Annual Technical Report

TITLE: Genetic variation in the foliar pathogen *Dothistroma septosporum* and relationship to toxin production

FSP PROJECT NUMBER: Y051203

ABSTRACT:

*Dothistroma septosporum* (= *Dothistroma pini*) is a foliar pathogen on many species of pine around the world. Recently there has been a large outbreak of *Dothistroma* in northwestern British Columbia that has been causing concern due to the extent of the outbreak as well as the severity of damage, which includes some stands with almost 100% mortality. Several factors may be contributing to the extent and severity of the outbreak. These include changing weather patterns, an increase in host availability, or more virulent strains of *Dothistroma* produced by sexual reproduction patterns of the fungus. The effects from this outbreak, and future outbreaks include mortality of pine in plantations, reforestation expenses, and reduced timber yields due to growth reduction from repeated years of defoliation.

In British Columbia, the sexual stage of the fungus is present, which may lead to an increase in genetic diversity. The levels of genetic diversity may correlate with increased virulence or dothistromin production by the fungus. As this toxin has been shown to have human mutagenic properties, it is important to know what risk it poses to forest workers as well as to our forests. Therefore, the objectives of this study are: 1) To explore the genetic variation of *Dothistroma septosporum* in northwestern British Columbia as it relates to location and environment; 2) To determine if the amount of dothistromin produced by strains of *D. septosporum* correlates to the genetic diversity present within the populations of fungi in northwestern British Columbia; and 3) To determine if the genetic variation and/or relatedness of *Dothistroma septosporum* is comparable or higher in the northwest portion of British Columbia in relation to other parts of the world.

Approximately 30 sites across 3-5 biogeoclimatic subzones (Interior Cedar Hemlock and Subboreal Spruce) will be sampled, eight trees per site and several branches per tree. Microsatellite markers and Amplified Fragment Length Polymorphisms (AFLP) will be used to compare genetic variation at different spatial scales, including some international samples. Dothistromin assessments on selected isolates will be carried out with collaborators from New Zealand.

INTRODUCTION:

*Dothistroma septosporum* (= *pini*) is a fungal pathogen belonging to the group ascomycota. It is an important pathogen on many species of pine worldwide. The fungus colonizes pine needles, and as a part of its life cycle produces dothistromin, a mycotoxin believed to be responsible for the disease, resulting in red banding on the needles, and eventually causing death of the needle and defoliation of the tree.
*Dothistroma septosporum* has been causing problems in exotic pine plantations in many countries of the Southern Hemisphere since the late 1950s (Gibson, 1972). Outbreaks of the disease followed the widespread planting of exotic *Pinus radiata* monocultures in large-scale forestry operations (Gibson, 1972). *Dothistroma septosporum* has been found in some countries of the Northern Hemisphere, but past outbreaks have not been as severe or caused as much concern as they have in the south.

*Dothistroma septospora* was identified on pine species in British Columbia in the mid 1960s (Funk & Parker, 1966, Parker & Collis, 1966). The fungus was found on three native pine species and on six exotic species (Parker and Collis, 1966). The teleomorph was identified on foliage from several British Columbia localities in 1964 and 1965 (Funk and Parker, 1966). Early studies describe the teleomorph and the life cycle in British Columbia (Funk and Parker, 1966). Further studies on the disease in British Columbia are limited, because it has not been a major cause for concern as outbreaks have been small, have gone unnoticed or have been kept under control by natural factors such as weather.

More recently there has been a large outbreak of *Dothistroma septosporum* in Northwest British Columbia that has been causing a lot of concern due to the extent of the outbreak as well as the severity, which includes stands with almost 100% mortality (Woods, 2003). Over 90% of lodgepole pine plantations have suffered some damage in the current outbreak. The damage ranges in these plantations from low levels of infection to nearly 100% mortality in some (Woods, 2003). In central and Northern British Columbia, lodgepole pine (*Pinus contorta*) is the major species of pine, and is economically important as it is the main species planted in reforestation and is one of the major species harvested. The financial impacts of this type of disease outbreak can be quite severe. In many cases, these severely infected stands have previously reached free to grow standards and responsibility of the land has been returned to the government. Now these plantations are becoming under stocked and must be reforested and managed (Woods, verbal communication).

Several factors may be contributing to the extent and severity of the outbreak. These factors include conducive weather patterns, - warm, moist summers and cool wet falls-, current forest practices leading to an over abundance of host, or a decrease in vigor of the host, and a more virulent strain of the pathogen itself (Woods, 2003).

The current disease outbreak in British Columbia is unique due to the severity and extent, as well as the host being a native *Pinus* species. These circumstances have not been previously encountered in northern countries. The favourable conditions for disease, as well as the existence of the teleomorph in British Columbia, may have led to a case where sexual reproduction has allowed for rapid evolution of the pathogen population. Rapid evolution may have given rise to a more virulent strain of the fungus itself. Therefore, the purpose of this study is to explore the population genetic structure of *Dothistroma septospora* populations in Northwest British Columbia, to relate population structure to the contributions of reproductive strategies and to current forest practices, and to relate population genetic structure to toxin producing abilities of the pathogen.
METHODS

Field Collections
Foliar samples from lodgepole pine will be collected from trees showing symptoms of infection by *Dothistroma septosporum*. Trees will be sampled across thirty sites with approximately 6 sites in each of 5 subzones (Figure 1). Site selection criteria include: spatial coverage of the subzones studied; moderate to high levels of disease; and accessibility by vehicle or limited helicopter use.

Eight trees will be sampled within each site. The closest tree to a location determined from randomly selected compass bearing and a randomly selected distance from 0 to 50 meters will be tree 1. The remaining 7 trees will be sampled systematically from that point, with 30m between trees (Figure 2). Three samples will be taken from each tree where possible. One within the lower branches, one mid tree, and one in the mid to upper foliage.

This sample design allows for analysis of genetic variation in the fungus at multiple spatial scales: tree; site; within subzones; and between subzones. In addition, samples will be solicited from several international laboratories, including the University of Pretoria in South Africa, and Massey University in Palmerston, North New Zealand.

Laboratory Procedures
Needles showing symptoms and fruiting bodies of *Dothistroma* needle blight will be surface sterilised and placed on water agar. After two to three days, a conidial mass from one fruiting body will be scooped up using a dissecting needle, and streak plated on a new dish of water agar. After the conidia start to germinate, an individual colony originating from one or a few conidia, will be selected and placed on Dothistroma media consisting of 5% (w/v) malt extract, 2.3% (w/v) nutrient agar (Bradshaw et. al., 2000). The single colony will be allowed to grow up to fill a petri plate.

For growth rate measurements, a 0.5cm diameter plug of mycelium will be removed from the original culture plates and placed onto new Petri plates containing Dothistroma media. From these plates, qualitative assessments will be made for toxin production, and measurements will be made periodically to obtain growth rates. DNA will be extracted using a phenol-chloroform method modified from that developed by Al-Samarrai and Schmid, (2000).

DNA will be amplified using a polymerase chain reaction following methods of Gangley and Bradshaw (2001). Microsatellite loci will be used to measure variation in genetic diversity. Some primers are already available for use with *Dothistroma septosporum*, and using microsatellites will enable us to better compare our results to those from other studies that have been done or are underway. Gangley and Bradshaw (2001) have developed primers for five loci. Two of the primers have two alleles, two primers with three alleles, and one primer with four alleles. Additional microsatellite primers are being developed in laboratories in South Africa and New Zealand and will be utilized if possible.

PCR products will be multiplexed and viewed on an automated DNA sequencer following protocols by Moon et al. (1999). A fluorescent phosphoramidite dye will be attached to the 5’ end of the microsatellite primers. These can be read at 750-770 nm, 685-706 nm, and 650-670 nm.
The PCR products will be mixed with fluorescently labelled size standards and a loading buffer. Samples will be electrophoresed through a polyacrylamide gel in a Bec CEQ 8000 DNA sequencer.

If there is little to no variation in the microsatellite loci amplified by these primers, then sequencing of the ITS region between the nuclear 18S and 5.8S rRNA genes will be used as an alternative method. Methods of Bradshaw et al. (2000) or Barnes et al. (2004) will be adapted and modified as necessary.

The chosen method will be coupled with amplified fragment length polymorphism (AFLP) using methods developed by Vos et al., 1995. Amplified fragment length polymorphism is a technique for generating DNA fingerprints using random fragments of genomic DNA. It couples restriction fragment length polymorphism with polymerase chain reaction to generate unique banding patterns for genetically distinct individuals. AFLP is a useful method that requires no prior sequence knowledge and the number of fragments can be manipulated by selection of specific primer sets. They have some advantage over other methods in their reliability due to stringent PCR conditions (Vos et. al., 1995).

DNA will be subjected to restriction reactions following protocols by Vos et al. (1995). DNA will be incubated with restriction enzymes, and after one-hour reagents, including adapters specific to the enzyme and a DNA ligase will be added to the reaction and incubation will continue. AFLP adapters consist of a core sequence and an enzyme specific sequence.

DNA will then be subjected to PCR with AFLP primers that correspond to the enzymes used in the restriction reaction. Primers for AFLP reactions consist of a core sequence, an enzyme specific sequence and a selective extension. PCR conditions will be optimised for each of the primers. If necessary, a preamplification step will precede using the same general conditions except primers will have a single selective nucleotide versus a longer selective extension. The resulting products would then be used as a template for a second amplification cycle. DNA fingerprints will then be visualised on a 5% polyacrylamide gel.

**Data analysis**

Data will be converted into a file with each individual as one case, and data will consist of zeros representing no band and ones representing a band present for each of the alleles detected. Gene frequencies and standard population genetic statistics will be calculated using POPGENE (Molecular Biotechnology Centre, University of Alberta, Canada). The proportion of polymorphic loci and average marker diversity will be calculated for each population. An analysis of molecular variance (AMOVA) will be performed and genetic diversity will be partitioned into within population and among population diversity. Significant differences will be assessed at \( \alpha = 0.05 \). Wright’s F-statistics will be calculated to look at population differentiation, specifically \( F_{st} \) which relates subpopulations to the total population. A neighbour-joining tree will be generated to analyse and visually represent population divergence for the *Dothistroma septospora* populations in Northwest BC.
Dothistromin extraction and quantification

For variation in toxin production, dothistromin will be extracted and quantified in New Zealand at Dr. Rosie Bradshaw’s lab. Methods for extraction will follow those in Bradshaw et al. (2000), and Jones et al. (1993). A correlation will be used to determine if a relationship exists between the amount of genetic diversity and amount of toxin produced by isolates.

RESULTS TO DATE

Foliage samples with symptoms of disease have been collected from 19 sites over four subzones (Fig. 1). Isolations have been attempted from all of these samples and successful isolations have been placed into pure culture on Petri dishes. Slant cultures have also been prepared for each isolate for longer term storage purposes. Colony morphology and growth rate observations have been completed or are ongoing for each isolate.

DNA extraction protocols have been developed using a sub-sample of isolates. We have been able to achieve adequate amounts of DNA using extraction procedures modified from Al-Samarrai and Schmid, (2000). DNA has been extracted from 20 isolates and the remaining extractions are ongoing.

Microsatellite primer sequences have been obtained from collaborators and are being tested with a sample of our current isolate collection. Figure 3 is an example of pcr products from one microsatellite primer.

DISCUSSION – not applicable

CONCLUSION AND MANAGEMENT IMPLICATIONS – not applicable

LITERATURE CITED


Figure 1. Approximate sample sites for *Dothistroma septosporum*, in the Interior Cedar Hemlock and Sub-boreal Spruce Biogeoclimatic Zones.
Figure 2. Sample tree grid layout. The tree closest to each point is the sample tree.

Figure 3. Example of the pcr products from a microsatellite primer.