Study Plan - Examination of genetic diversity of the fungal pathogen *Inonotus tomentosus* across forest stand types in the sub-boreal spruce zone of British Columbia

Research Summary

Inonotus tomentosus is a fungal pathogen of commercially valuable tree species in British Columbia and one of the most important biotic disturbance agents in sub-boreal and boreal forests in Canada. Although ubiquitous across North America, little is known about mode of infection and relative roles of basidiospores and root contact in disease spread. An investigation into the diversity of genotypes of *I. tomentosus* will help to determine whether infection is due to spread by root contact, by basidiospores, or a combination of the two and if forest management affects the mode of disease spread. This information is essential to properly manage the disease and to understand the potential impact on timber supply from second growth stands. The results from this project will provide forest managers with important information on disease spread from which effective management strategies can be developed. Using three different techniques that detect polymorphisms within the genome of this species, we will determine the level of genetic variation occurring in natural stands and plantations within BC and make comparisons between the two types of stands. An initial pilot study will be carried out to illustrate the efficacy of SSCP in assessing the genetic variation within populations of *I. tomentosus*.

Research Objectives

1. To carry out a pilot study in which a protocol for SSCP analysis, specific to *Inonotus tomentosus* is established.

2. (i) To examine the level of genetic variation of *Inonotus tomentosus* in natural old growth forests in BC.

   (ii) To examine clone size and distribution of *Inonotus tomentosus* within mortality centers of old growth forests in BC.

3. (i) To examine the level of genetic variation of *Inonotus tomentosus* in BC plantations.

   (ii) To examine clone size and distribution of *Inonotus tomentosus* within mortality centers of BC plantations.

4. To compare patterns in genetic variation and clone size of *I. tomentosus* between natural old growth stands and plantations in BC, and from these patterns make inferences concerning the mode of disease spread and the impacts, if any, of forest management on this pathogen.

Methods

We have chosen to use three methods to assess genetic diversity. Vegetative compatibility testing, random amplified polymorphic DNA (RAPD) analysis and PCR-SSCP (single strand conformation polymorphism) will be employed in this study. Vegetative compatibility (VC) is a self/nonself recognition system in fungi based on polymorphisms at one or potentially several different loci within the genome, revealing phenotypic variation between fungal isolates within a population (Milgroom and Cortesi 1999). VC analysis has been successfully applied in previous studies dealing with *I. tomentosus* to illustrate variation within populations and has been found to be a useful tool for indicating genetic variation (Lewis and Hansen 1991a).

RAPDs are widely used to illustrate intraspecfic variation in fungi. This technique utilizes multiple short unspecific primers that have many possible binding sites in the genome. The resulting amplification products are separated by gel electrophoresis and reveal banding patterns unique to each isolate. This technique has been used in numerous studies of fungal plant pathogens and has provided a suitable estimation of intraspecific genetic variation in fungal populations on a small scale (Hamelin *et al.* 2000, Hamelin 1998, Gogglioli 1998, Printzen *et al.* 1999).

SSCP is a highly effective method of genetic analysis that differentiates between closely related individuals based on sequence differences in the genetic code within the amplified regions (Orita *et al.* 1989). This has been proven to be a highly effective and reliable means of differentiation between fungal isolates, without the need for direct sequencing (Hegedus and Khatchatourians 1996, Kjoller and Rosendahl 2000). Double stranded DNA is heated in a formamide solution that denatures the DNA, producing single strands. The strands, in solution, are loaded into a non-denaturing low-temperature gel. When they are no longer in a denaturing environment, the strands will form hydrogen bonds within themselves, creating secondary structures unique to each particular base sequence. The strands will migrate through the gel and across a gradient at different rates due to these secondary structures. This technique has not been used widely with *I. tomentosus*, therefore it is necessary to develop and test a protocol to establish whether this method will be suitable for further analysis of this fungus.

The purpose of using these three methods is to verify, compare and correlate results obtained using each technique. VC testing examines variation at a number of loci within the genome that are related to somatic compatibility, SSCP will examine fine scale variation within specific targeted genes and RAPDs will provide an overall picture of variation throughout the entire genome. As such, this study will also provide a novel comparison of the power of resolution of each method for the purpose of examining fungal population genetics.

Pilot Study to Develop SSCP Protocol

1. Sample Collection

Inonotus tomentosus sporocarps were collected from two mixed spruce-pine stands located in the Prince George and Kispiox forest districts. Two pairs of sporocarps were chosen from each site. The pairs consist of two sporocarps growing within 5 m of each other, and each pair was approximately 100 m apart.

2. DNA extraction and amplification

Extraction of DNA from sporocarp tissue that has been stored at -20° C was performed using the methods adapted from Germain *et al.* (unpublished). Diatomaceous earth was used to grind approximately 10-20 mg of tissue in 1.5 ml microcentrifuge tubes. Samples were suspended in 100 ul of Quiagen extraction buffer (100 mM Tris HCL, 2 % CTAB buffer, 1.4 M NaCl, 1 % PEG 8000 and 20 mM EDTA) + .25% beta-mercapthenol, incubated at 65° C for an hour and vortexing every 15 minutes. To emulsify the mixture, phenol: chloroform: isoamyl alcohol (25:24:1) was added to each sample, followed by centrifugation at 1300 rpm for five minutes. The upper phase was transferred to a clean microcentrifuge tube and precipitated with ammonium acetate and cold isopropanol. DNA was washed with 70% ethanol and re-suspended in 20 ul of TE-8 buffer. Dilutions (1:10) were prepared for PCR.

DNA was amplified using a 25µl volume containing 10x PCR buffer, 50 µM $MgCl_2$; pH 8.3, 2 µM of each of dNTP's, 1unit each of 25uM primers (see table 1), 1 unit of Platinum Taq polymerase and 5µl of DNA template. DNA amplification was carried out on an MJ Research Programmable Thermocycler under the following conditions for the ITS primers: 35 cycles of 30 sec. at 92 °C, 30 sec. at 50 °C, 30s at 72 °C, preceded by a 3 minute denaturation step (94 °C) and completed with a final extension time of 5 minutes (72 °C), with a hot start. The presence of good PCR product was verified by running amplified samples through electrophoresis on a .7% agarose gel. For the mitochondrial and nuclear markers, primer conditions are as follows: 36 cycles of 30 sec. at 92° C, 45s. at (*see table 1.) °C, 1 minute at 72 °C, precluded by a 3 minute denaturation step (92 °C) and with a final extension time of 10 minutes (72 °C), with a hot start.

3. Primer Selection

A screen for SSCP markers was performed by H. Germain and M.J. Bergeron at the Laurentien Forestry center in Quebec with PCR products generated by a series of primers already available. The first set of PCR primers found suitable for this analysis was ML 5 (CTCGGCAAATTATCCTCATAAG) and ML 6 (CAGTAGAAGCTGCATAGGGTC) (White *et al.* 1990), coding for the large mitochondrial ribosomal sub-unit. The second set of primers was MS 1 (CAGCAGTCAAGAATATTAGTCAATG) and MS 2 (GCGGATTATCGAATTAAATAAC) (White *et al.* 1990), coding for the small mitochondrial ribosomal sub-unit.

Primers it-BT-15-f (GGAGCCAGCAGTACCGTG) and it-BT-490-rc (CGTGAAGTATGCGTTAGC) were designed by Hugo Germain from beta-tubulin sequences obtained from amplification and sequencing with universal primers BT-1a (TTCCCCCGTCTCCACTTCTTCATG) and BT-1b (GACGAGATCGTTCATGTTGAACTC) (Glass and Donaldson, 1995). From the sequences obtained with primers developed by Hugo Germain, a new set of primers, ittub164f (GTAGAAGAGCAGATGCAGAATGT) and ittub387rc (TGGGTGTACCAATGCAAGAA)were developed by Marie-Josee Bergeron, as well as primers for the gene coding for actin from the universal primer actin 2: it112.31act2501f (GTGAAATTGTGCGCGACATC) and it112.31act2700rc (AACACGCCGCAAGTCAAC).

High levels of variation were observed in both actin and beta tubulin markers when tested with isolates of *I. tomentosus*, although lower levels were expected, as both are highly conserved "house-keeping" genes. The beta-tubulin and actin markers each span an intron of about 100 bp, which may explain this observed variation. The mitochondrial markers illustrate less variation than the two nuclear markers, yet reveal sufficient variation to provide information regarding genetic patterns within the population. Mitochondrial genes are always found in the haploid state in fungi as mitochondria are assumed to be transmitted by only one parent. As a result of this characteristic, all individuals appear to be homozygous at all mitochondrial loci, complementing the information obtained from nuclear markers that detect individuals in the heterozygous state.

We will be using two ITS primers for identification of *Inonotus tomentosus* on root samples (ITS209f, ITS700rc). To ensure that the primers are specific to *Inonotus* we performed PCR using DNA from a variety of reference samples of basidiomycetes and ascomycetes to illustrate negative results with these species. Unexpected amplification of some reference samples did occur. This is being examined in more detail by researchers at the Laurentian Forestry Centre in Quebec. The ITS primers have been shown to be specific to *I. tomentosus* in relation to other closely related species and genera (*Inonotus leporinus, Inonotus cuticulatus* (Bull.:Fr.) P. Karst, *Inonotus radiatus* (Sowerby:Fries) Karsten, *Inonotus rheades* (Pers.) Bong & Singer, *Inonotus. glomeratus* (P.K.) Murr., and *Phellinus pini*). To avoid contamination from non target species in the genetic variation study we will culture isolates from root samples collected in the field and from these we will extract DNA and perform VC testing.

Primer Name	**Sequence (5'-3')**	**Length of amplified fragment (bp)**	**Annealing Temp**
ITS209F	GCTAAATCCACTCTTAACAC	450	50
ITS700rc	AGGAGCCGACCACAAAACAT		
IT 112.31ACT2501F	GTGAAATTGTGCGCGACATC	199	65
IT112.31ACT2700RC	AACACGCCGCAAGTCAAC		
ITTUB164f	GTAGAAGAGCAGATGCAGAATGT	223	58
ITTUB387rc	TGGGTGTACCAATGCAAGAA		
MS 1	CAGCAGTCAAGAATATTAGTCAATG	600	55
MS 2	GCTGATTATCGAATTAAATAA		
ML 5	CTCGGCAAATTATCCTCATAAG	800	55
ML 6	CAGTAGAAGCTGCATAGGGTC		

Table 1. Primers for SSCP analysis

5.*SSCP analysis*

Samples were prepared for loading by adding 2 ul of PCR product to 5ul of loading buffer containing 95% formamide, 0.05% xylene cyanol and 0.05% bromophenol blue. Samples were heated at 95% for three minutes, quenched on ice and loaded.

A 5% polyacrylamide gel was cast with 30% acrylamide solution (34:1 acrylamide:bisacrylamide) and electrophoresed in .5x Tris-Glycine buffer (Bio-Rad Laboratories, Mississauga, Ont.). Using an upright protein gel electrophoresis unit (16 x 16 x 0.75 cm) (Protean II xi. System, Bio-Rad Laboratories, Mississauga, Ont.), with a cooling system to maintain a constant temperature of 4° C. The gel was run under a variety of conditions for each marker to attain optimal conditions for each. All runs were duplicated to ensure reproducible results. SSCP were visualized using a silver stain kit (Bio-Rad Laboratories, Mississauga, Ont.) Digitized images of the gels were captured using a Chemi-Imager 5500 (Alpha-Innotech Corp.).

6.Results and Discussion

Appropriate conditions for SSCP that provide sufficient separation of bands has been attained for each marker (table 2). Variation was observed in nuclear markers, but not in mitochondrial markers, which was expected due the small sample size used for the pilot study. The clarity of the bands is not yet adequate, but this problem will be addressed by testing a higher-grade substrate (MDE gel, Amersham Biosciences Inc.), in place of our original polyacrylamide matrix, as it has been shown to have superior resolution and clarity when used for SSCP (Hamelin 2002 pers. comm.).

Previous research illustrating the effectiveness and the broad applications of this technique lead us to anticipate that PCR-SSCP analysis would be an effective method of identifying differentiation between isolates of *Inonotus tomentosus* (Spinardi *et al.* 1991, Hegedus *et al.* 1996, Michaud *et al.* 1992). In light of the results that we have obtained through this pilot study we believe that it is appropriate technique for the purpose of examining genetic variation within populations of *I. tomentosus* in old growth forest stands and plantations in British Columbia.

Marker	**Voltage**	**Temp.**	**Time**	**Gel Conc.**
SSUmtDNA	150V	4º	14h	5%
LSUmtDNA	150V	4º	16h	5%
Actin	100V	4º	5h	6.5%
Beta-tubulin	100V	4º	5h	6.5%

Table 2. SSCP conditions

Genetic Variation Study

1. Study Sites

1a. Old Growth Stands

Three old growth stands[1] in the sub boreal spruce (SBS) zone of central BC have been selected for our study. Two sites are located in forest stands approximately 80 km northeast of Prince George off of the Beaver-Bowron Forest Service Road (UTM coordinates: 544815). The third old growth site is located off highway no. 16 bridge over Bowron River, 5.8 km west of Purden Lake Resort (UTM Coordinates: 665725)

"Walk through" surveys were conducted in each stand to identify and locate an area with several putative disease centers. Stand composition is mainly spruce or mixed spruce and sub-alpine fir. Twenty to thirty diseased trees from each stand will be sampled. All trees within the diseased area will be described and stem mapped. All trees over 10 cm in diameter will be referenced on the map by distance and bearing. Dead and symptomatic trees will be noted on the map and described based on indicators such as crown health, foliage color and reduction in terminal growth.

1b. Plantations

The oldest plantations in the Prince George Forest District were located using records from the Ministry of Forests. All three sites are located within forest stands off Highway No. 16, 13.7 km west of Purden Lake Resort, 8.6 km east of Wansa Creek, 7.9 KM West of Bowron River Bridge and 0.4 km east of Small Creek (UTM coordinates: 603728). "Walk-throughs" were done to locate and identify infected areas in these old plantations. These stands are composed mainly of spruce with multiple disease centers and were planted in 1969-1970. Twenty to thirty trees will be sampled using the same method as in old growth sites.

2. Sample Collection, Isolation and Culturing

One cross section of root will be cut from each diseased tree using a Pulaski. Samples will be brought back to the university to be stored at 4º C. Using a surface sterilized hatchet, each root will be partially split open and pulled apart to reveal the infected core of the root. The root will be taken to a laminar flow hood from which three small chips of wood from the infected area will be cut out and transferred to 3% malt extract agar plates using a sterilized scalpel and forceps. Cultures will be incubated at room temperature and checked every two to three days for contamination, and replated as necessary. To positively identify *Inonotus tomentosus* in culture, mycelia from cultures

[1] For this study, "Old growth" stands are seral, between the ages of 80-120 years old. OG1 is in the true old growth phase, while stands OG2 and OG3 are in the understory reinitiation phase, as defined by Oliver and Larson (1996).

will be examined for the presence of chlamydospore-like structures[2]. This will be done by preparing a squash mount for each isolate and observing the mycelia using a compound microscope at 400 x magnification.

3. DNA extraction and PCR amplification

Isolates of *I. tomentosus* will be grown in culture for the purposes of obtaining pure samples for DNA extraction and VC testing. When pure cultures have been established and DNA has been extracted from every isolate, root samples will be discarded. *I. tomentosus* will be isolated from infected root tissue samples and will be cultured on 3% MEA. DNA extraction and amplification for all samples will be carried out using the methods described in section 4.1.2 and 4.1.3. Concentration of DNA will be quantified by comparing band intensity on an agarose gel of DNA extracts to that of a DNA marker with a known concentration.

4. Vegetative Compatibility analysis

Sub samples from each culture obtained from infected roots will be plated and paired in all combinations. Small (8 mm) plugs will be placed 1 cm apart with hyphae down. The plates will be incubated at room temperature and examined at 4, 6 and 8 weeks for development of a reaction line and changes in morphology. Lines will be rated as 0 (no reaction), .5 (no line, no intermingling of hyphae – uncertain reaction), 2 (light/raised line – not compatible), and 3 (definite raised/dark line – not compatible). Images of pairing in culture will be captured using a scanner, and cultures will be diagrammed and described throughout the incubation process.

5.RAPD Analysis

Two kits consisting of 20 RAPD primers each (Quiagen - Operon Technologies) will be tested to obtain 5-10 primers that produce repeatable banding patterns for 10 isolates. DNA amplification will be performed using reagents and protocols recommended with the Operon Kit. We will adjust the recommended protocol, if necessary, to suit our equipment and conditions. RAPD markers will be selected only if they are repeatable distinct bands, and will be scored as separate putative loci. Bands will be scored as present (1) or absent (0) in a data matrix of samples x markers.

6. SSCP analysis

SSCP protocols established during the pilot study, described in section 4.1.4, will be applied.

[2] Chlamydospore-like structures are small globose hyphal swellings unique to I. tomentosus, distinguishing it from other fungi that have similar morphology in culture (Nobles, 1948)

7. Data Analysis

Reactions of the pairings done for VC testing will be scored in tabular format for each site. The number of vegetatively compatible groups within each site will be calculated. This data will be compared within and between stand types to obtain an average number of genetically distinct individuals based on compatibility at VC loci.

For SSCP, variation between genotypes will be assessed visually using standards created from samples with known conformation. Samples that show similar banding patterns across gels will be run together to confirm differences or similarities. Bands will be scored as 1 (present) or 0 (absent) in a data matrix (isolate x allele). RAPD data will be scored in the same manner for all clear repeatable polymorphic bands. Following the method used by Hamelin (1995), in which populations were examined using RAPD markers only, all loci will be scored as separate putative loci, with a dominant allele (a band present), and a null allele (band not present). The data from both sets of analysis will be imported into Arlequin 2.000 software for population genetics analysis (Excoffier 2000). Analysis of molecular variance (AMOVA) will be used to separate the total variation into hierarchical categories of variation within stands, variation between stands of same type and variation between the two different stand types. This partitioning of the data set reflects the nested design of our experiment.

Stem mapping data will be plotted using ArcView. To this data, data layers for tree description will be added, as well as layers for genotyping data derived from each of the three types of analysis (VC, RAPD, SSCP). Clone size will be calculated by measuring the area encompassed by each genetically distinct individual (the area covered by the associated infected tree(s) to half way between an infected tree and the closest healthy tree), for all infected trees in the plot. Clone size will be calculated separately for each method of analysis for each site.

8. Anticipated Timeline from March 2003

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March 2003

- Continue DNA extractions
- Continue to monitor VC reactions
- Initiate SSCP and RAPD analyses

May 2003

- Continue all laboratory analyses above
- Field sample remaining site (plantation)
- Fill sample other sites where necessary as determined by VC, RAPD and SSCP analyses
- Process new field samples: isolation, slant culture, DNA extraction, VC pairings

September 2003

- Complete laboratory analyses (SSCP, RAPD, VC)
- Merge spatial data and lab results using GIS format

October 2003

- Report and thesis preparation
- Extension publications and presentations
- Web site update

January 2004

9. Literature Cited

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