphere	14	2	11%	1	25%	12	64%	226	0.358	0.001*

[1]P-value based on 1 000 randomizations.

*The null hypothesis of no population structure is rejected at P<0.01.

Table 6: Pairwise population differentiation (above the diagonal) as measured by θ and number of migrants measured as M=[(1/θ) - 1]/2 (below the diagonal). Theta estimates in bold were significantly greater than zero (P<0.05) based on permutation tests

	RSA	KENYA	ECUADOR	CHILE	N. ZEALAND	AUSTRALIA	POLAND	AUSTRIA	CZECH	SLOVAKIA	HUNGARY	ROMANIA	BHUTAN	USA
RSA	-	**0.333**	**0.502**	**0.530**	**0.515**	**0.557**	**0.343**	**0.291**	**0.358**	**0.351**	**0.347**	**0.326**	**0.453**	**0.507**
KENYA	1.00	-	**0.580**	**0.604**	**0.579**	**0.616**	**0.388**	**0.283**	**0.357**	**0.348**	**0.362**	**0.419**	**0.487**	**0.592**
ECUADOR	0.50	0.36	-	0.007	0.557	0.580	0.467	0.338	0.431	0.417	0.437	0.536	0.526	0.680
CHILE	0.44	0.33	76.42	-	0.614	0.618	0.492	0.356	0.447	0.440	0.453	0.570	0.530	0.702
NEW ZEALAND	0.47	0.36	0.40	0.31	-	0.040	0.437	0.310	0.377	0.357	0.398	0.461	0.515	0.661
AUSTRALIA	0.40	0.31	0.36	0.31	12	-	0.494	0.356	0.425	0.418	0.435	0.573	0.515	0.704
POLAND	0.96	0.79	0.57	0.52	0.64	0.51	-	0.114	0.156	0.166	0.165	0.210	0.424	0.457
AUSTRIA	1.22	1.27	0.98	0.90	1.11	0.90	3.89	-	0.004	0.071	0.029	0.014	0.264	0.387
CZECH	0.90	0.90	0.66	0.62	0.83	0.68	2.71	113.14	-	0.077	0.032	0.070	0.316	0.455
SLOVAKIA	0.92	0.94	0.70	0.64	0.90	0.70	2.51	6.54	5.99	-	0.099	0.130	0.372	0.439
HUNGARY	0.94	0.88	0.65	0.60	0.76	0.65	2.53	16.74	15.13	4.55	-	0.039	0.360	0.457
ROMANIA	1.03	0.69	0.43	0.38	0.58	0.37	1.88	35.21	6.64	3.35	12.32	-	0.437	0.474
BHUTAN	0.60	0.53	0.45	0.44	0.47	0.47	0.68	1.39	1.08	0.84	0.89	0.64	-	0.622
USA	0.53	0.45	0.44	0.47	0.47	0.68	1.39	1.08	0.84	0.89	0.64	1.08	0.84	-

Figure 1. Multiplexed allele sizes and range of loci generated with 15 fluorescently labelled microsatellite markers designed by Ganley *et al.* (2001) and Barnes *et al.* (2008b). Combined allele sizes, in base pairs, were obtained by screening the microsatellite markers on a few isolates from Chapter 4 and from this chapter.

100 110 120 124 130 140 142 147 149 151 156 160 170 175 177 178 180 182 184 186 187 188 190 192 194 200

A DS1 DCB2 F MB1 G2 J O

182 184 186 188 190 194 199

200 204 210 222 228 234 242 243 244 246 248 250 258 280 290 300

E M S

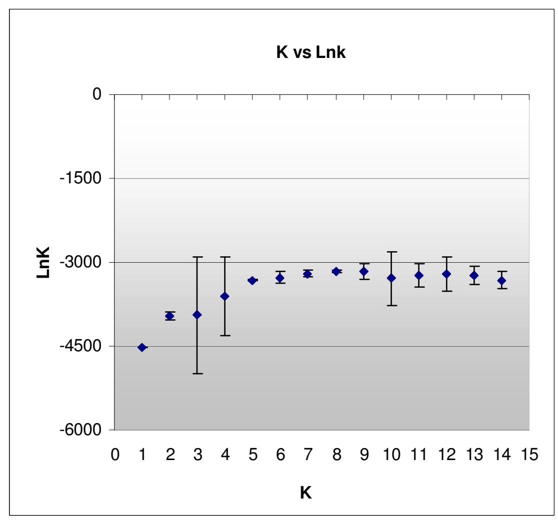
214 227 235 241 243 247 258 262 264 266 268 270 272

300 303 305 307 309 318 321 325 330 334 338 351 353 360 370 383 385 396 400 401 413

I K L DS2 L

334 354 356 358 360 362 366 375 379 381 383 385 395 397 398 402 407

Figure 2: Optimal number of populations (K=5) as determined by the LnK and DeltaK values obtained from STRUCTURE analyses using the admixture model with correlated allele frequencies.


K vs Lnk
LnK
0
-1500
-3000
-4500
-6000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
K

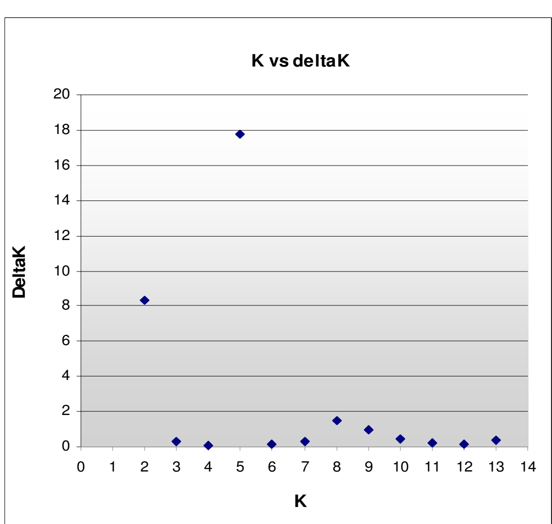

K vs deltaK
DeltaK
20
18
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
K

Figure 3: Assignment of individuals into K=2 populations (above), that separate most of the Northern and Southern Hemisphere isolates, and the optimal population size of K= 5 (below), using the program STRUCTURE.

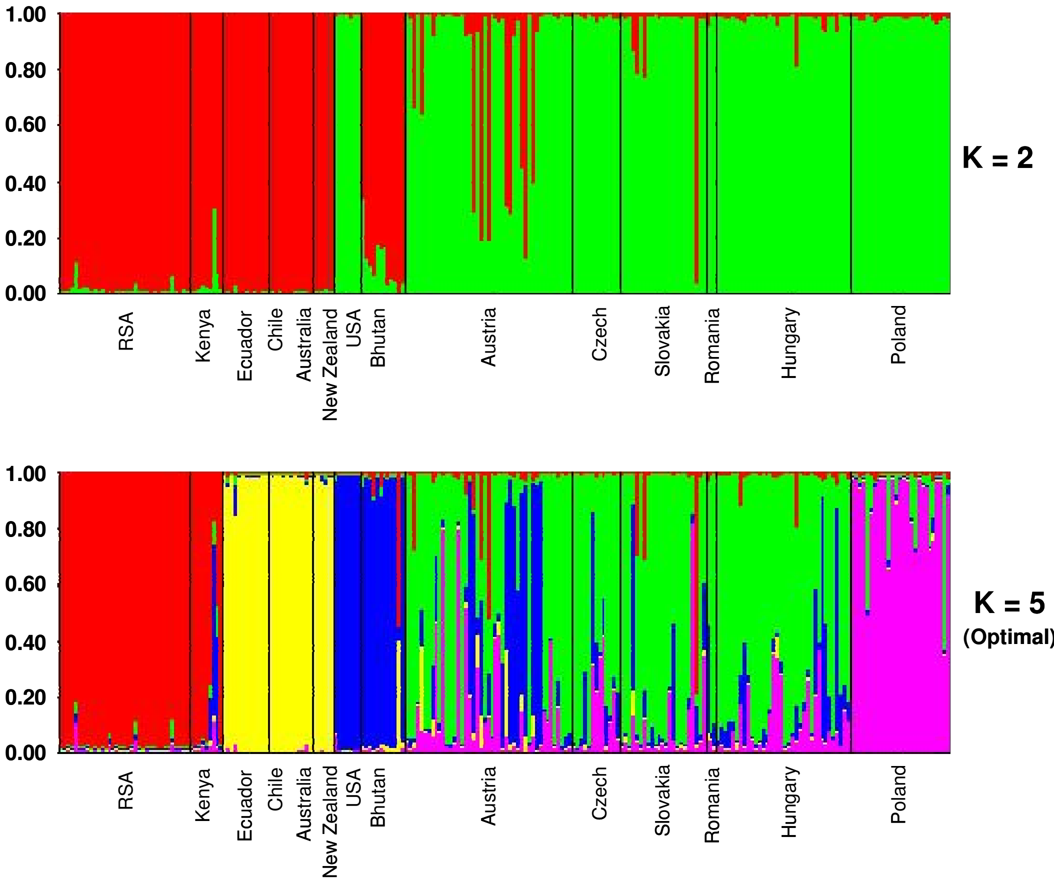

1.00
0.80
0.60
0.40
0.20
0.00
K = 2
RSA
Kenya
Ecuador
Chile
Australia
New Zealand
USA
Bhutan
Austria
Czech
Slovakia
Romania
Hungary
Poland
1.00
0.80
0.60
0.40
0.20
0.00
K = 5
(Optimal)
RSA
Kenya
Ecuador
Chile
Australia
New Zealand
USA
Bhutan
Austria
Czech
Slovakia
Romania
Hungary
Poland

Figure 4: Proportion of individuals within countries assigned to one of five clusters as identified by STRUCTURE analyses.

RSA Kenya

1
2
3
4
5

AFRICA

Chile Ecuador Australia New Zealand

SOUTH AMERICA
&
AUSTRALASIA

USA Bhutan

NORTH AMERICA
&
ASIA

Austria Czech Slovakia Romania Hungary Poland

EUROPE

Chapter 6

Needle blight caused by two species of Dothistroma in Hungary

ABSTRACT

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

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

Sampling

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

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

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

Isolations and DNA extractions

Diszel and Russia

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

Csabrendek

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

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

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

DNA analyses

Sequencing

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

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

Primer Doth_A

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

RFLP

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

Mating type

The DNB species-specific mating type primers (Groenewald *et al.* 2007) were utilised to confirm the identity of the DNB isolates as determined using the ITS-RFLP patterns. They were also used to determine the mating type of the isolates. The species-specific primers amplify regions of approximately 820 bp and 480 bp for MAT1 and MAT2 in *D. pini* and 823 bp and 480 bp for MAT1 and MAT2 in *D. septosporum*. PCR reactions and conditions were the same as those described by Groenewald *et al.* (2007).

RESULTS

Isolations and DNA analyses

Diszel and Russia

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

Csabrendek

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

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

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

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

Mating type

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

a

d

e

b

c

f

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

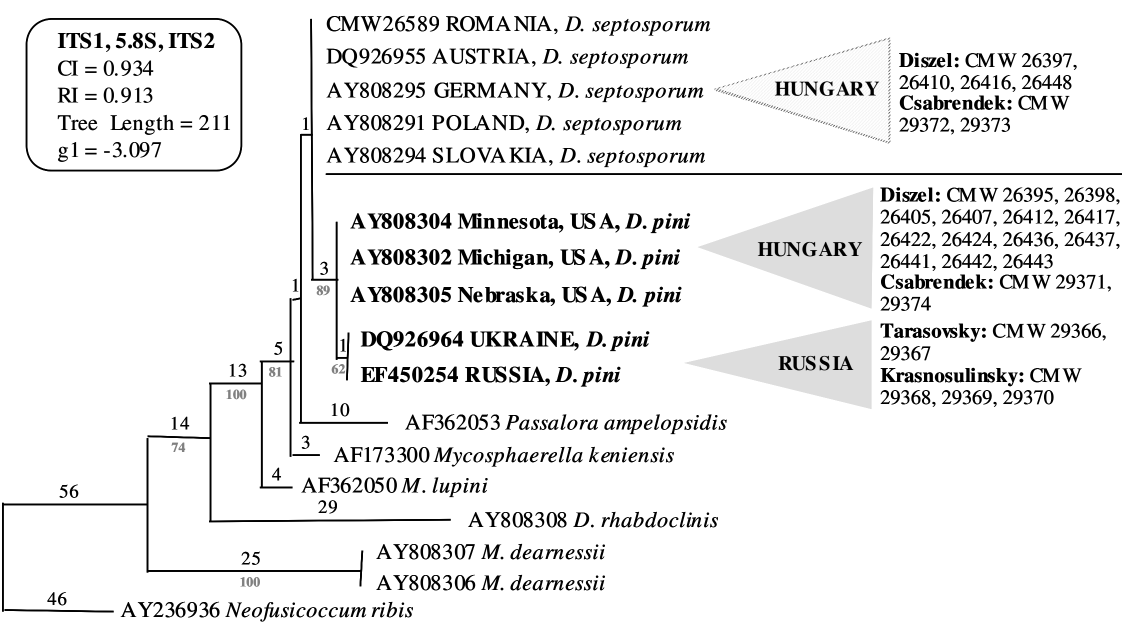
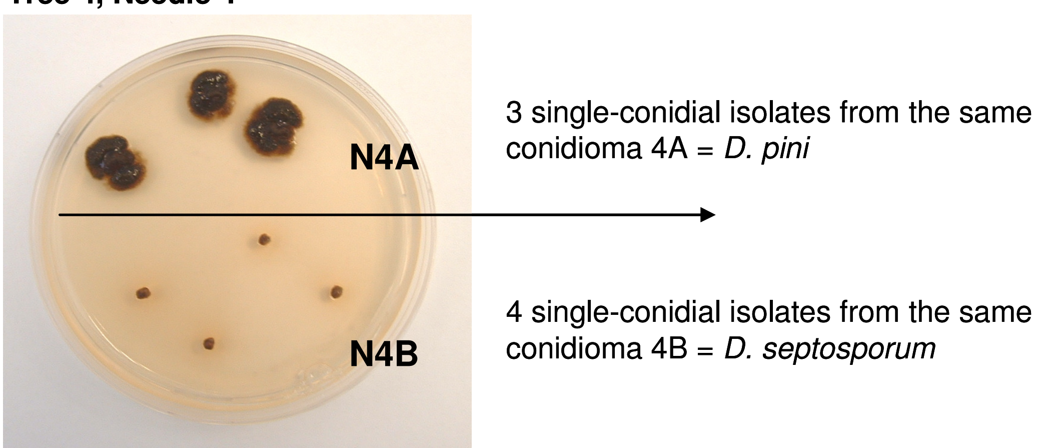
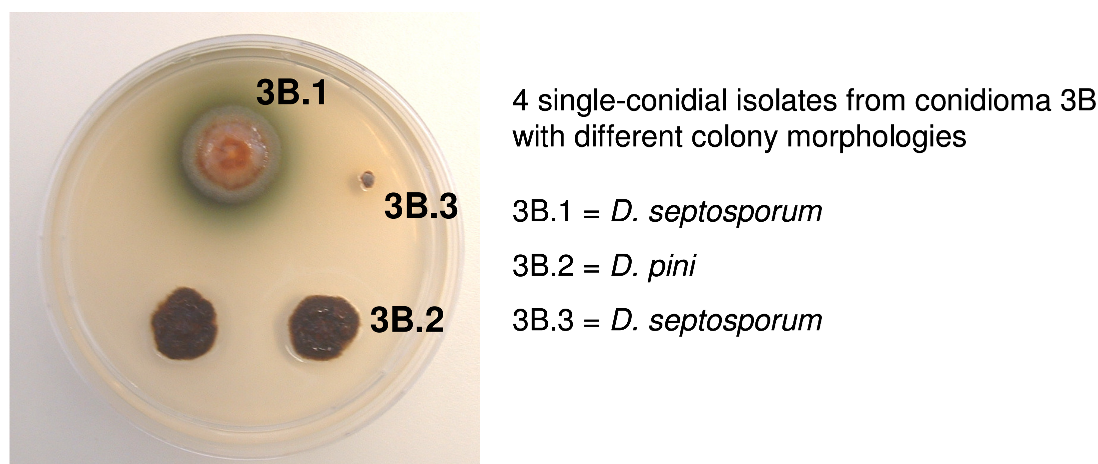

ITS1, 5.8S, ITS2
CI = 0.934
RI = 0.913
Tree Length = 211
g1 = -3.097
CMW26589 ROMANIA, D. septosporum
DQ926955 AUSTRIA, D. septosporum
AY808295 GERMANY, D. septosporum
AY808291 POLAND, D. septosporum
AY808294 SLOVAKIA, D. septosporum
HUNGARY
Diszel: CMW 26397, 26410, 26416, 26448
Csabrendek: CMW 29372,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

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

Tree 4, Needle 4



Tree 8, Needle 3



SUMMARY

Student: Irene Barnes

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

Supervisors: Professor Brenda Diana Wingfield
Professor Michael John Wingfield

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

Degree: Ph.D

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

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