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Genetic diversity and colonization patterns of *Onnia tomentosa* and *Phellinus tremulae* (Hymenochaetaceae, Aphyllophorales) in the boreal forest near Thunder Bay, northwestern Ontario

by

Zachary R.W. Hoegy

A Graduate Thesis Submitted in

Partial Fulfillment of the Requirements for the

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ABSTRACT

Hoegy, Z.R.W. 2016. Genetic diversity and colonization patterns of *Onnia tomentosa* and *Phellinus tremulae* (Hymenochaetaceae, Aphyllophorales) in the boreal forest near Thunder Bay, northwestern Ontario. 90 Pp. + x Pp.

Keywords: genets, heart rot, root rot, somatic incompatibility, spatial mapping, white rot

Forest health is impacted greatly by fungi, particularly those that cause disease in living trees. By examining genetic diversity within populations of pathogenic fungi and their patterns of colonization it is possible to gain a greater understanding of host-pathogen interactions. Two common pathogens in the boreal forest are *Onnia tomentosa*, causal agent of a root-rot disease in spruce known as stand opening disease, and *Phellinus tremulae*, causal agent of white heart rot in stems of trembling aspen. Both fungi are members of the Hymenochaetaceae in the Basidiomycota.

Two black spruce (*Picea mariana*) plantations located north of Nipigon were examined for *Onnia tomentosa*. Spatial coordinates of 124 basidiomata were taken, and the basidiomata collected from plots that had received different commercial thinning treatments. Using extracted DNA from each of the basidiomata, it was possible to measure genetic diversity and consequently genet size. One hundred and sixteen genetically distinct individuals were found suggesting that the majority of the basidiomata represented unique genets. The distribution pattern was mapped. Stand thinning appears to negatively impact colonization of spruce by *O. tomentosa* compared with that observed in unthinned control stands.

In an ancillary study, a stand of trembling aspen (*Populus tremuloides*) located at Silver Mountain (74 km SW of Thunder Bay) was examined for *Phellinus tremulae*. Four infected trees were harvested and each stem cut into 50 cm sections with the top 5 cm from each section removed as a cookie. From each cookie, isolations of *P. tremulae* were made onto agar media and somatic compatibility techniques were utilized to determine size and distribution of genets in each tree. Two trees contained two genets of *P. tremulae*, one tree contained a single genet, while the remaining tree failed to yield any isolations of *P. tremulae* at all.

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GENERAL INTRODUCTION

The boreal forest stretches across North America, Europe, and Asia south of the arctic tundra, and is dominated primarily by conifers. This forest provides benefits in the form of air and water filtration, carbon sequestration, oxygen production, and habitat for various plant and animal species. The boreal forest also provides economic opportunity for many communities.

Although many factors affect forest health, including fire, weather, and insect pests, it is the role of fungi that has the greatest impact. Forest health is positively affected by those fungi that form a symbiotic association with tree roots known as mycorrhizae. This association benefits trees by increasing the efficiency of nutrient and water uptake, provides protection of roots from soil-borne root pathogens, and provides protection from elevated levels of heavy metals and other toxins in the soil. Other fungi are important in decomposing wood and woody debris and aid in the creation of the organic layer in forest soils. However, some fungi are serious pathogens which can detrimentally affect individual trees, or entire forest stands and as a result, can have a negative ecological effect on the ecosystem as well as a negative economic effect on the forestry sector.

Two common and widespread disease-causing fungi found in the boreal forest are *Onnia tomentosa* (Fr.) P. Karsten and *Phellinus tremulae* (Bond.) Bond. & Borisov. Both species belong to a group of bracket fungi placed by mycologists in the Family Hymenochaetaceae within the Division Basidiomycota. *Onnia tomentosa* causes a white pocket rot in the roots and butt of living conifers, particularly spruce (*Picea* spp.), eventually leading to blow down of the infected trees. *Phellinus tremulae* causes a white pocket rot in the heartwood of living poplars (*Populus spp.*), particularly trembling aspen (*Populus tremuloides* Michx.).

The objectives of this study was to map the basidiomata of *Onnia tomentosa* found in two black spruce plantations and determine genetic diversity and subsequently genet size through extraction and analysis of DNA from the fruiting bodies. The result would determine if the genets were small, indicating recent colonization of the spruce stand, or were large thus suggesting the genets were present for many years. Comparisons between different thinning treatments and unthinned controls would also help towards the creation of a management strategy. As an ancillary study, four trees of trembling aspen infected with *Phellinus tremulae* were cut, and isolations of the fungus made onto agar media. Genetic diversity was determined through the pairing of isolations utilizing the somatic incompatibility technique. This would determine whether each tree was colonized by a single individual of *P. tremulae* or several.

This thesis is divided into three chapters: 1) a literature review of existing research related to the biology and pathology of *Onnia tomentosa*; 2) an examination of genetic diversity and colonization patterns of *Onnia tomentosa* in plantations of black spruce; 3) an examination of genetic diversity of *Phellinus tremulae* in stems of four individual trembling aspen.

CHAPTER 1:

BIOLOGY AND PATHOLOGY OF *ONNIA TOMENTOSA* –

A LITERATURE REVIEW

The Boreal Forest

In the northern hemisphere, the boreal zone covers 552 million hectares, of which 307 million hectares is forested (Natural Resources Canada 2016a). In North America, the boreal zone stretches from the western shores of Alaska to the east coast of Newfoundland and Labrador, a distance of over 10,000 kilometres. The boreal zone covers approximately 53% of Canada, and represents 28% of the world's total boreal zone (Naturals Resources Canada 2015; Natural Resources Canada 2016a). It provides habitat for many organisms such as mammals, birds, insects, fungi, and other microorganisms and provides space for important lakes and wetlands. The region is home to several coniferous species of trees such as black spruce (*Picea mariana* (Mill.) Britton, Sterns & Poggenburg), white spruce (*P. glauca* (Monench) Voss), jack pine (*Pinus banksiana* Lamb.), balsam fir (*Abies balsamea* (L.) Mill), tamarack (*Larix laricina* (Du Roi) K. Koch), and eastern white cedar (*Thuja occidentalis* L.) (Archibold 1995). It is a valuable resource for the forest industry; in 2013, 148 million cubic metres of industrial roundwood was harvested in Canada. This wood was mainly used for lumber production but also for plywood, veneer, and oriented strand board, as well as pulp and paper products (Natural Resources Canada 2016c). That 148 million cubic metres represents 0.3% of Canada's total standing wood, 47 billion cubic metres (Natural Resources Canada 2016c). Canadian forest exports contribute 17.1 billion dollars in net trade, and approximately 47% of total exports are made up from three key products; northern bleached softwood kraft pulp (NBSK), newsprint (Canada is the largest producer worldwide with having 12% of the world's total), and softwood lumber (accounts for 20% of the value of Canada's forest exports) (Natural Resources Canada 2016c).

The boreal forest covers 74% of Ontario's land mass and is 65.2% productive. In 2013, 115,358 hectares were harvested in Ontario and in 2014, trade exports resulted in over 3 billion dollars (Natural Resources Canada 2016c). The harvesting of timber is done sustainably within Canada and if the timber is cut on public land (which is about 94% of Canada's forests) the trees must either be replanted or allowed to grow back naturally so that deforestation does not occur (Natural Resources Canada 2016b).

The boreal forest is a disturbance driven system that is greatly affected by wildfire, insects, and disease. Between 2012–2014, 200,378 hectares of forested area were burned in Ontario from a total of 2404 fires (Ontario 2015; Natural Resources Canada 2016c). Fire disturbance is a natural occurrence in the forest, and is required for nutrient cycling, influencing habitat for various species, as well as other ecological processes (McCullough *et al.* 1998). For renewed life to occur, plants such as jack pine (*Pinus banksiana)* have adapted to fire occurrence, and require the heat to open their cones and allow the seeds to disperse. The effects of a large, stand-replacing fire is comparable to clearcutting in that, tree species adapted to the occurrence of fire will have an advantage once the over story is opened up to allow light through the canopy. Fire not only affects the trees which it uses for fuel (McCullough *et al*. 1998) but can also affect insects either by directly killing them or altering variables such as over or understory vegetation, soil composition (i.e. sand, silt, clay), tree density, or other aspects (Lyon *et al.* 1978; Martin and Mitchell 1980). However, fire can also predispose surviving trees to attacks by bark beetles or wood-borers (McCullough *et al.*1998) depending on the severity of the fire.

Beyond fire, insects are another major disturbance vector in the boreal forest. Epidemic insect populations can represent a threat to the sustainability of the forest ecosystem. Insect damage can cause large disturbances within the boreal forest due to severe defoliation and tree

wounding that can lead to tree death. Across Canada between 2012 and 2013, 839,848 hectares were either defoliated by insects or contained beetle-killed trees (Natural Resources Canada 2016c). An estimated 416,503 hectares of trees in Ontario were killed by major insects in 2013 (National Forestry Database 2016). Insects not only create volume loss due to their feeding and the mechanical destruction of the tree, but also act as vectors for fungi by providing openings in the tree for the spores of pathogenic fungi to enter through.

Disease pathogens are useful for effective control of plant species (Dinoor and Eshed 1984) especially at the regeneration layer (Grubb 1977). Disease pathogens, however, are normally viewed negatively with regards to economically important hosts. For example, white pine blister rust (*Cronartium ribicola* J.C. Fisch.) has negatively influenced the distribution of eastern and western white pine (*Pinus strobus* L. and *Pinus monticola* Douglas ex. D. Don, respectively) (Manion 1991). Beech bark disease is the result of an interaction between a scale insect (*Cryptococcus fagisuga* Lindinger) and the fungus *Nectria coccinea* Desm., which has led to significant impacts on populations of the American beech (*Fagus grandifolia* Ehrh.). This disease causes mortality in clumps due to the limited dispersal abilities of the causal agent (Houston *et al.* 1979). Fungal pathogens can also have such a devastating impact on their target host, that it can result in functional extinction. For example, chestnut blight is caused by *Cryphonectria parasitica* (Murrill) Barr. The American chestnut (*Castanea dentata* (Marsh.) Borkh.) was the dominant tree species in the Appalachian mountain range until the mid-1900s when the blight was introduced and eliminated it from the forest canopy (Keever 1953; Mackey and Sivec 1973; McCormick and Platt 1980), resulting in various other tree species replacing the chestnut's dominant/codominant role (Stephenson 1986).

Impact of Root-rotting Fungi

Disease causing fungi enter through wounds on a tree and are able to begin the breakdown of the tree for their own growth and eventual reproduction. Root rot alone represents more than 74% of the annual economic loss in black spruce (Basham 1994). Over 90% of all merchantable trees that were sampled in Ontario showed evidence of root decay (Whitney 1988). Wood decay is an important nutrient cycling process where nutrients are returned to the soil, and become available to be used by other organisms. Fungi are essential for the task, but not all fungi are saprophytes (feeding on dead organic matter). Some fungi attack living trees to obtain essential nutrients. Specifically, root rot diseases impact the structural integrity, mortality, and overall health of trees (Cruickshank *et al*. 1997; Garbelotto *et al*. 1999; Dettman and van der Kamp 2001; Heinzelamm *et al.* 2012; Travadon *et al*. 2012). Root rotting pathogens play an integral role in forest dynamics by killing individual trees, which allows nutrients that are locked in the host tree to be released by the forest fungal community.

The interaction of these key disturbance mechanisms (fire, insects, and root rotting pathogens) all play a role on one another. When a disturbance caused by one of these occurs, it opens up opportunities for the other two in stand dynamics and regeneration. For example, if a tree is structurally weakened by root rot, the infected tree collapses and can damage other trees as it falls. If the stand density is high this can lead to more infection points for pathogenic spores (Whitney 1976, 1988, 2000). Eventually this leads to dried, dead wood on the forest floor (weather permitting) - a perfect fuel source for fire (McCullough *et al.*1998). A fire can either be a stand replacing fire that allows for faster growing pioneer tree species to regenerate or it can weaken non-killed trees, predisposing them to attacks by insects, continuing the cycle.

There are three major pathogens of root rot in North American forests (Whitney 1985). The first, *Armillaria mellea* (Vahl: Fr.) Kummer, is the most often identified species. However, it has been discovered that several closely related species of *Armillaria* (known as biological species) can cause *Armillaria* root rot in stands of both coniferous and deciduous trees and are thus referred to collectively as the *Armillaria mellea* complex (Williams *et al.* 1986; Dumas 1988). The spread of the majority of *Armillaria* species is mainly through vegetative mycelial growth in the form of rhizomorphs along the root systems of susceptible hosts (Kile *et al.* 1991). The pathogen then grows radially outwards creating distinct patches of dead and decaying hosts known as infection centres and the size of these centres can range from a single tree to tens of hectares (Kile *et al*. 1991, 1986; Korhonen 1978; Rishbeth 1978, 1991; Ullrich and Anderson 1978; Smith *et al*. 1992, 1994; Worrall 1994; Rizzo *et al*. 1995; Legrand *et al.* 1996). *Armillaria*'s infection strategy is based on clonal vegetative spread rather than dissemination through basidiospores (which is considered a rare event) (Redfern and Filip 1991; Taylor *et al.* 1999).

The second major root rot pathogen is *Heterobasdion annosum* (Fr.) Bref. (formerly *Fomes annosus* (Fr.) Karst.) which is the causal agent of annosum root rot and is most prevalent on pine species (Barnard 1979; Swedjemark and Stenlid 1993) throughout the temperate regions of the world (Hodges 1969). A species complex consists of three intersterility groups (S, F, and P) which are separated by their host affinity. The three main phylogenetic clades are European isolates (F group), Eurasian isolates (S group), and North American isolates (S group) (Johannesson and Stenlid 2003). Symptoms of *H. annosum* are thin, discoloured crowns, windthrown trees, and discolouration, staining, or decay of tree roots. Advanced decay is noted by white pocket rot sometimes with attendant black spots or flecks (Barnard 1979).

Basidiospores germinate on the surface of cut stumps of susceptible species and proceed to colonize the stump (Hodges 1969), entering into the root system where it can enter the healthy root systems of adjacent trees (Barnard 1979; Whitney 1988). Infection can be prevented by denying access to the host through chemical treatment of the stumps (Hodges 1969), through the use of biological control agents applied directly to the stump (Whipps and Lumsden 1989) or by limiting root contacts between susceptible species by mixing host trees with non-host trees (Hodges 1969; Menges and Loucks 1984). Both *Armillaria* and *Heterbasidion annosum* can cause extensive damage to forest stands through root degradation thus increasing susceptibility to fire, wind, insects and other pathogens.

Onnia tomentosa

The third major root rot pathogen in North America is *Onnia tomentosa* (Fr.) P. Karsten (formerly known as *Inonotus tomentosus* (Fr.) Teng, or as *Polyporus tomentosus* Fries) (Whitney 1962, 1980, 1993, 1995; Bernier and Lewis 1999; Germain *et al*. 2002, 2009). It is a basidiomycetous fungus that forms stalked fruiting bodies on the ground above infected roots of its host, or can form bracket shaped fruiting bodies on the butt of the tree (Whitney 1962, 2000; Germain *et al.* 2009) (Fig. 1.1). Above ground fruiting is not apparent until many years after infection. So, accurate disease recognition involves labour intensive root sampling to find the characteristic white pocket rot (Lewis 1997).

Figure 1.1 Basidioma of *Onnia tomentosa* in a plantation of black spruce.

The taxonomic history of *Onnia tomentosa* is both varied and confusing because the first description (Fries 1821) was based on the nature of the tissue context and the pore layer depth which is highly variable (Haddow 1941). The distinction between *O. tomentosa* and the similar *Onnia circinata* (Fr.) P. Karst. (formerly *Inonotus circinatus* (Fr.) Teng) is based on macro-morphological features of the basidioma such as central or lateral stipe, colour of the pileus, tube layer thickness, setae (quantity, presence, and whether or not they are hooked or straight), basidia, and host preference (Gilbertson and Ryvarden 1986). However, these features are so similar when comparing the two species against each other that it can easily attribute to misdiagnoses of occurrence and/or pathogenicity.

Although *Onnia tomentosa* can be found on several conifer species (Farr *et al.* 1989), it is most damaging on spruce and pine species (Whitney 1977; Germain *et al*. 2009). It can be found

in Europe (Breitenbach and Kranzlin 1986), Asia (Teng 1996), as well as North America where it occurs throughout the commercial range of native spruce in the boreal forest east of the Rocky Mountains in Canada (Whitney 1977, 1988; Gilbertson and Ryvarden 1986). As well, it is considered an important disease-causing agent on inland spruce in the British Columbia interior (Hunt and Peet 1997; Whitney 2000). *Onnia tomentosa* is also found at higher elevations in southern North America (Gilbertson and Ryvarden 1986; Farr *et al*. 1989).

Pathology

Onnia tomentosa causes a characteristic white pocket rot in infected roots. In the United States, the disease is known as tomentosus root rot, but in Canada it is commonly known as stand opening disease (Whitney 1962, 2000). *Onnia tomentosa* is able to affect both the root and the butt of a host tree, weakening it's structural integrity, and thereby predisposing it to other environmental factors. This results in the eventual decline of the infected tree. Hosts of *O. tomentosa* suffer ecological and economic losses because of reduced growth and the devaluation of the log due to butt cull, windthrow damage, and tree mortality (Whitney 1977, 1995; Lewis 1997).

Lewis *et al*. (1992) showed that intrabark mycelium of *O. tomentosa* only directly penetrates roots that are less than 4 centimetres in diameter while xylem infection occurs through the feeder roots or small junctions. The disease will then spread and decay the small living roots and then progress to the bark. However, when roots are greater than 5 centimetres in diameter, the fungus is unable to readily grow in the bark which causes the hyphae from *O. tomentosa* to advance through the heartwood until the tree dies, at which point it will then begin decaying the stained wood (Lewis *et al*. 1992).

Onnia tomentosa commonly infects older, more mature trees (around the age of 50) and has been known to infect trees as young as 19 years (Whitney 1977). Once roots are infected, the fungus can extend upwards into the butt of the tree. The heartwood is the primary area being attacked (Whitney 1977, 1988). The time it takes for the rot to extend radially from the heartwood into the sapwood is relatively long compared to how rapidly it moves up the heartwood longitudinally (Whitney 1977; Hunt and Peet 1997). Due to the extensive rot through the heartwood, the central column of stain and decay is able to extend upwards from ninety to one hundred and fifty centimeters from the base of the tree (Whitney 1962, 1977). The actual time it takes to go from initial infection to tree death and eventual spread to another tree is dependent on tree health at time of infection, as well as other factors that may significantly weaken the trees health during the infection process by *O. tomentosa*.

Onnia tomentosa is capable of persisting for decades (up to 30 years) in the remains of spruce and pine trees (Whitney 1962, 1977; Lewis and Hansen 1991a; Bernier and Lewis 1999). Lewis and Hansen (1991a) postulated that viability and therefore, infectivity could extend beyond the 30 year period. When stumps that were infected with the fungus were examined, it was noted that the fungus moved to a position within the stump ready to infect another host as soon as the tree roots made contact (Lewis and Hansen 1991a).

Spread within host

As *O. tomentosa* spreads throughout the trunk of the tree, its hyphae also radiate outwards from the point of infection through the roots. This infection of the roots will hinder the tree's growth because even if only one root is infected with tomentosus root disease, it can reduce the radial growth of the host (Lewis 1994; Hunt and Peet 1997). The estimated spread rate of *O. tomentosa* was calculated by Hunt and Peet (1997) to vary between 12 cm year^{-1} to 25

cm year^{-1}. This rate of spread is relatively slower compared to other important root rotting fungi such as *Armillaria ostoyae* (Romagn.) in Douglas-fir (van der Kamp 1993), *Phellinus weirii* (Murrill) Gilb. on Douglas-fir (Bloomberg 1988; Nelson and Hartman 1975), and *Heterobasidion annosum* (Fr.) Bref. in northeastern California (Slaughter and Parmeter 1995) with mean spread rates of 22 cm year^{-1}, 25-35 cm year^{-1}, and 22-39 cm year^{-1} respectively. Hunt and Peet (1997) also noted that once *O. tomentosa* becomes established, growth rate increases to a rate that is more than double the rate measured by Whitney (1962) of 12.4 cm year^{-1}. The rate at which *O. tomentosa* spreads is affected by the weather, temperature, and age of establishment. In areas where temperatures are cooler, it results in a negative impact on the growth of *O. tomentosa* (Hunt and Peet 1997).

Dissemination and establishment of new infection points

Onnia tomentosa has two modes of dissemination throughout the boreal forest. The first is by spore inoculation, similar to other fungi in the Basidiomycota. *Onnia tomentosa* releases basidiospores from pores on the underside of the basidiomata and these are carried by the wind where they land on susceptible hosts (Whitney 1962; Germain *et al*. 2009). The basidiospores can travel up to several kilometers (Pedgley 1986; Lewis and Hansen 1991a; Germain *et al*. 2009). Once released from the fruiting body, the basidiospores can then enter the tree through wounds caused by insects, wind, etc., before germinating. Germinating spores of *Onnia tomentosa* produce hyphae which commonly enter wounds in roots 1 cm or less in diameter (Whitney 1962), although it is inconclusive as to which specific wounds the hyphae from germinating spores usually enter. Whitney (1961, 1962) ruled out weevil (*Hylobius* sp.) feeding sites as infection sites, but did note that the wounding caused by the weevil predisposes the roots to infection, allowing *O. tomentosa* to infect the susceptible host.

The second mode of dissemination is through root contact. This is where a root from an infected tree comes into contact with a root from an uninfected tree of the same species and the fungus spreads via vegetative growth from tree to tree (Whitney 1988; Myren and Patton 1970; Lewis *et al*. 1992). The mycelium from the first host will spread into the second host where it will begin the process of decay (Whitney 1988; Lewis and Hansen 1991a). This second mode of dissemination is the major cause of *O. tomentosa*'s spread throughout the forest (Whitney 1962, 1980, 1988; Lewis and Hansen 1991a).

Formation of basidiomata

After infection by the fungus has occurred and weather conditions are optimal (moist weather in August and September, within stand openings), basidiomata (fruiting bodies) will be produced (Whitney 1962). As the disease progresses into later stages within the host, more basidiomata will occur on infected areas. Observations by Whitney (1962, 1977, 1995) have shown the fruiting body will appear with either a pileus and stalk originating from the infected roots or as a bracket from the infected areas of the trunk (Whitney 1962, 2000). However, host trees can be infected with *O. tomentosa* for several years before exhibiting above ground symptoms (Myren and Patton 1970; Lewis 1997). Not only are optimal weather conditions required for the forming of the fruiting bodies but soil and site conditions also play an integral role in the spread of *O. tomentosa*.

Influence of soil characteristics

Previous studies and observations have indicated that a relationship between ecosystem characteristics and the presence of *O. tomentosa* may exist (Hobbs and Partridge 1979; Merler 1984; Oulette *et al*. 1971; van Groenewoud and Whitney 1969; Bernier and Lewis 1999). Studies

conducted by Whitney (1976, 1980) and Bernier and Lewis (1999) suggested factors that may affect root rot include moisture, pH, nutrients in the soil, slope gradient, aspect, slope position, soil texture, effective rooting depth, depth to seepage water, humus depth and form, and predominant tree species. Observations by Bernier and Lewis (1999) showed the largest quantity of *O. tomentosa* was present in their driest moisture regime, however, if this regime was compared, on an absolute scale to the rest of the sub-boreal spruce zones, it would be considered moist relative to the other sites. This result would indicate that *O. tomentosa* prefers moist sites compared to dry conditions but if the site becomes wet to very wet, it will negatively impact the incidence of *O. tomentosa*. Bernier and Lewis (1999) were also able to obtain statistically significant impacts of pH on incidence of *O. tomentosa*. The range found by them (pH of 3.0 to 7.0) was found by Whitney (1962) to be uninhibiting to *O. tomentosa* grown *in vitro*. Others (van Groenewoud and Whitney 1969) determined that the disease did not occur in soils with pH higher than 7. However, Lewis *et al*. (2004) contradicted what was previously stated by Bernier and Lewis (1999) and reported that pH does not have a strong influence on the root disease. Unlike pH, factors such as moisture, nutrients, slope, soil texture, etc., seem to have a greater impact on the tree species present, which indirectly affects *O. tomentosa* (Bernier and Lewis, 1999; Whitney 1976).

Another factor thought to play a role in the spread of *O. tomentosa* is stand density or basal area. Researchers van Groenewoud and Whitney (1969) reported a positive relationship between disease incidence and stand density, however, Lewis *et al*. (2004) found that disease incidence of *O. tomentosa* was not related to spruce density, supporting earlier work done by Bernier and Lewis (1999). The surest way to determine if the fungus is primarily spreading by root contact or by spores within a forest stand is to determine the size of *O. tomentosa* genets

(clones) since each basidiospore should be the outcome of a unique recombination event (Germain *et al*. 2009).

Genetics and genets

In many Homobasidiomycetes, inbreeding is prevented courtesy of a heterothallic tetrapolar mating system (Peterson 1995). This system involves two different alleles on two different genes and when a basidiospore germinates and produces haploid monokaryotic hyphae it will only anastomose or fuse with haploid monokaryotic hyphae that possess differing alleles on each of the two genes (i.e. A_1B_1 will anastomose with A_2B_2 but not with A_1B_1, A_1B_2, or A_2B_1) (Peterson 1995; Germain *et al*. 2009). This is known as sexual incompatibility. This new dikaryotic hyphae will grow vegetatively and anastomose with other individuals of the same genetic background (Germain *et al*. 2009).

Somatic incompatibility (sometimes known as vegetative incompatibility) is where dikaryotic mycelium will not anastomose with genetically different dikaryotic mycelium of the same species (rejection of genetically dissimilar, usually dikaryotic mycelia) (Worrall 1997). The individuals are known as genets (Worrall 1997; Germain *et al*. 2009). What somatic incompatibility is and how it differs from sexual incompatibility is that it is associated with genetic difference, therefore it is a heterogenic incompatibility system (whereas sexual incompatibility is a homogenic incompatibility system) (Worrall 1997). The function of somatic incompatibility is to maintain individuality of mated mycelia through prevention of genetic exchange. The role this incompatibility serves is to maintain phenotypic diversity among genotypically distinct individuals in a population as well as avoiding infection (such as by mycoviruses) (Worrall 1997).

To understand how an organism is infecting an area, genet studies can be extremely useful. Genet studies have been conducted on *Armillaria gallica* Marxm. & Romagn. (formally *A. bulbosa* (Barla) Kile & Watling) (Smith *et al*. 1992), *Heterobasidion annosum* (Swedjemark and Stenlid 2001), as well as other important soil-inhabiting fungi (not necessarily all root rotting). Most pertinent to the case of *O. tomentosa* is the study conducted by Germain *et al*. (2009). Through somatic incompatibility one could calculate spread of infection allowing for the approximate determination of the point of infection, how many distinct individuals there are, and how large of an area an individual covers. Determining area covered by the mycelium of an individual, one can determine how long it took to cover that area by dividing that area by the colony growth rate of that particular species of fungus. Somatic incompatibility can also be used to determine whether a fungus spreads vegetative or by basidiospores (Worrall 1997).

The conclusions made by Germain *et al*. (2009) showed that spruce stands in Western Quebec were initially colonized by spore inoculation followed by vegetative expansion and those basidiospores were an important mode of infection. This was similar to what Lewis and Hansen (1991b) had observed in north central British Columbia. Genet size (similar to overall incidence of *O. tomentosa*) was found to vary extensively and is likely to depend on age of colonization, site quality, tree density, etc. (Germain *et al*. 2009). Compared to other studies, genets of *O. tomentosa* were small in comparison to some tree pathogens (various species of *Armillaria*, *Heterobasidion annosum, Phellinus tremulae*), yet similar in size to other tree-associated basidiomycetes (such as ectomycorrhiza-forming fungi).

What Germain *et al*. (2009) alluded to was that since there was no recorded history of *O. tomentosa* prior to the spruce plantation where their study took place, they assumed that the pathogen was patchy and not abundant because it does not infect deciduous trees. However, once

a plantation had been established, and the stand ages and matures it then becomes more susceptible to *O. tomentosa* infection (Germain *et al*. 2009). A previous study by Dahlberg and Stenlid (1994) on the ectomycorrhiza-forming fungus *Suillus bovinus* (L.) Roussel*,* observed that as the forest matures the number of individual genets decreased, although the size of the surviving genets will increase. Germain *et al*. (2009) anticipated the same outcome to occur within their study area.

Onnia tomentosa is a major player in the boreal forest as it is a very significant pathogen of spruce, pine, and other conifer species. It targets hosts when they reach maturity and predisposes them to other factors that can cause tree mortality in addition to the decay they cause within the host. The root rotting pathogen is able to survive in infected stumps for decades, ready to infect another host as soon as contact is made (Whitney 1962; Lewis and Hansen 1991a). Debate is ongoing regarding its most significant mode of dissemination. Regardless though, when it is present in a stand, it is able to stay there for as long as there are optimal hosts for it to colonize. It can be speculated that site and soil characteristics play a larger role on the pathogen's host rather than directly affecting the pathogen, which is likely true for most pathogens. Unlike some other major root rotting fungi of conifer forests (e.g. *Armillaria* spp.), *O.tomentosa* appears to produce many small genets that, over time, will be reduced, but surviving genets will grow to larger size (Lewis and Hansen 1991b; Germain *et al*. 2009). More studies with *O. tomentosa* with regards to genet size is required to confirm these assumptions.

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CHAPTER 2:

GENETIC DIVERSITY AND COLONIZATION PATTERNS OF *ONNIA TOMENTOSA* IN TWO PLANTATIONS OF BLACK SPRUCE (*PICEA MARIANA*) IN NORTHWESTERN ONTARIO FOLLOWING DIFFERENT LEVELS OF THINNING

Abstract

Onnia tomentosa (Fr.) Karst. is prevalent throughout North America, Europe, and Asia and is responsible for causing a significant root-rot disease of conifers commonly known as stand-opening disease. Although the disease infects both spruce and pine, it is more severe on the former. In the late summer of 2014, the spatial coordinates of 124 basidiomata were taken, and the basidiomata collected from two black spruce plantations near Limestone Lake (Boom Lake planted in 1960 and the Airstrip planted in 1962), north of Nipigon, Ontario that had undergone thinning treatments six years prior. The three thinning treatments were light thinning (25% basal area removal), heavy thinning (45% basal area removal), and control (no thinning occurred). There was also a clear cut treatment, however, no basidiomata of *O. tomentosa* were found. Using extracted DNA from each of the basidiomata, single strand conformational polymorphism polymerase chain reaction (SSCP-PCR) of two nuclear loci were conducted, and DNA sequencing of two mitochondrial loci were used to measure genetic diversity and consequently genet size in order to see if stand density had an effect on *O. tomentosa*'s colonization patterns. There were 116 genetically distinct individuals identified, suggesting that the majority of the basidiomata represented unique genets. Stand thinning appeared to negatively influence *O. tomentosa* colonization, however, it is inconclusive whether the light or heavy thinning treatments are better at countering the fungal pathogen.

Introduction

Onnia tomentosa (Fr.) P. Karsten is a widespread, root-rotting pathogen found throughout North America, Europe, and Asia (Whitney 1962; Breitenbach and Kranzlin 1986; Teng 1986). Its preferred hosts are conifer tree species, but is particularly severe on spruce (Whitney 1977; Germain *et al*. 2009). Once infected, the roots of the tree begin to decay, forming the characteristic white pocket rot which leads to the eventual degradation of the tree roots and butt (Whitney 1988; Lewis 1997). This makes the host susceptible to blow down, hence the common name for the disease – Stand Opening Disease.

Onnia tomentosa has two modes of dissemination; (i) release of basidiospores formed by the basidioma and (ii) vegetative growth from extramatrical mycelial expansion or root contact (Germain *et al.* 2009). Each basidioma is a unique recombination event (Pedgley 1986). Fruiting bodies (basidiomata pl., basidioma sing.) do not begin to form until significant infection of the host has occurred. These basidiomata greatly enhance the range that the disease can spread through the release of basidiospores.

The sexual recognition system is under the control of two different mating-type loci which is referred to as the heterothallic tetrapolar mating system (Germain *et al.* 2009). Each basidiospore produces monokayotic hyphae which will anastamose with another monokaryotic hyphal strand of the opposite mating type forming dikaryotic hyphae. This dikaryotic mycelium grows vegetatively and can anastamose with hyphae of the same individual (or hyphae that has identical genetic loci controlling somatic compatibility) (Peterson 1995; Worrall 1997; Germain *et al.* 2009). It is the dikaryotic mycelium that infects rootlets and spreads via root contact which results in an infection centre that consists of a single somatic-compatibility genotype- or genet (Whitney 1962; Lewis and Hansen 1991b; Lewis *et al.* 1992; Germain *et al.* 2009). If

colonization occurs by basidiospores, a genetic mosaic (meaning multiple genets) will result since each colonization event is the result of recombination. The size of the genets positively correlates to the age of colonization, meaning large genets are expected to be older than small genets (Germain *et al.* 2009).

Spreading either by basidiospores or vegetatively, *O. tomentosa* can cover and infect multiple hosts in a relatively short time. Stand density is reported to have a positive relationship on disease incidence (van Groenewoud and Whitney 1969). Although, Lewis *et al.* (2004) stated that disease incidence was not related to stand density.

In 2006-2007, the Centre for Northern Forest Ecosystem Research (CNFER) secured funding to establish a commercial thinning trial in black spruce (*Picea mariana* (Mill.) B.P.S.) plantations that were established by George Marek (former district forester) in the early 1960s near Limestone Lake near Nipigon, Ontario to examine the effects of thinning. Early density regulation is not only important for disease disruption either through planting or juvenile spacing, but is essential for ensuring that stands meet the age, live crown, and health criteria that makes them suitable for commercial thinning (McKinnon *et al.* 2006). Between 1993 and 2003, approximately one million hectares of Ontario's boreal forest was regenerated to conifer plantations, mostly black spruce and jack pine (*Pinus banksiana* Lamb.) (CCFM 2003).

In 2008, thinning plots were established. Sixteen one-hectare experimental thinning plots and 10.8 hectares of operational thinning plots were installed. The treatments represent a range of intensity based on the amount of basal area removed; light thin (LT 25% removed), moderate quality thinning (QT 35% removed), heavy thinning (HT 45% removed), clear cuts (CC 100% removed), and un-harvested controls.

In the summer of 2013, it was noted that basidiomata of *O. tomentosa* were beginning to fruit. Whitney and Fleming (2005) discovered that even though the basidiomata of *O. tomentosa* fruit episodically, their presence correlates with root rot in white spruce plantations and was useful in predicting losses. The above ground presence of basidiomata represents approximately the occurrence of below ground mycelial structure (Germain *et al.* 2009). Traditional methods involved pairing dikaryotic cultures derived from basidiomata on agar to observe somatic compatibility/incompatibility to determine genet size (Worrall 1997). However, molecular techniques allow for generating a larger sample size and prevents misidentification of *O. tomentosa* with other morphologically similar species (e.g. *Onnia circinata* (Fr.) P. Karst.). Single-strand conformational polymorphism (SSCP) PCR was the chosen method to observe genetic differences among *O. tomentosa* populations. SSCP was chosen because it allows for the screening of mutations in a specified DNA region by choosing DNA primers that span that region and SSCP PCR allows for the screening of a large number of samples due to the simplicity and quickness of the technique. The purpose of this study was: a) to map the occurrence of basidiomata of *O. tomentosa* in two plantations of black spruce, b) determine the size and distribution of individual genets of *O. tomentosa* in the two plantations, and c) determine whether operational thinning in the black spruce plantations affects colonization by *O. tomentosa*. It is hypothesised that the incidence of *O. tomentosa* in thinned treatment plots will be less compared to control plots due to reduction in root contacts.

Materials and Methods

Fungal Sampling

One hundred and twenty-four basidiomata of *Onnia tomentosa* were collected in the late summer of 2014 on the forest floors of a 56 and 54 year old black spruce (*Picea mariana*) plantations (Fig. 2.1) known as Boom Lake and Airstrip respectively, located near Limestone Lake (49º6′36″N, 88º9′41″W), Ontario, Canada. The soil characteristics and climate factors for the two plantations are provided in Table 2.1. The area had undergone randomized blocking of four treatments: control (no basal area removal), light thin (25% basal area removal), heavy thin (45% basal area removal), and clear cut (100% basal area removal). However, only the light thin, heavy thin, and control plots were used to sample from since we knew there was *O. tomentosa* present in those plots. Clear cuts were not sampled from because the host is not present for *O. tomentosa* to fruit from. Plots that were used for sampling were systemically assessed and all plots received a complete examination of the entire plot. Basidiomata gathered from control, light thinned, and heavy thinned all had spatial coordinates (x, y) recorded using a 62s Garmin GPS unit (Garmin Inc.). Samples were then numbered, placed into paper bags, and then stored at -15ºC until DNA extractions were completed in 2015.

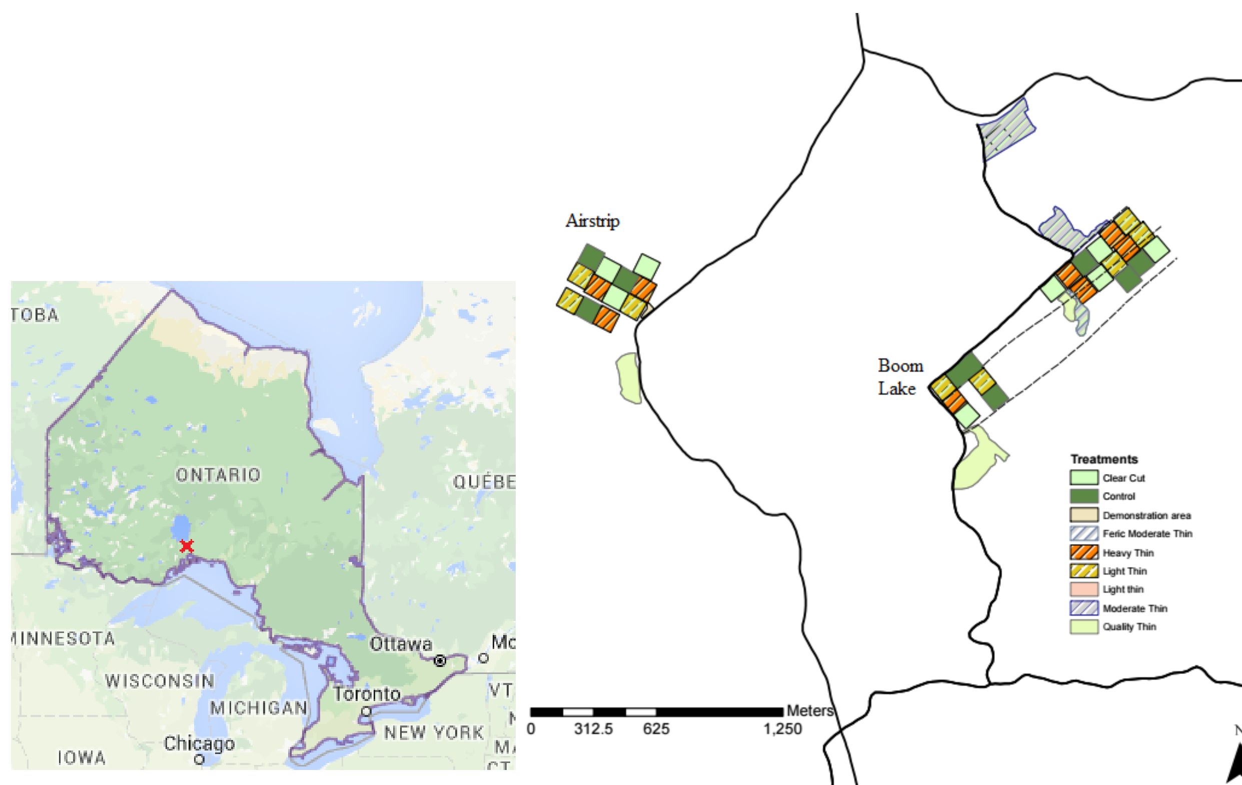

Figure 2.1 Location of study site in Ontario, and layout of thinning treatment plots.

Table 2.1 Variables between Airstrip and Boom Lake plantations.

	Airstrip	Boom Lake
Soil Type	Calcareous clay	Sandy Loam with a loess silty cap
Moisture Regime	4	1
Drainage Class	4	1
Annual Mean Temperature (ºC)	2.7	2.7
Annual Mean Precipitation (mm)	668	668
Site Index (m)*	14.71	15.12

*Index age for site index = 50 years at breast height age.

DNA Extraction

Samples were brought out of -15ºC storage and a section of the pileus (or stipe if pileus was too degraded) was removed and weighed to approximately 5 mg. To limit contamination from the environment, the piece was aseptically removed from the interior of the pileus or if the

piece was taken from the stipe it was taken from the middle area after the outer area was removed.

All work involving DNA extraction was undertaken at the Lakehead University Paleo-DNA Laboratory. DNA was extracted by placing each sample in a separate sterile 0.2 mL tube where one stainless steel bead was also placed. The 0.2 mL tube containing both sample and bead was then placed into the TissueLyser LT adapter (QIAGEN Inc.). The lid was then securely fastened once all sample tubes had been placed in the rotator. The TissueLyser LT ran for one minute at 50 Hz. When the tissues had been completely homogenized they were removed and placed into a test tube rack until the next step. Samples that were not homogenized completely were run again for another minute at 50 Hz. No samples were homogenized for more than two minutes.

Using the DNeasy® Plant mini kit (QIAGEN Inc.) 400 µL of AP1 buffer and 4 µL of RNase A stock solution (100 mg/mL) were added to the samples and then vortexed vigorously. To reduce precipitates (if any), buffers were warmed before use. The AP1 buffer and RNase were not mixed before use. The mixture was then incubated for 10 minutes at 65ºC using a Thermomixer R (Eppendorf Ltd.). Tubes were inverted 3 times throughout the 10 minute incubation period to lyse the cells. After the incubation was completed, 130 µL of AP2 buffer was then placed on ice for five minutes, which precipitates polysaccharides, detergent, and proteins. Tubes were then centrifuged for five minutes at 13,000 rpm using a 5415D model centrifuge (Eppendorf Ltd.). The solution was then pipetted out and moved to QIAcube specific 2 mL tubes (QIAGEN Inc.) and placed in the rotator of the QIAcube (QIAGEN Inc.). Samples then underwent the QIAcube protocol for plant DNA which is provided in the following steps: lysate was pipetted into the QIAshredder Mini spin column (QIAGEN Inc.) and then placed into

a 2 mL collection tube and centrifuged for 2 minutes at 13,000 rpm. The flow through fraction was then transferred into a new tube (without disturbing the pellet). 1.5 mL of Buffer AP3/E (QIAGEN Inc.) was pipetted into the cleared lysate followed by 650 µL of the flow through fraction into a DNeasy Mini spin column (QIAGEN Inc.) in a 2 mL collection tube. This was then centrifuged for 1 minute at 8000 rpm. The remaining sample then had another 650 µL of flow through fraction pipetted in and was then again centrifuged for 1 minute at 8000 rpm. The DNeasy Mini spin column (QIAGEN Inc.) was then transferred to a new 2 mL collection tube and 500 µL of Buffer AW (QIAGEN Inc.) was added and was centrifuged for 1 minute at 8000 rpm. The flow through was discarded and another 500 µL of Buffer AW (QIAGEN Inc.) was then added to the DNeasy Mini spin collection tube. This was then centrifuged for 2 minutes at 13,000 rpm to dry the membrane, and then transferred to a 2 mL tube. Finally, 100 µL of Buffer AE (QIAGEN Inc.) was then pipetted directly to the DNeasy membrane, which was then incubated for 5 minutes at 20ºC, and then centrifuged for 1 minute at 8000 rpm to elute the solution.

Marker Selection

Screening for single-strand conformational polymorphism (SSCP) was done on amplicons created by four different pairs of primers (Table 2.1) specific to *Onnia tomentosa*. Two pairs, MS1 / MS2 (MS) and ML5 / ML6 (ML) (White *et al*. 1990) were selected to amplify mitochondrial genes. The it-BT-15-f / it-BT-490-rc was designed from an alignment of β-tubulin (BT) sequences that were from amplification with fungal primers BT1a / BT1b (Glass and Donaldson 1995). The fourth and final set of primers used were it112.31act2501f / it112.31act2700rc and it was designed from actin (ACT) and RNA polymerase II largest subunit (RPB1) aligned sequences which were obtained from amplification with degenerate primers

ACT2-F / ACT2-R (Germain *et al.* 2009). The latter two sets of primers were used to select and amplify nuclear genes specific to *Onnia tomentosa*. The four primers were chosen because of the work done by Germain *et al.* (2009) where a similar study was undertaken.

Table 2.2 Primer sequences, annealing temperatures, and amplification length for locus and allele specific amplifications at two mitochondrial and two nuclear loci in *Onnia tomentosa*.

Locus	Primers	Primer Sequence	Annealing Temperature (ºC)	Amplicon Length (bp)
Mitochondrial large subunit	ML5	5'-CTCGGCAAATTATCCTCATAAG-3'	59	778
	ML6	5'-CAGTAGAAGCTGCATAGGGTC-3'		
Mitochondrial small subunit	MS1	5'-CAGCAGTCAAGAATATTAGTCAATG-3'	59	619
	MS2	5'-GCGGATTATCGAATTAAATAAC-3'		
Beta-tubulin (specific)	it-BT-15-f	5'-GGAGCCAGCAGTACCGTG-3'	50	494
	it-BT-490-rc	5'-CGTGAAGTATGCGTTAGC-3'		
Actin (specific)	it112.31act2501f	5'-GTGAAATTGTGCGCGACATC-3'	59	200
	it112.31act2700rc	5'-AACACGCCGCAAGTCAAC-3'		

Genomic DNA Amplification

Nuclear DNA amplification was carried out using 5.0 µL 5x GoTaq Flexi Buffer, 0.5 µL of dNTP mix 10 mM, 2.0 µL of magnesium chloride 25 mM, 0.25 µL of each primer, 0.13 µL of GoTaq DNA polymerase, 14.9 µL of double distilled H_2O, and 2.0 µL of sample at 1.0 ng concentration for a total 25 µL volume. Samples were then placed in the PTC-225 Peltier ThermoCycler (BioRad Laboratories Inc.) and had a hot start at 95°C for 2 minutes, then denatured at 95°C for 30 seconds, followed by an annealling step at 50°C for BT primers and 59°C for ACT primers for 1 minute, and extended at 72°C for 2 minutes for 40 cycles. The final extend was at 72°C for 5 minutes and the holding step at 7°C.

Mitochondrial DNA amplification required 2.5 µL of 10x PCR buffer (minus Mg), 0.5 µL of dNTP mix 10 mM, 1.0 µL of magnesium chloride 25 mM, 0.25 µL of each primer, 0.1 µL of Platinum Taq (5U/µL), 15.4 µL of double distilled H_2O, and 5.0 µL of 1.0 ng concentration sample of DNA for a 25 µL volume. PCR was carried out using a PTC-225 Peltier ThermoCycler using the following protocol: hot start (94°C for 2 minutes), denature (94°C for 30 seconds), anneal (59°C for 1 minute), extend (72°C for 2 minutes), repeated for 50 cycles and then held for 7°C.

Single Strand Conformational Polymorphism - PCR

Nuclear DNA was run through a 6% PAGE (polyacrylamide gel electrophoresis) gel to detect polymorphisms. An 8% PAGE gel was used for detecting polymorphisms within mitochondrial DNA. The 8% PAGE gel was made from 1.0 mL 5xTBE buffer (1.1M Tris; 900 mM Borate; 25 mM EDTA; pH 8.3), 1.0 mL of acrylamide, 5.2 mL of H_2O, 10 µL of TEMED, 100 µL of 10% ammonium persulfate, and 0.7 mL of glycerol.

Nuclear DNA samples (5 µL) were mixed with 3 µL of DNA gel loading dye (6x) (Thermo Fisher Scientific Inc.) and then loaded into 6% PAGE gels which ran in 1xTBE buffer for 45 minutes at 118 volts at room temperature. Mitochondrial samples were placed in 0.5 mL tubes using 5 µL of sample, 5 µL of Formamide, and 3 µL of DNA gel loading dye (6x) (Thermo Fisher Scientific Inc.). Tubes were heated for 7 minutes at 95°C (standard heatblock, VWR Scientific Products) then immediately placed on ice for 5 minutes. After which, tubes were vortexed and spun down and then placed back on ice while the gel was being loaded. 8% PAGE gels ran in 1xTBE buffer that was refrigerated until the buffer was approximately 4°C and the entire gel casing was placed in a Styrofoam cooler with ice packs lining the inside to keep the entire unit around 4°C. The gel ran for 180 minutes at 140 volts. The X Cell SureLock

(Invitrogen Canada Inc. Burlington, ON) gel container was powered by ACCU power (VWR Scientific Products).

After gels (both 6% and 8% PAGE gels) were finished running, they were then separated from their gel cassette and the polyacrylamide gels were placed into a staining bath filled with ethidium bromide solution. Gels were left in the bath for 15 minutes then removed and placed onto the transilluminator (MultiImage Light Cabinet, Alpha Innotech Corperation, San Leandro, CA). The gel was photographed and removed from the transilluminator. The gel was then analyzed using AphaEaseFC 4.0 software (ProteinSimple, San Clara, CA).

Mitochondrial DNA Sequencing

Mitochondrial DNA samples were sequenced, as inconclusive results were derived from the gels. Samples were purified by adding 4 µL of shrimp alkaline phosphatase (SAP) and 2 µL of Exonuclease I to 20 µL PCR reaction. Everything was done on ice. The mixture was then vortexed and incubated at 37°C for 15 minutes then at 80°C for 15 minutes using the Mastercycler (Eppendorf Scientific Inc.) This procedure inactivates the enzymes and the PCR product is now purified.

For sequencing, all reagents were placed into a 0.2 mL PCR tube: 3.0 µL of sample, 4.2 µL of sterile water, 2.0 µL of 5x sequencing buffer, 0.3 µL of 10 µM primer, and 0.5 µL Big Dye Terminator Ready Reaction Mix v3. The mixture was vortexed and briefly centrifuged (approximately 20 seconds) to settle the solution into each tube. Tubes were then placed into the thermocycler Mastercycler (Eppendorf Scientific Inc.). The protocol for the thermocycler was as follows: initial denatured at 96°C for 60 seconds, denatured at 96°C for 10 seconds, annelled at 50°C for 5 seconds, extension at 60°C for 75 seconds, repeated for 15 cycles, denatured at 96°C for 10 seconds, annealled at 50°C for 5 seconds, extension at 60°C for 90 seconds, repeated for 5

cycles, denatured at 96°C for 10 seconds, annealled at 50°C for 5 seconds, extension at 60°C for 120 seconds, repeated 5 cycles, held at 7°C. After cycling, samples were then cleaned using a sodium acetate/ethanol precipitation.

A sodium acetate/ethanol solution (3M sodium acetate, pH 5.4 - 3.0 µL and 95% ethanol 62.5 µL) was prepared in 0.5 mL tubes and vortexed briefly (~10 seconds). 24.5 µL of sterile water was added to the sequencing reaction. The diluted sequencing reaction was transferred to a tube containing ethanol/sodium acetate mixture. Tubes were vortexed and sat at room temperature for 20 minutes. Tubes were then placed in a microcentrifuge (Eppendorf centrifuge 5415D) and ran for 20 minutes at 13000 rpm (inserts for 0.5 mL tubes were required for the centrifuge). The supernatants were then aspirated with a separate pipette for each sample. 250 µL of 70% ethanol was added to the tube and vortexed for 20 seconds. Tubes were placed back into the centrifuge in the same orientation as they were the first time and spun for 5 minutes at 13000 rpm. The supernatants were aspirated again and the tubes were dried in a vacuum centrifuge (Eppendorf concentrator 5301) for 12 minutes.

After purification and desiccation, samples were then suspended in 15 µL of Hi-Di formamide. Samples were vortexed for 1 minute, the formamide being kept near the base of the tube. Samples were then heated at 95°C for 3 minutes and then placed on ice for 2 minutes after which they were vortexed again to ensure formamide was at the base of the tube. Samples were then pipetted into an ABI plate which was then placed in an Eppendorf centrifuge 5804 to ensure the product was at the base of the wells. The ABI plate was then placed on a Hitachi Applied Biosystems 3130x1 Genetic Analyzer. Sequences were analyzed using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI).

Spatial Mapping

The spatial maps of the basidiomata of *O. tomentosa* were done using ArcMap 10.1 (Esri's ArcGIS software). The points were taken using a 62s Garmin GPS unit (Garmin Inc.) and where clusters of basidiomata occurred, a single GPS point was taken and then the basidiomata had their distance and bearing taken from where the GPS point was.

Results

Genet Composition and Distribution

One hundred and twenty-four basdiomata were collected from 12 treatment plots (4 control, 4 light thin, and 4 heavy thin): 3 replications were undertaken in the Airstrip plantation (clay site) while only 1 set of treatment plots was undertaken in the Boom Lake plantation (sandy loam site). Two basidiomata had missing data at more than one locus and were removed from the study. The remaining 122 basidiomata were plotted spatially (Fig. 2.2 and 2.3) and where a genet with more than one baisdiomata occurred, a unique symbol was used to represent the basidioma belonging to the same multilocus genet (MLG) (Fig. 2.4-2.10). One hundred and sixteen MLGs were discovered, 110 of which were represented by a single basidioma (Table 2.2). For genets represented by more than one basidiomata, the average diameter of the genet was 0.55 m.

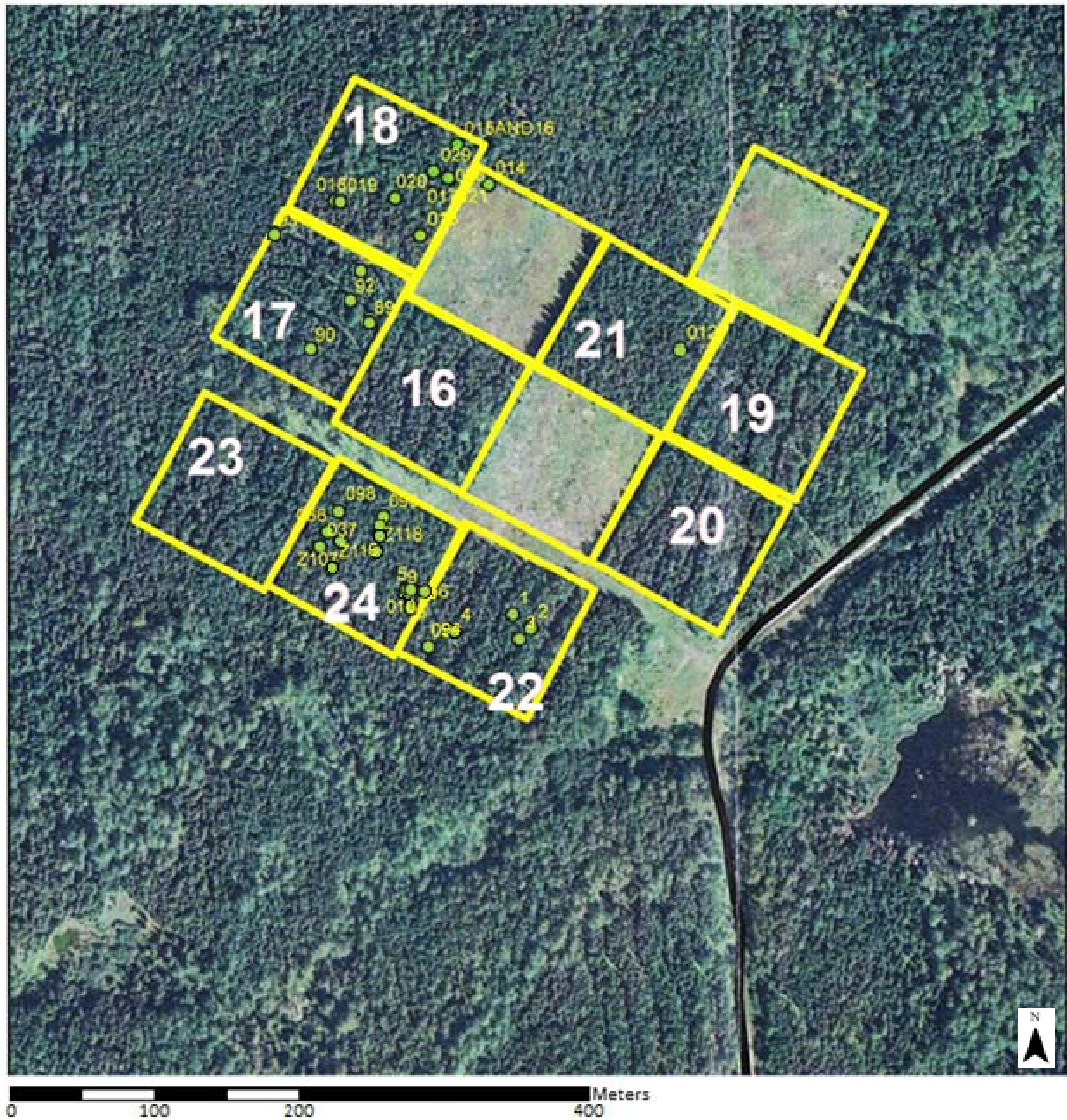

Figure 2.2 Map of the Airstrip plantation and location of *Onnia tomentosa* basidiomata that were collected from control treatment (18, 21, and 24), light thin (17, 20, and 23), and heavy thin (16, 19, and 22) treatment plots.

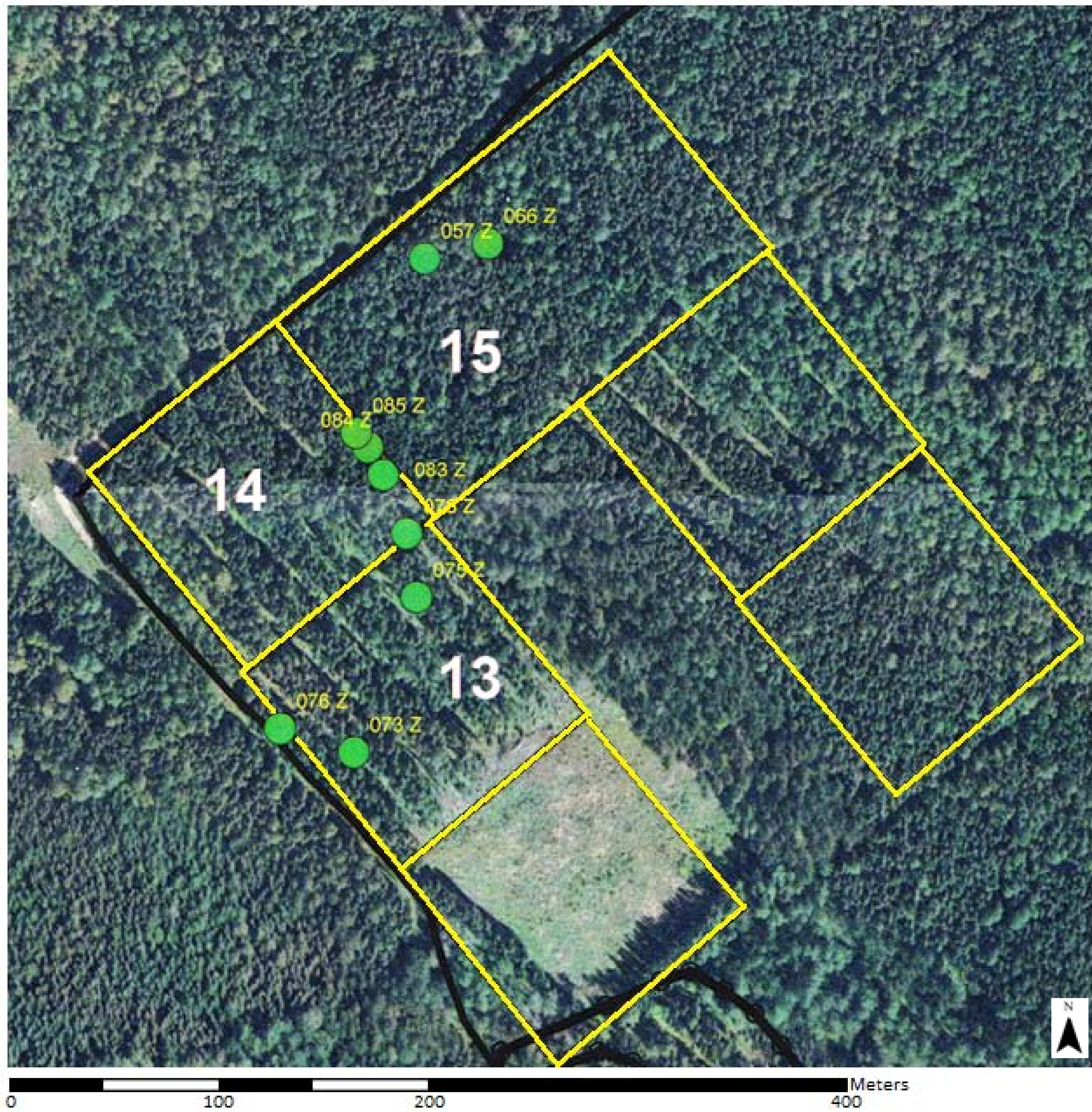

Figure 2.3 Map of the Boom Lake plantation and location of *O. tomentosa* basidiomata that were collected from control (15), light thin (14), and heavy thin (13) treatment plots.

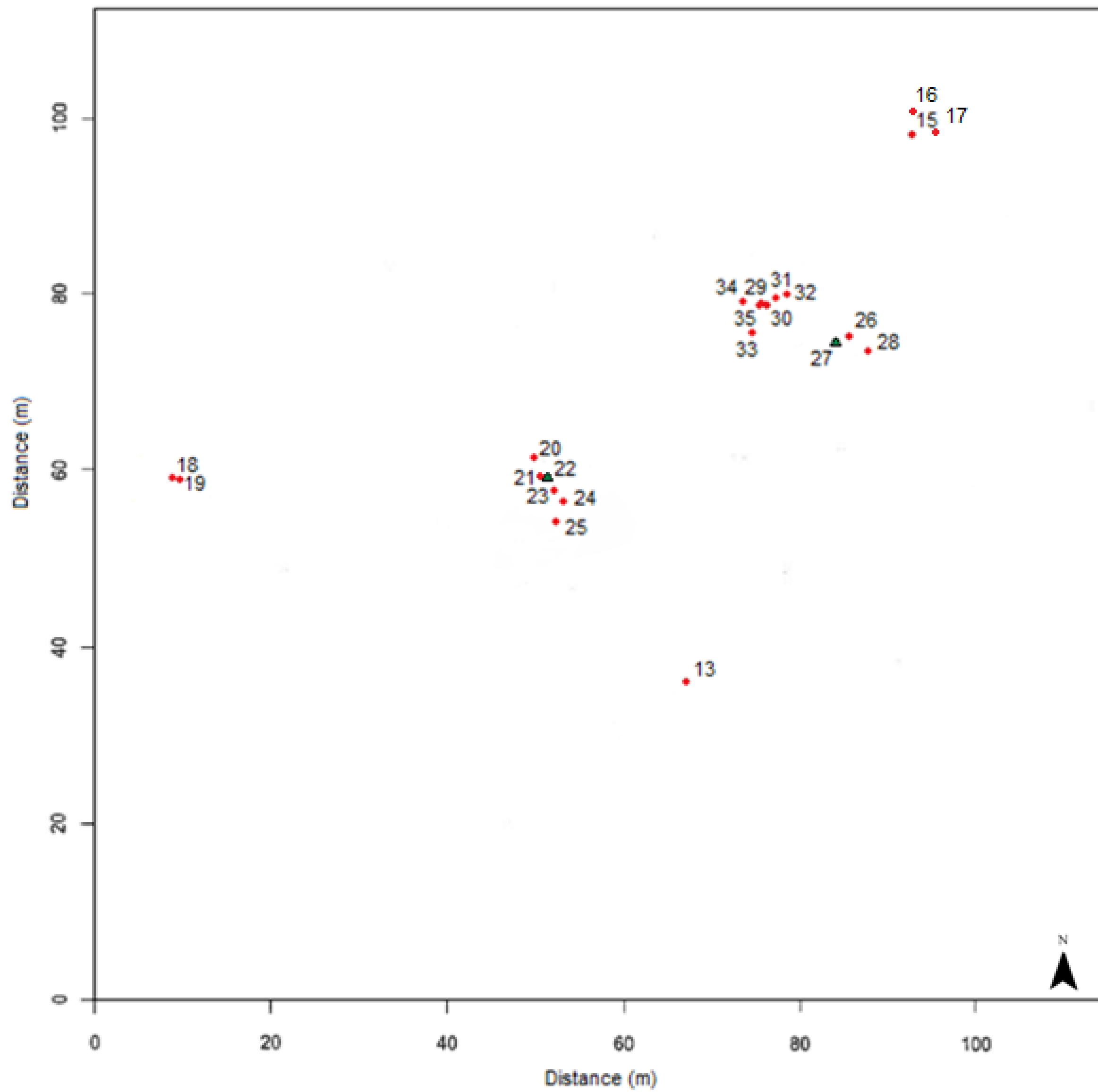

Figure 2.4 Spatial distribution of *Onnia tomentosa* in control plot 18. Each genet is represented by a single basidioma, each having a unique multilocus genotype (MLG). Points denoted with a Δ, indicate those genets represented by more than one basidioma.

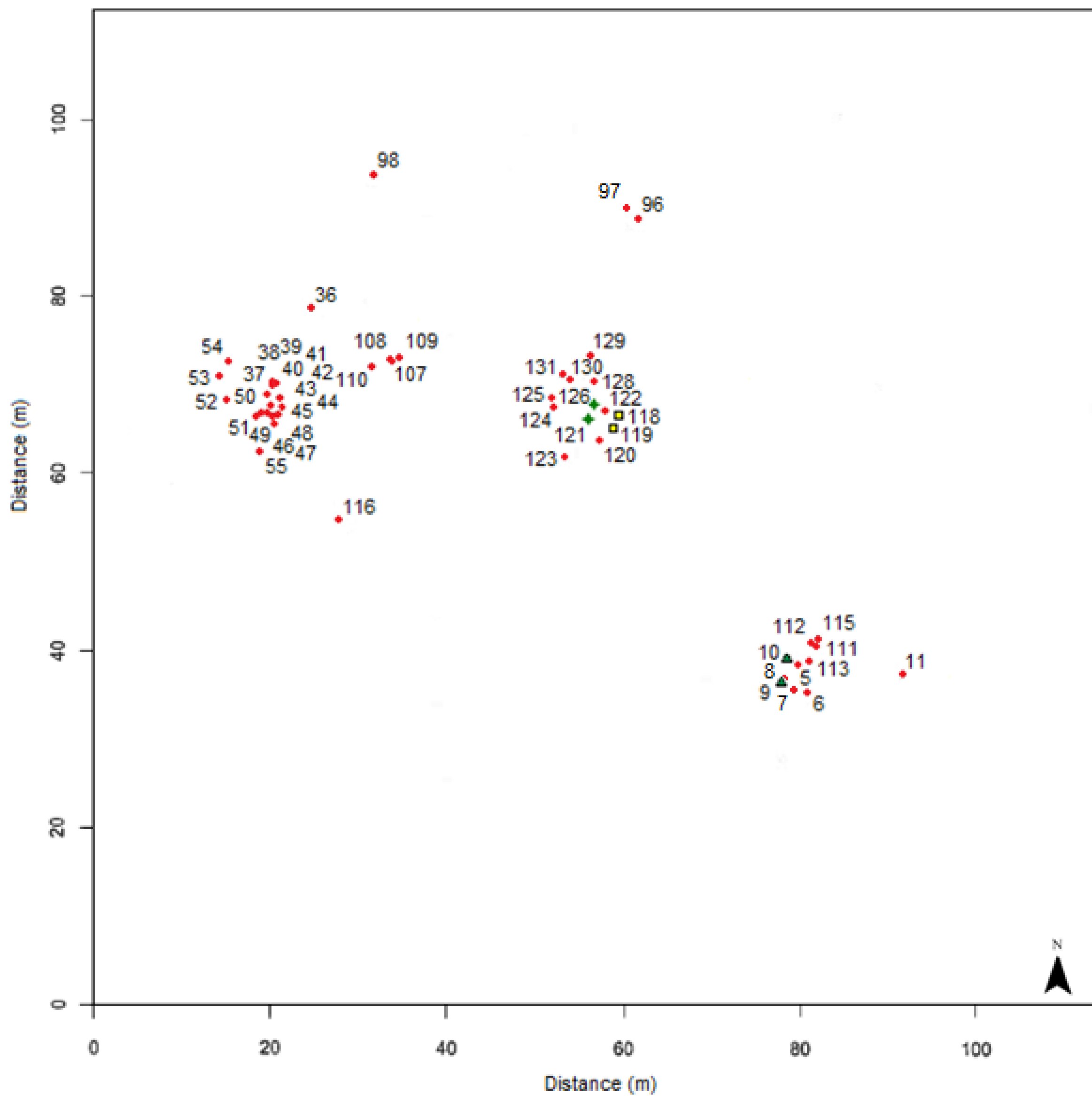

Figure 2.5 Spatial distribution of *Onnia tomentosa* control plot 24. Each genet is represented by a single basidioma, each having a unique multilocus genotype (MLG). Points denoted with various symbols (+, Δ) indicate those genets represented by more than one basidioma.

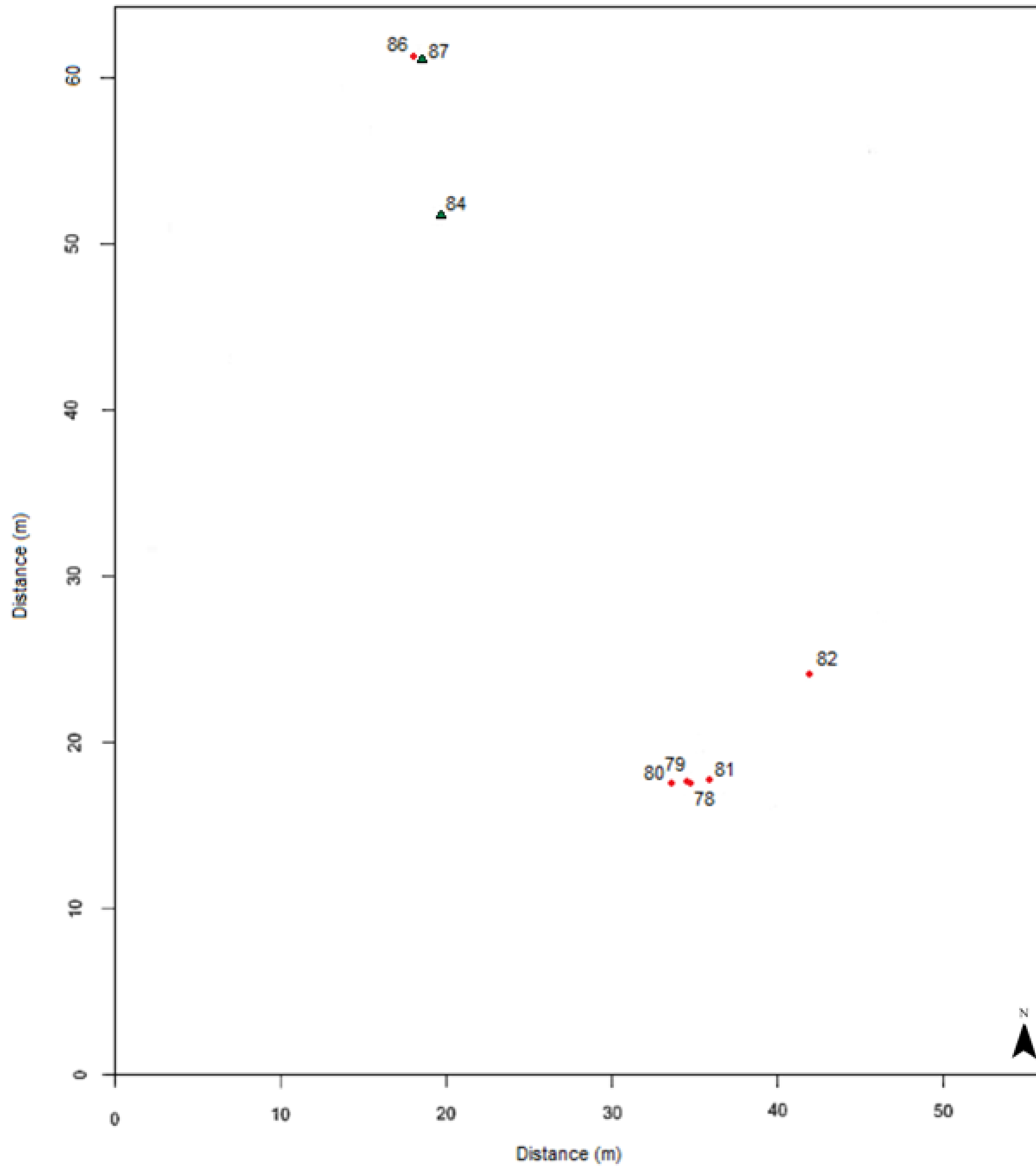

Figure 2.6 Spatial distribution of *Onnia tomentosa* in light thin treatment (25% basal area removal) plot 14. Each genet is represented by a single basidioma, each having a unique multilocus genotype (MLG). Points denoted with a Δ indicate those genets represented by more than one basidioma.

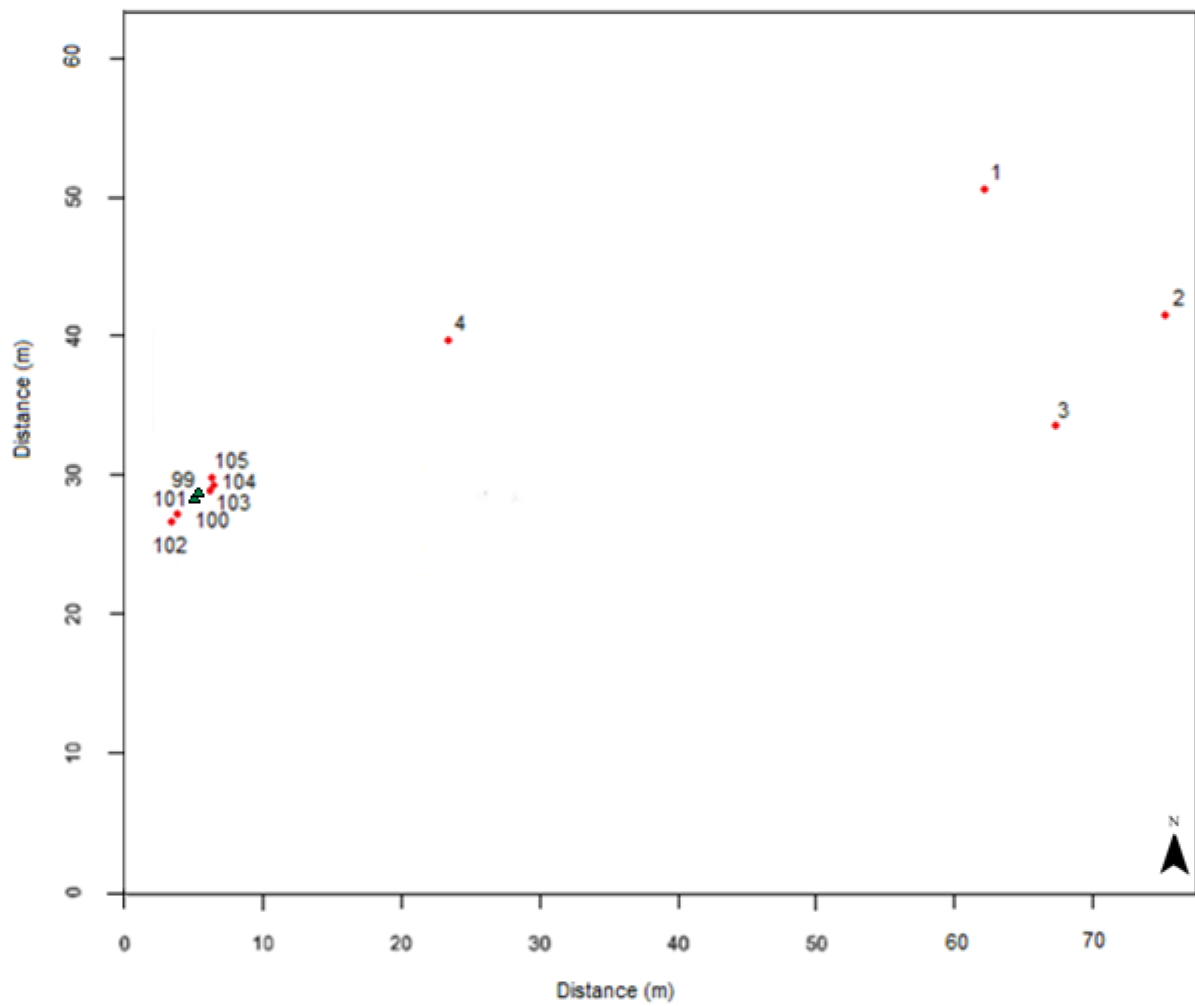

Figure 2.7 Spatial distribution of *Onnia tomentosa* in heavy thin treatment plot (45% basal area removal) 22. Each multilocus genet is represented by a single basidioma, each having a unique multilocus genotype (MLG). Points denoted with a Δ indicate those genets represented by more than one basidioma.

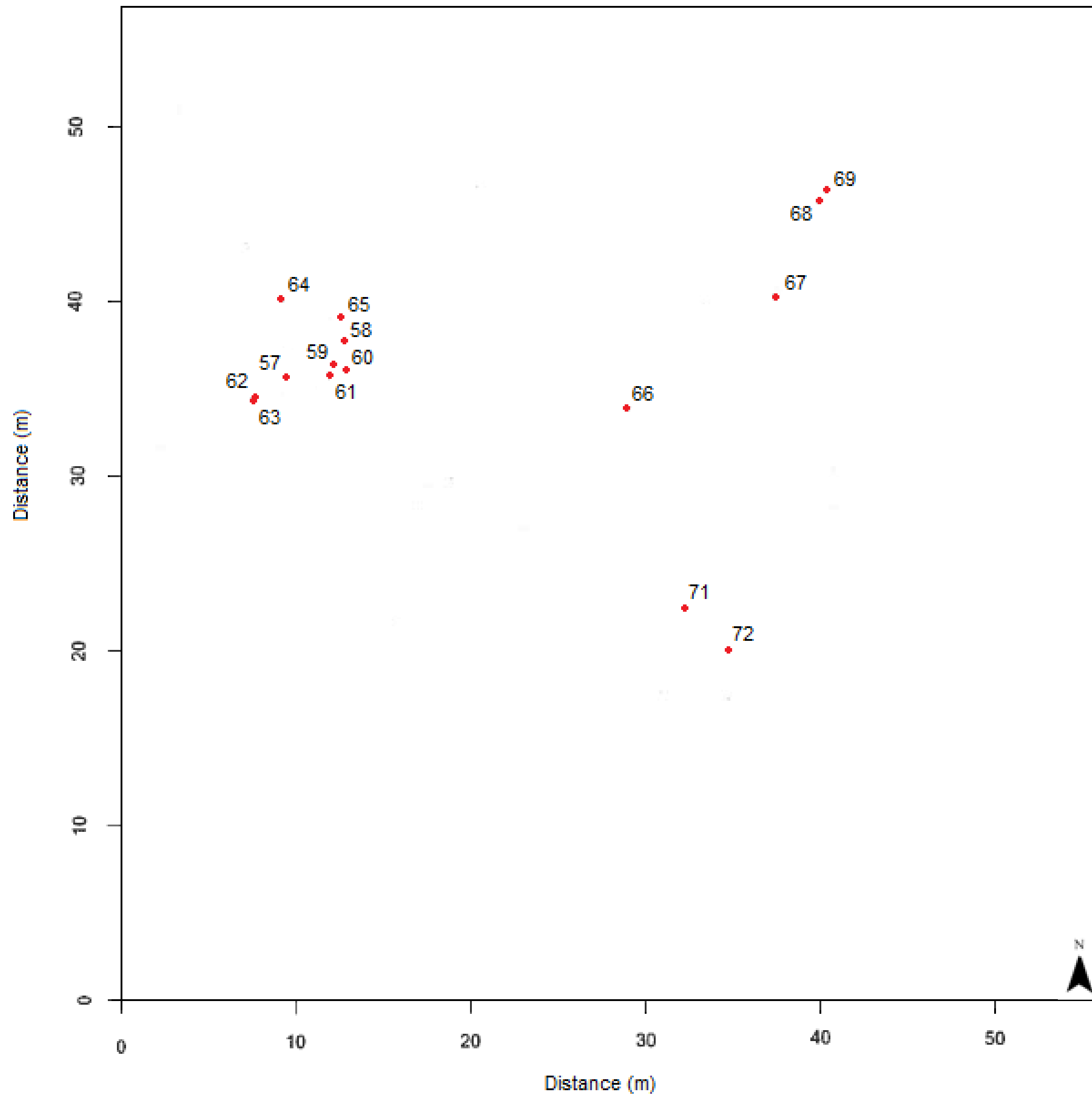

Figure 2.8 Spatial distribution of *Onnia tomentosa* in control plot 15. Each genet is represented by a single basidioma, each having a unique multilocus genotype (MLG).

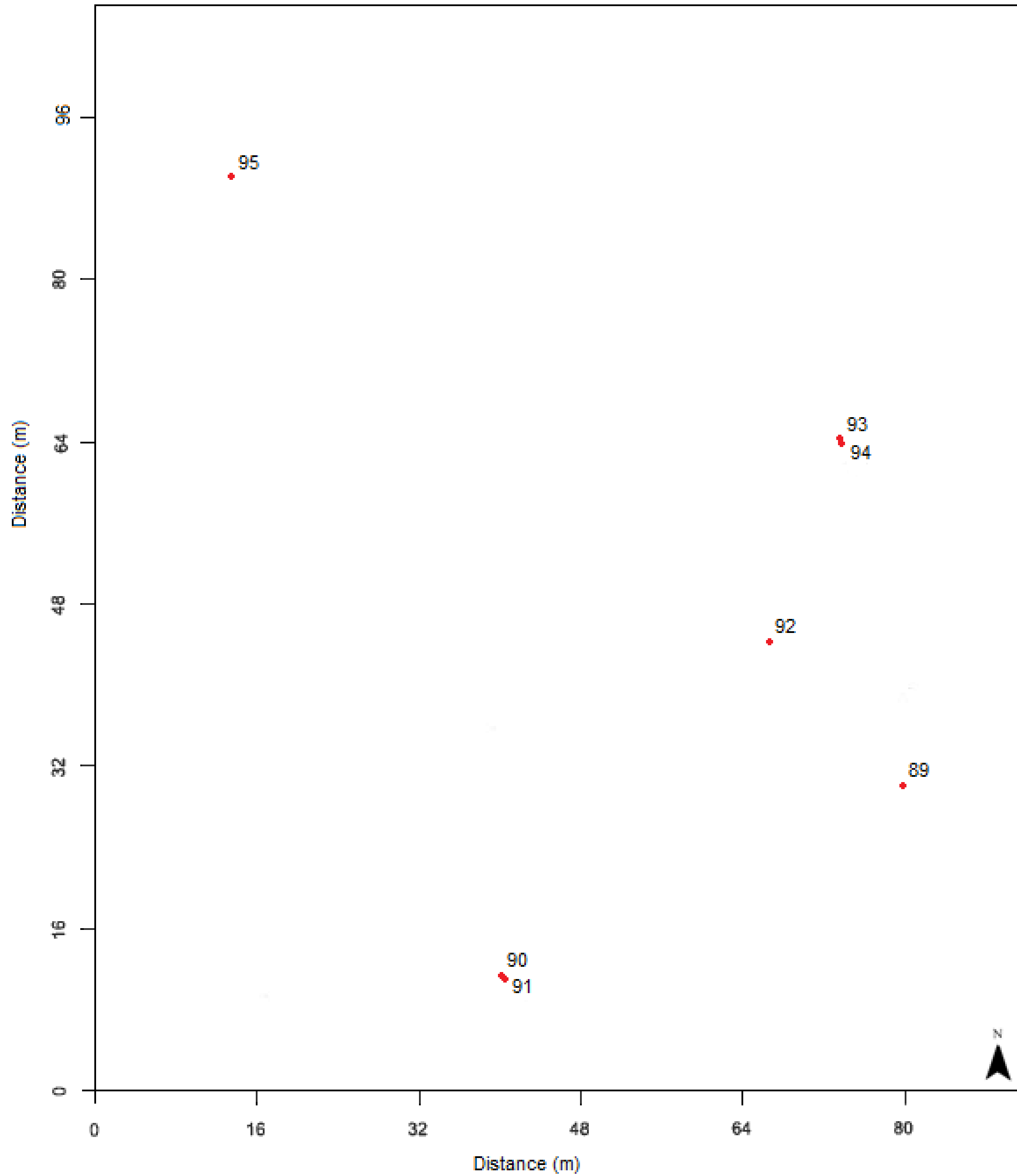

Figure 2.9 Spatial distribution of *Onnia tomentosa* in light thin treatment (25% basal area removal) plot 17. Each genet is represented by a single basidioma, each having a unique multilocus genotype (MLG).

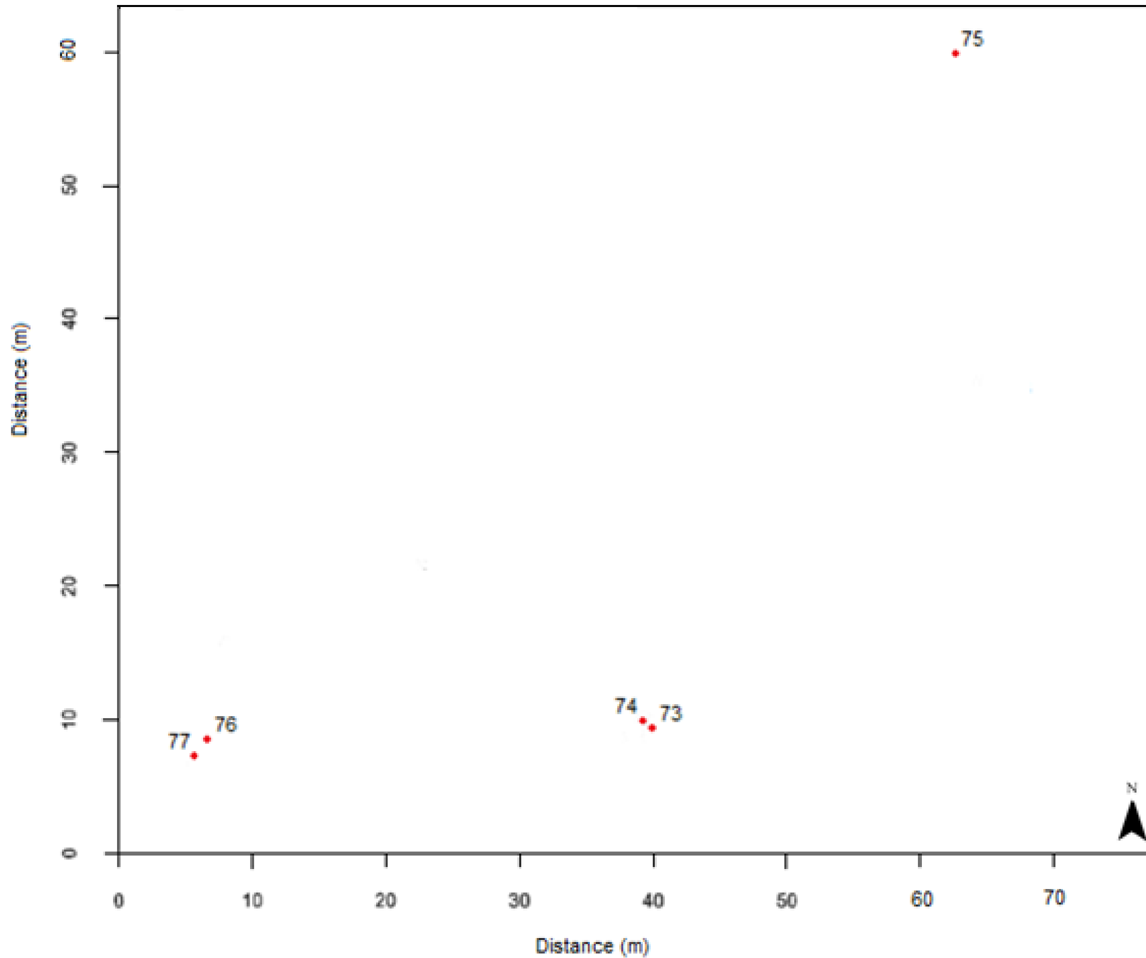

Figure 2.10 Spatial distribution of *Onnia tomentosa* in heavy thin treatment (45% basal area removal) plot 13. Each genet is represented by a single basidioma, each having a unique multilocus genotype (MLG).

Multi-locus Genet Analysis

The DNA analysis was done from both the PAGE gels (e.g. Fig. 2.11) and DNA sequencing, with each sample being scored at each locus to congregate samples that had the same scoring for each locus into a MLG (Table 2.3).

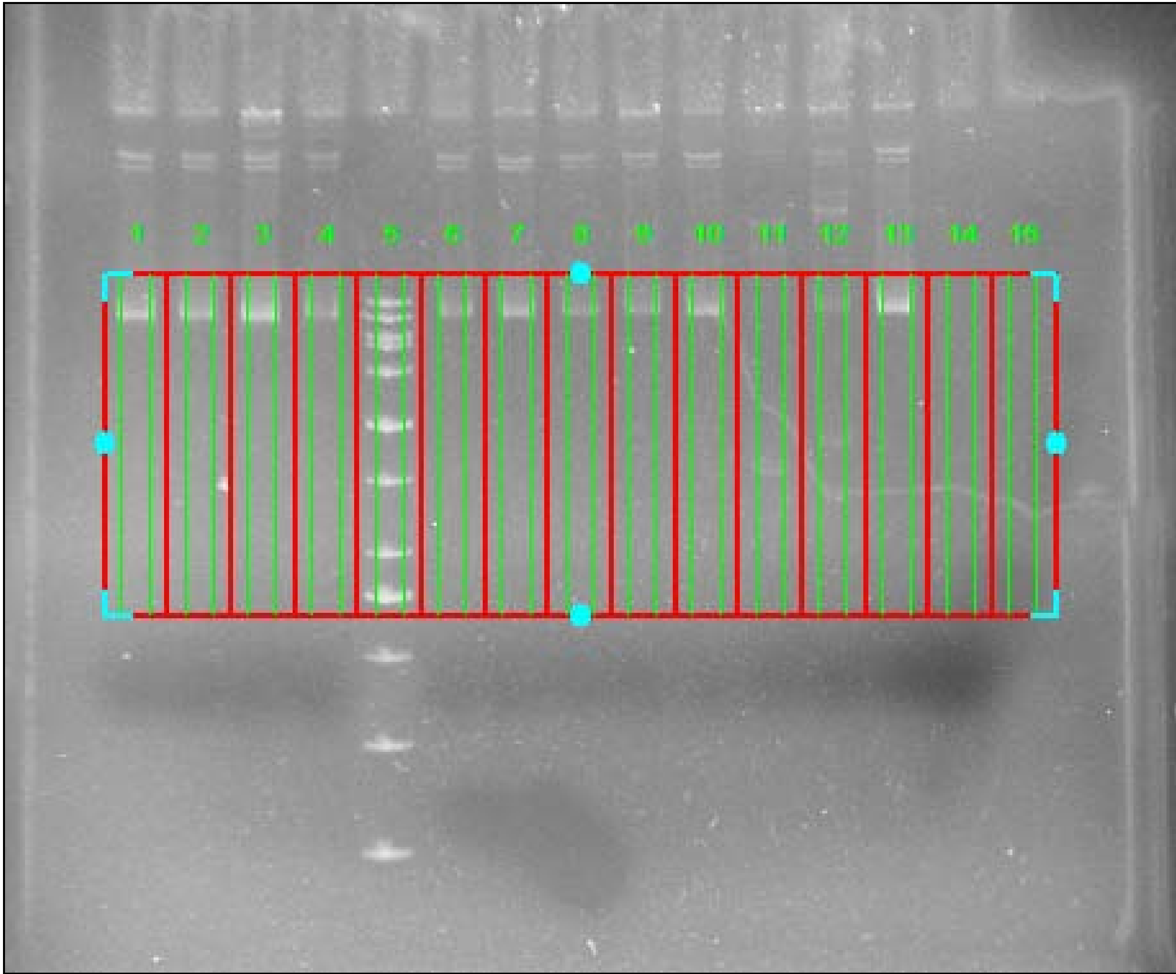

Figure 2.11 Gel chromatography image of genetic samples observed with mitochondrial large subunit primer. The grid overlay is part of the AlphaEaseFC software to measure polymorphisms.

Table 2.3 Genotype of each discrete multilocus genet of *Onnia tomentosa* identified.

			Locus			
Genet #	N*	Size (cm)	MS	ML	ACT	BT
1	1	-	A	A	FF	B
2	1	-	A	A	H	A
3	1	-	C	-	H	B
4	1	-	A	A	A	AA
5	1	-	B	A	D	A
6	1	-	B	A	FF	W
7	1	-	B	A	T	X
8	1	-	B	A	FF	S
9	2	480	B	A	CC	R
11	1	-	D	A	FF	N
12	1	-	A	A	FF	R
13	1	-	A	A	T	N
15	1	-	A	A	N	V
16	1	-	A	A	EE	P
17	1	-	A	A	K	T
18	1	-	A	A	R	S
19	1	-	A	A	P	I
20	1	-	A	A	P	L
21	1	-	A	A	V	E
22	2	3600	A	A	X	D
23	1	-	A	A	Z	GG
24	1	-	A	A	Z	A
25	1	-	A	A	X	A
26	1	-	A	A	V	B
28	1	-	A	A	V	A

29	1	-	A	A	Z	K
30	1	-	A	A	H	T
31	1	-	A	A	C	Y
32	1	-	A	A	C	Q
33	1	-	A	A	B	V
34	1	-	A	A	S	T
35	1	-	A	A	Y	S
36	1	-	A	A	Z	U
37	1	-	A	A	F	W
38	1	-	A	A	I	I
39	1	-	A	-	S	Z
40	1	-	A	A	K	W
41	1	-	A	A	S	R
42	1	-	A	A	S	K
43	1	-	A	-	W	Q
44	1	-	A	A	R	D
45	1	-	A	A	S	E
46	1	-	A	A	U	K
47	1	-	A	-	R	K
48	1	-	A	A	P	FF
49	1	-	A	A	K	O
50	1	-	A	A	H	M
51	1	-	A	-	K	R
52	1	-	A	A	K	M
53	1	-	A	A	K	L
54	1	-	A	A	P	O
55	1	-	C	-	H	O
57	1	-	A	A	H	L

58	1	-	A	A	C	M
59	1	-	A	A	C	J
60	1	-	A	A	A	M
61	1	-	A	A	F	M
62	1	-	A	A	F	U
63	1	-	A	A	H	S
64	1	-	A	A	N	T
65	1	-	A	A	K	U
66	1	-	A	A	P	V
67	1	-	A	A	K	GG
68	1	-	B	A	D	I
69	1	-	B	A	D	K
71	1	-	B	A	B	N
72	1	-	B	A	F	P
73	1	-	A	A	D	P
74	1	-	A	A	A	P
75	1	-	C	A	B	DD
76	1	-	A	A	A	V
77	1	-	A	A	A	U
78	1	-	A	A	A	S
79	1	-	A	A	DD	S
80	1	-	A	A	R	J
81	1	-	A	A	L	C
82	1	-	A	A	V	D
84	2	1980	A	A	P	J
86	1	-	A	A	O	F
89	1	-	C	A	H	DD
90	1	-	C	-	T	BB

91	1	-	C	A	V	CC
92	1	-	A	A	T	F
93	1	-	C	A	P	CC
94	1	-	C	A	V	R
95	1	-	A	A	T	C
96	1	-	C	A	J	N
97	1	-	C	A	J	GG
98	1	-	A	-	P	C
99	2	30	A	A	M	E
101	1	-	A	A	M	G
102	1	-	A	A	G	G
103	1	-	A	A	E	I
104	1	-	A	A	E	G
105	1	-	A	A	C	G
107	1	-	A	A	DD	G
108	1	-	A	A	W	O
109	1	-	A	A	S	I
110	1	-	A	A	V	I
111	1	-	D	A	W	I
112	1	-	D	A	W	J
113	1	-	D	A	Y	M
115	1	-	D	A	BB	O
116	1	-	C	-	Y	EE
118	2	70	A	A	V	P
120	1	-	A	A	Q	S
121	2	300	A	A	G	L
122	1	-	A	A	E	L
123	1	-	A	A	B	H

124	1	-	A	A	G	M
125	1	-	A	A	I	J
127	1	-	A	A	I	R
128	1	-	A	A	D	M
129	1	-	A	A	E	P
130	1	-	A	A	G	J
131	1	-	A	A	I	K

*Number of basidiomata per genet. Loci were groupings based on genetic differences observed at each specific primer. Mitochondrial small subunit primer (MS), mitochondrial large subunit primer (ML), β-tubulin primer (BT), actin primer (ACT).

Allelic Diversity within Onnia tomentosa

The alleles discovered by nuclear primers showed a high level of diversity at both loci. The ACT locus had 31 alleles and the BT locus had 33 alleles (Table 2.4). Genetic samples observed at both loci were grouped by a single base pair differences among PAGE gels. After sequencing the samples, the mitochondrial large subunit loci observed a single grouping among *O. tomentosa* samples and mitochondrial small subunit loci observed 4 groupings among *O. tomentosa* samples.

Table 2.4 Allele frequency at two nuclear loci genotyped in *Onnia tomentosa.*

	Locus	
Allele	ACT	BT
A	0.049	0.041
B	0.033	0.025
C	0.041	0.025
D	0.041	0.033
E	0.033	0.033

F	0.033	0.016
G	0.041	0.041
H	0.066	0.008
I	0.033	0.057
J	0.016	0.057
K	0.066	0.049
L	0.008	0.049
M	0.025	0.066
N	0.016	0.033
O	0.008	0.041
P	0.074	0.057
Q	0.008	0.016
R	0.033	0.057
S	0.049	0.057
T	0.041	0.033
U	0.008	0.033
V	0.074	0.033
W	0.033	0.025
X	0.025	0.008
Y	0.025	0.008
Z	0.033	0.008
AA	--	0.008
BB	0.008	0.008
CC	0.016	0.016
DD	0.016	0.016
EE	0.008	0.008
FF	0.041	0.008
GG	--	0.025

Colonization and Distribution under Thinning Effects

Thinning treatments seemed to negatively impact the presence of *Onnia tomentosa* as fewer basidiomata were collected from plots that were thinned (Fig. 2.12 and Fig. 2.13). The average basidioma count for the control was 24.33 compared to the light and heavy thinned treatments which had 2.33 and 3.67 respectfully in the Airstrip plantation (Fig. 2.12) however, a one-way ANOVA was done and confirmed no significant differences among treatments, *p*-value was 0.165. Boom Lake plantation had more basidiomata in control (15) but the light thinning and heavy thinning treatments did have basidiomata present as well, 9 and 7 respectively (Fig. 2.13), however, it is important to note that these basidiomata were collected from thinning treatments without replicates.

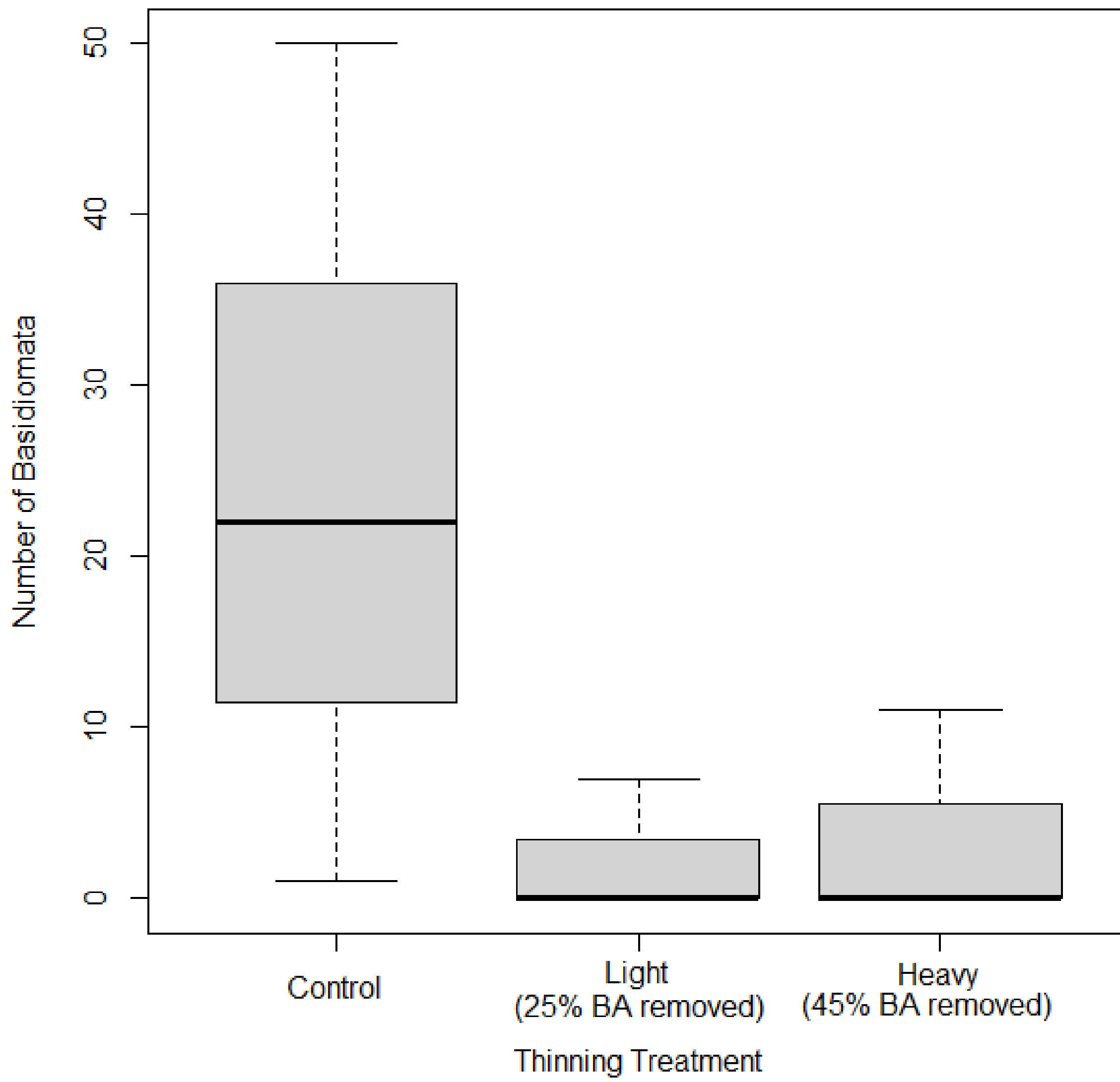

Figure 2.12 Box plot of total basidiomata collected from each treatment within the Airstrip plantation.

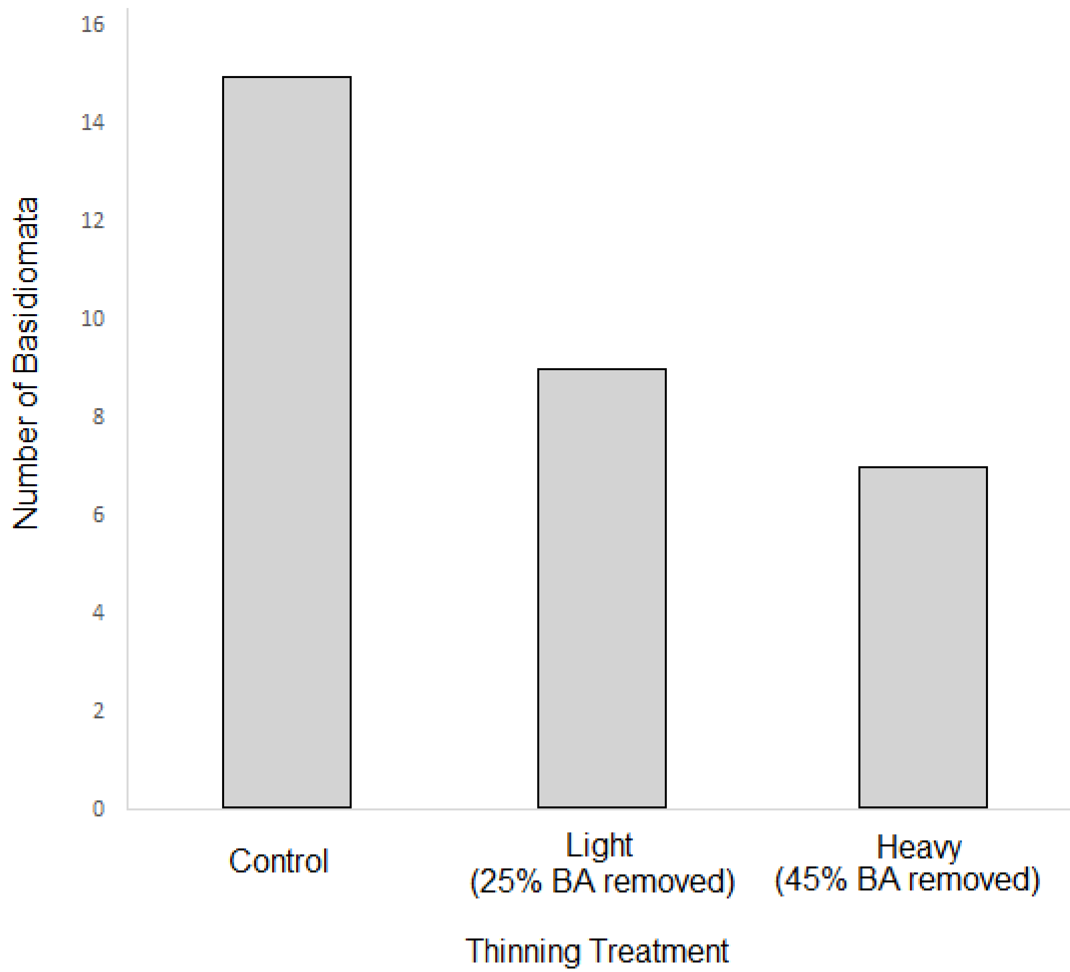

Figure 2.13 Bar graph of total basidiomata collected from each treatment at Boom Lake plantation.

Discussion

The plots underwent thorough examination for basidiomata of *O. tomentosa* and each treatment was surveyed equally. Using both spatial coordinates and discrete MLGs it was determined that thinning operations did have a negative effect on the colonization and spread of *Onnia tomentosa*. However, it is difficult to say definitively if it was the thinning treatments or it some other factor that caused the reduced presence of basidiomata since there was no significant differences among the two thinning treatments and control at the Airstrip plantation (Fig. 2.12). Only one plot representing each thinning treatment was sampled from the Boom Lake plantation and this was done simply because it was known that *O. tomentosa* was present in that specific area and the collections were biased in favour of collecting basidiomata for DNA sampling. It is known that the basidiomata appear episodically (Whitney and Fleming 2005; Germain *et al.* 2009) and that the basidiomata do not appear until after the pathogen is already well established in its host. The episodic appearance makes it difficult to ascertain the full scale of the *O. tomentosa* presence when doing above ground sampling.

The heavy thinned treatment at the Airstrip plantation had a slightly larger basidioma count of *O. tomentosa* compared to the light thinned treatment, but that was largely a function of a single plot (plot 22, Fig. 2.7) which was a heavy thinned treatment adjacent to a control plot (plot 24) that had a very large number of basidiomata. Perhaps the two adjacent areas are over an epicentre that was in the process of colonization by *O. tomentosa*, resulting in a cluster that contributes greatly to the heavy plot basidiomata count. Also, Lewis *et al.* (2004) and previous work by Bernier and Lewis (1999) suggested that disease incidence is not related to host density when studying *O. tomentosa*. Although fewer basidiomata were collected within plots that underwent thinning treatments versus the control, whether or not thinning treatment actually

affects the spread of *O. tomenotsa* will have to be tested in the future when the fungus is allowed to spread via root contact. The high variability between the control plots can also attest to the work of Lewis *et al.* (2004).

It is apparent, based on the genetic analysis of the basidiomata that the pathogen is in the early stages of colonizing the plantation. This is from the fact that out of the 122 basidiomata analyzed, there were 116 genets, 110 of which were represented by a single basidioma. Also, based on the spatial patterns observed in Fig. 2.4 – Fig. 2.10, the treatment plots were inoculated by basidiospores and that these observations were made at the start of the fungus colonizing the area. It is anticipated that future collections of basidiomata followed by analysis will probably show the growth of the genets as they vegetatively spread and compete with one another. Some genets may be overtaken, but the genets that do thrive will prosper and become larger. This decrease of population diversity and increase of genet size as stands age has been documented with ectomycorrhiza-forming fungi such as *Suillus bovinus* (L.) Roussel (Dahlberg and Stenlid 1990).

Genets, once established will spread radially outwards from the point of colonization, with the age of the genet being calculated through the use of growth rates from published estimates. The growth rate of *O. tomentosa* is subjected to factors such as age of the stand, soil temperature, weather, etc. Due to the fact the study occurred in northwestern Ontario where the average annual temperature is 2.7ºC in the Limestone Lake area, it is justifiable to use the lower growth rate presented by Hunt and Peet (1997) of 12 cm year^{-1}. The two plantations were planted in 1960 (Boom Lake) and 1962 (Airstrip), so genets would have to be 12 metres in diameter to be as old as the plantation itself. In fact, if we look at genet size represented by two or more basidiomata the average diameter size was 55 cm. Most genets were represented by a single

basidioma and thus were significantly smaller. However, two larger sized genets did occur and they were represented by #22 and #27 and #84 and #87 that were 36 m and 19.8 m in diameter, respectively (Table 2.3). These two rather large genets could however have had their sizes skewed because the distance measured between the two basidiomata for each was based on their reference points on the spatial charts (Fig. 2.4 and Fig. 2.6, respectively). There can be measurement error from the GPS unit (+/- 5 m) as well as human error. Ideally, to obtain an accurate diameter measurement of each genet one would have to physically measure the distance between the genetically identical basidiomata. However, since basidiomata were removed for genetic analysis this was not possible. In retrospect, numbered markers could have been placed in each location of a basidioma.

Despite the two larger genets found, the other genets were substantially smaller, with the third largest genet being 3.0 m and the next largest being only 0.48 m (Table 2.3). Germain *et al.* (2009) found the average genet size of *O. tomentosa* in their white spruce plantation in Quebec to be 3.42 m which, when compared to the genets found by this study are larger. They also found a greater abundance of genets that contained more than a single basidioma. However, this is likely due to the white spruce plantation having the pathogen well established for a greater amount of time compared to the Limestone Lake plantations where a variety of harvestings, wildfires, and pesticide treatments occurred between 1937 and 2014. Harvesting took place at Limestone Lake between 1937 to 1940 followed by wildfire which occurred over the next 8 years. The Boom Lake plantation was established in 1960 with seedlings from the Fort William Nursery and the same was done in the Airstrip plantation in 1962. In 1964 Airstrip was also planted with seedlings from Swastika Nursery. Hand cleaning occurred at the Boom Lake plantation in 1966 then herbicide treatment was applied to the Airstrip plantation in 1969. Hand

cleaning occurred in both the Boom Lake and Airstrip plantations from 1971 – 1980. Both plantations were then treated to eradicate weevils (*Hylobius* spp.). Whitney (1961, 1962) ruled out weevil feeding sites as entry points for *O. tomentosa* but did note that the weevil can predispose the tree itself to infection by the fungus. Then in 1990 and 1991 the plantations underwent treatment to kill budworm (*Choristoneura* spp.). The two plantations underwent thinning treatments in 2008. Germain *et al*. (2009) also hypothesized that *O. tomentosa* was not necessarily abundant on living trees as it was likely present in the stumps of white spruce trees that occupied the stand before clear cuts occurred in their study site in Quebec.

It has been documented that *O. tomentosa* can survive in infected stumps for up to 30 years (Lewis and Hansen 1991a; Tkacz and Baker 1991). Germain *et al.* (2009) also stated that the stand they studied was previously a mixwood stand, containing both deciduous and coniferous trees resulting in a patchy appearance of *O. tomentosa*. It was not noted whether *O. tomentosa* was present within the Limestone Lake plantation before it was replanted to black spruce in the early 1960s but if it is likely that there were patches of it residing in the soil or infected material (stump or roots) it is only now beginning to colonize the maturing black spruce host.

Onnia tomentosa genet size observed here was substantially smaller compared with studies investigating other root rotting pathogens such as species within the *Armillaria mellea* (Vahl) P. Kumm. complex, *Heterobasidion annosum* (Fr.) Bref. as well as to some tree-associated basidiomycetes that form ectomycorrhizas (Germain *et al.* 2009). Genets for *Armillaria* species were found ranging from 1 hectare to 37 hectares (Coetzee *et al.* 2001; Dettman and van der Kamp 2001a, b; Bendel *et al*. 2006), and in the case of *Armillaria gallica* Marxm. & Romagn a single genet was found to be 635 m across (about 1,500 years old) (Smith

et al. 1992). *Heterobasidion annosum* genets were smaller, around 18 to 21 m in diameter (Vasiliauskas and Stenlid 1998). *Lactarius xanthogalactus* Peck, *Amanita franchetii* (Boud.) Fayod, and *Russula cremoricolor* Earle are ectomycorrhiza-forming fungi which were reported to have genets that covered 9.3, 1.5, and 1.1 m^2, respectively (Redecker *et al.* 2001). It is anticipated, now that the population of *O. tomentosa* is establishing itself that some will grow to form genets in size comparable to those genets found by Germain *et al.* (2009) and the ectomycorrhiza-forming fungi.

There was also prolific diversity of nuclear alleles. This high diversity also suggests that this population is just starting their colonization. As the genets increase in size, the number of genets is likely to be reduced, which, in turn will reduce this nuclear allele diversity. Germain *et al.* (2009) also noted high allelic diversity at the two nuclear loci. In this present study, the two nuclear loci were used to form MLGs to give us an idea of how the plantation was being colonized. However, in future studies of this population, it would be beneficial to sequence the PCR derived segments to confirm the SSCP analysis. Sequencing each PCR derived segment would also clarify whether introns (non-coding sections of the DNA strand) are contributing to the polymorphism differences observed in the PAGE gels or not, as introns can be absent or present which contributes to size variation within the gels.

The use of universal BT and ACT primers in combination with the specific nuclear primers in future studies would allow for the determination of whether or not the population is at a Hardy-Weinberg equilibrium and is experiencing hetero- and homozygous deficiency/excess. An example of another nuclear locus that could have been examined would be the RNA polymerase II largest subunit (specific) (Germain *et al.* 2009). It would also be useful in future studies to compare back to the present day population and determine what genets increased in

size and which ones, if any, were out competed. It will be important to revaluate the study site again to see if the thinning treatments did indeed have an effect on the spread of *O. tomentosa* now that vegetative spread will likely play a larger role since the original colonization event occurred.

Molecular techniques other than SSCP-PCR that could be applied to this type of study in the future could be simple sequence repeat markers (SSR) or microsatellites to examine genetic populations of *O. tomentosa*. The use of microsatellites is useful when dealing with minute concentrations of DNA but are not ideal when dealing with samples larger then 350 base pairs. Three out of the four locus examined were greater than 490 base pairs (MS, ML, and BT) (Life Technologies 2012).

From this present study, it appears that tree thinning does negatively affect the presence of *O. tomentosa* but since it is still in the early stages of colonization, it is difficult to predict what the minimum amount of thinning is required to have an effect. Since the population of *O. tomentosa* is in the early stages of colonization, and since the basidiomata appear episodically it would be important to re-examine the plantation in the future to measure how population size and diversity is progressing. A more balanced sampling design over the two plantations with contrasting soil types will be critical to examine if soil is influencing disease incidence.

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CHAPTER 3:

THE OCCURRENCE OF GENETS OF *PHELLINUS TREMULAE* IN LIVING STEMS OF TREMBLING ASPEN (*POPULUS TREMULOIDES*) FROM A STAND IN NORTHWESTERN ONTARIO

Abstract

Phellinus tremulae (Bond.) Bond. & Borisov is a pathogen on trembling aspen (*Populus tremuloides* Michx.) that causes a severe heart rot which can extend out into the sapwood. Four infected trembling aspen were harvested in May 2015 from Lakehead University`s Silver Mountain property. Each tree stem was cut into 50 cm bolts with the top 5 cm from each cut into cookies. From each cookie, isolations of *P. tremulae* were initially made onto 2% malt extract agar while pairing cultures to demonstrate somatic incompatibility were carried out on carrot agar. One tree failed to yield *P. tremulae* while another tree contained a single genet. The remaining two trees possessed two genets each. This study confirms the work conducted by Holmer *et al.* (1994) that more than one genet of *P. tremulae* can occupy a single host.

Introduction

Phellinus tremulae (Bond.) Bond. & Borisov, a serious heart rot pathogen, can reduce the economic value of aspen for lumber (Niemelä 1974). The geographic range of *P. tremulae* is widespread across northern Europe, Siberia and North America (Breitenbach and Kranzlin 1986). *Phellinus tremulae* infects European aspen (*Populus tremula* L.), trembling aspen (*Populus tremuloides* Michx.), and large tooth aspen (*Populus grandidentata* Michx.) (Basham 1958; Thomas *et al.* 1960; Hiratsuka and Loman 1984), although in North America it occurs mainly on trembling aspen (Allen *et al.* 1996). Trembling aspen is one of the most widely distributed hardwood trees in North America and comprises a sustainable portion of wood volume in the boreal forest (Peterson and Peterson 1992; Mallett and Myrholm 1995).

Taxonomically, *P. tremulae* was initially considered as part of the *Phellinus ignarius* (L.: Fr.) Quél complex however, it was concluded that *P. tremulae* is host specific exclusively to species of aspen (Niemelä 1974, 1975; Allen *et al*. 1996). The basidiomata are perennial, woody conks that are triangular in shape (Hiratsuka and Loman 1984; Allen *et al.* 1996). The upper surface of the fruiting bodies are zoned with grey-black to black that become roughed with maturity, while the underside is brown and poroid (Allen *et al.* 1996) (Fig. 3.1).

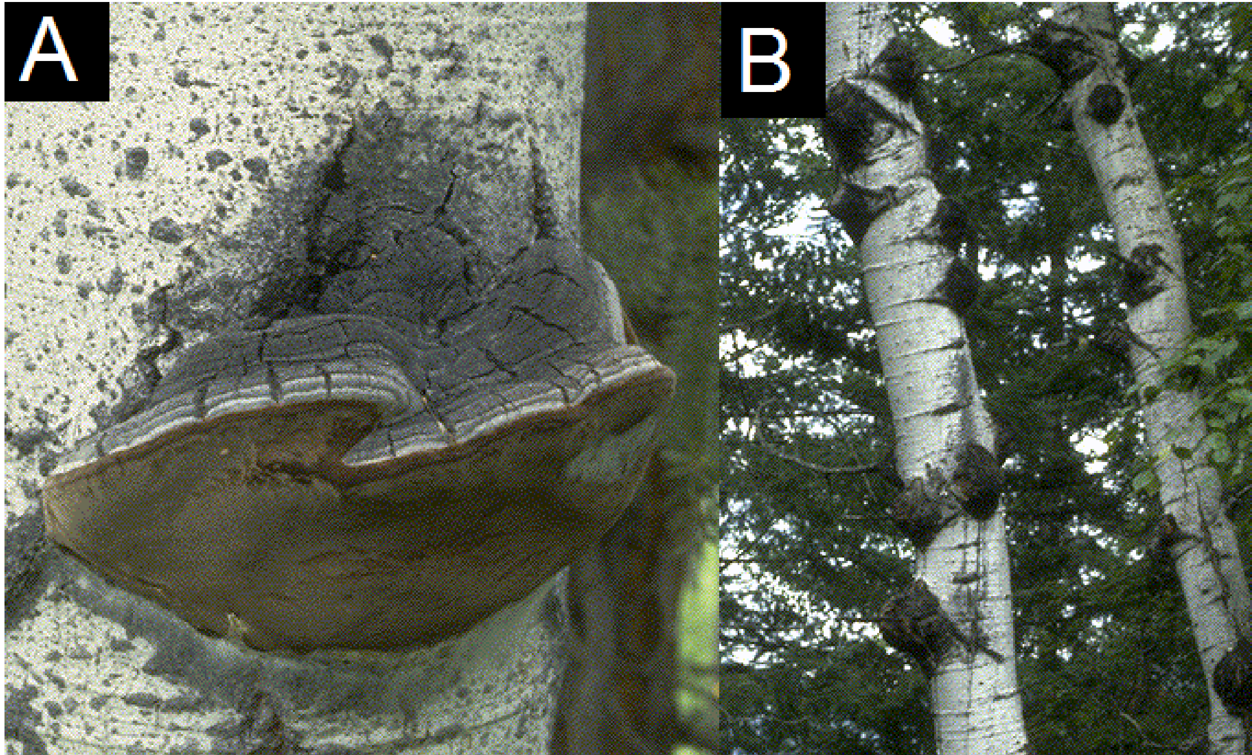

Figure 3.1 Basidioma of *P. tremulae* (A) and "sterile" conks on trembling aspen (B) (Allen *et al*. 1996).

The infection cycle of *P. tremulae* is not entirely clear, although it is theorized that the fungus enters into the heartwood of the host by using dead or broken branches as channels where basidiospores germinate (Brown and Merrill 1971; Wikstrom and Unestam 1976). However, Etheridge (1961) felt that the rot caused by *P. tremulae* were merely lateral extensions from the heartwood and not the other way around. Black, sterile mycelial masses commonly referred to as sterile conks, blind conks, or punk knots form at the branch scars alongside the basidiomata (Allen *et al.* 1996). Fire scars and insect injuries are other entry points through which *P. tremulae* basidiospores could inoculate a new host (Schmitz and Jackson 1927). The actual symptoms of an infected trembling aspen tree appear as a yellow-white zone within the heartwood that is surrounded by a yellow-green to brown margin. As the disease progresses, a soft yellow-white wood develops with fine black lines running throughout the area (Hiratsuka and Loman 1984; Allen *et al.* 1996).

Like most tree pathogenic Basidiomycota, there are no external symptoms (such as basidiomata) until well after the pathogen becomes established, therefore, estimating volume loss due to *P. tremulae* can be difficult. *Phellinus tremulae* can cause severe decay in an aspen tree and a single fruiting body can indicate as much as 82% gross volume loss (Allen *et al.* 1996). It is also noted that decayed wood of tremblng aspen infected with *P. tremulae* has a distinct wintergreen odour when cut (Allen *et al.* 1996).

Sexual and somatic (vegetative) compatibility studies have been conducted on various species of basidiomycetous fungi (Peterson 1995; Worrall 1997). Mating or sexual compatibility studies are conducted by examining monokaryotic hyphae and the interactions between other monokaryotic hyphae (Peterson 1995). Sexual incompatibility promotes outbreeding by allowing the monokaryons to have self-recognition and prevent inbreeding (Peterson 1995; Hiscock and Kües 1999). Whereas somatic incompatibility is determined by secondary mycelium (vegetative, dikaryotic mycelium) interacting with other dikaryotic mycelium and preventing anastomoses to occur. This is useful for the fungi as it prevents unwanted or unfit nuclei into the thallus, prevents transmission of mycoviruses, and helps maintain genetic heterogeneity in populations (Worrall 1997).

Somatic incompatibility studies have been used to examine populations of root rotting fungi, ectomycorrhiza-forming fungi, and other decay fungi (Childs 1963; Barrett and Uscuplic 1971; Adams 1974; Shaw and Roth 1976; Korhonen 1978; Kile 1983; Stenlid 1985; Dahlberg and Stenlid 1990; Stenlid and Holmer 1991; Germain *et al.* 2009). Pairing two dikaryotic cultures against each other on the same plate of agar is a classical method to determine genets among a population of a given species.

When *P. tremulae* is cultured on malt extract agar, two morphologies of mycelium form, referred to as a bleaching type (B-type) and the other referred to as a staining type (S-type) (Hopp 1936; Verrall 1937; Niemelä 1974; Holmer *et al*. 1994). The difference between the two types of mycelium is that the B-type produces aerial mycelium that grows faster compared to the S-type which does not produce aerial mycelium and grows out as a flat brown-red culture that secretes dark pigments into the agar (Holmer *et al.* 1994). When the bleaching type is plated on the same agar medium as another, but genetically distinct, culture of *P. tremulae* it will form brown-black pigmentation at the interaction zone where the two colonies come into contact (Rayner and Boddy 1988; Hiorth 1965; Holmer *et al.* 1994). Using this phenomenon, it is possible to distinguish genets of *P. tremulae* within a population.

Is there only one genet of *P. tremulae* in a single aspen or is there more than one genet per host? The purpose of this study was to examine the population structure of *P. tremulae* in trembling aspen by using the classical somatic incompatibility technique. This will allow for a comparison with a study conducted in Sweden by Holmer *et al.* (1994) on *P. tremulae* genets in European aspen (*P. tremula*). It is hypothesized that if the trees are infected with *P. tremulae* then more then one genet will be within the host.

Materials and Methods

Harvest

Four trembling aspen trees were harvested in May 2015 from Lakehead University's Silver Mountain property, which covers approximately 287 hectares (Fig. 3.2). The property is located off highway 593, about 3 kilometers south from Silver Mountain station and it is 74 km southwest from Thunder Bay, Ontario (latitude 48º 14' 44" North and longitude 89º 52' 36" West). The annual precipitation for this area is 469 mm and the average temperature for the month of May (when the trees were harvested is 8ºC). Trees were selected that had numerous basidiomata present on the stems (Fig. 3.3) and were within one hectare of each other. The trees were cut as close to ground level as possible which resulted in a stump, the height of the stump varied because of the severity of the rot which was deemed unsafe to cut through. Each log was then painted with a vertical line to help orientate each cookie cut with each other. Bolts were cut at 50 cm intervals and the top 5 cm of each bolt were cut as cookies (Table 3.1) and marked with a "T" to signify top end orientation. Each cookie was bagged and labelled (e.g. 1-1, 1-2, etc.) to signify tree identity and position along the stem (Appendix I). The cookies were then brought back to the lab and were placed in a freezer at -15°C.

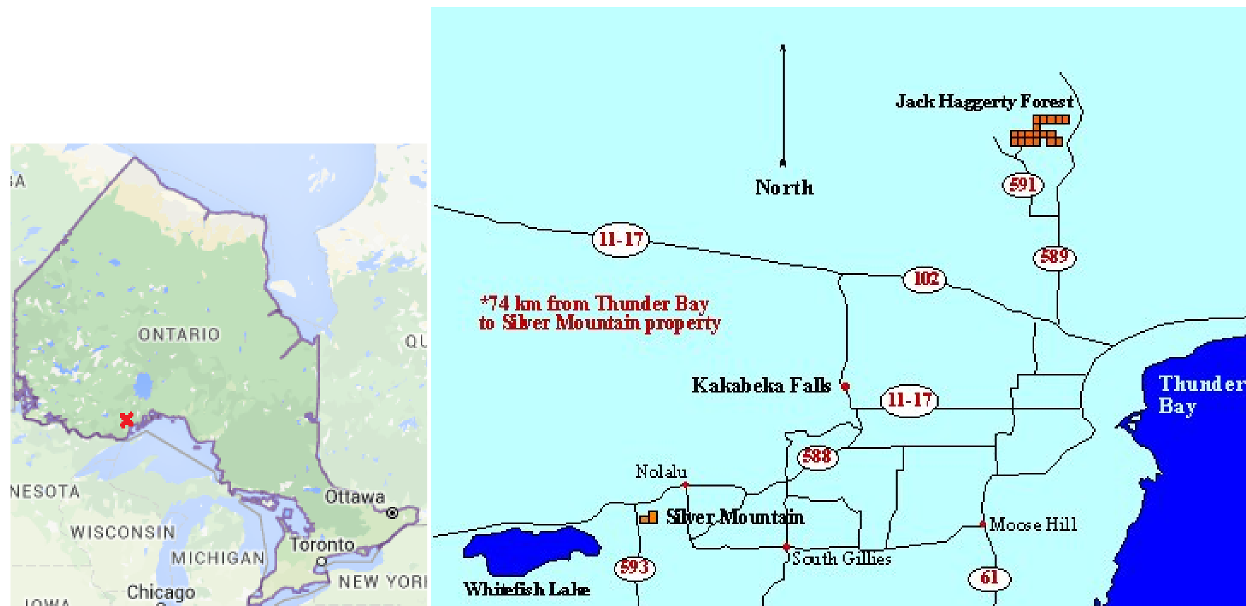

Figure 3.2 Map of Silver Mountain property, located 74 km southwest from Thunder Bay, Ontario. Retrieved from http://flash.lakeheadu.ca/~fluckai/PMsilver.html

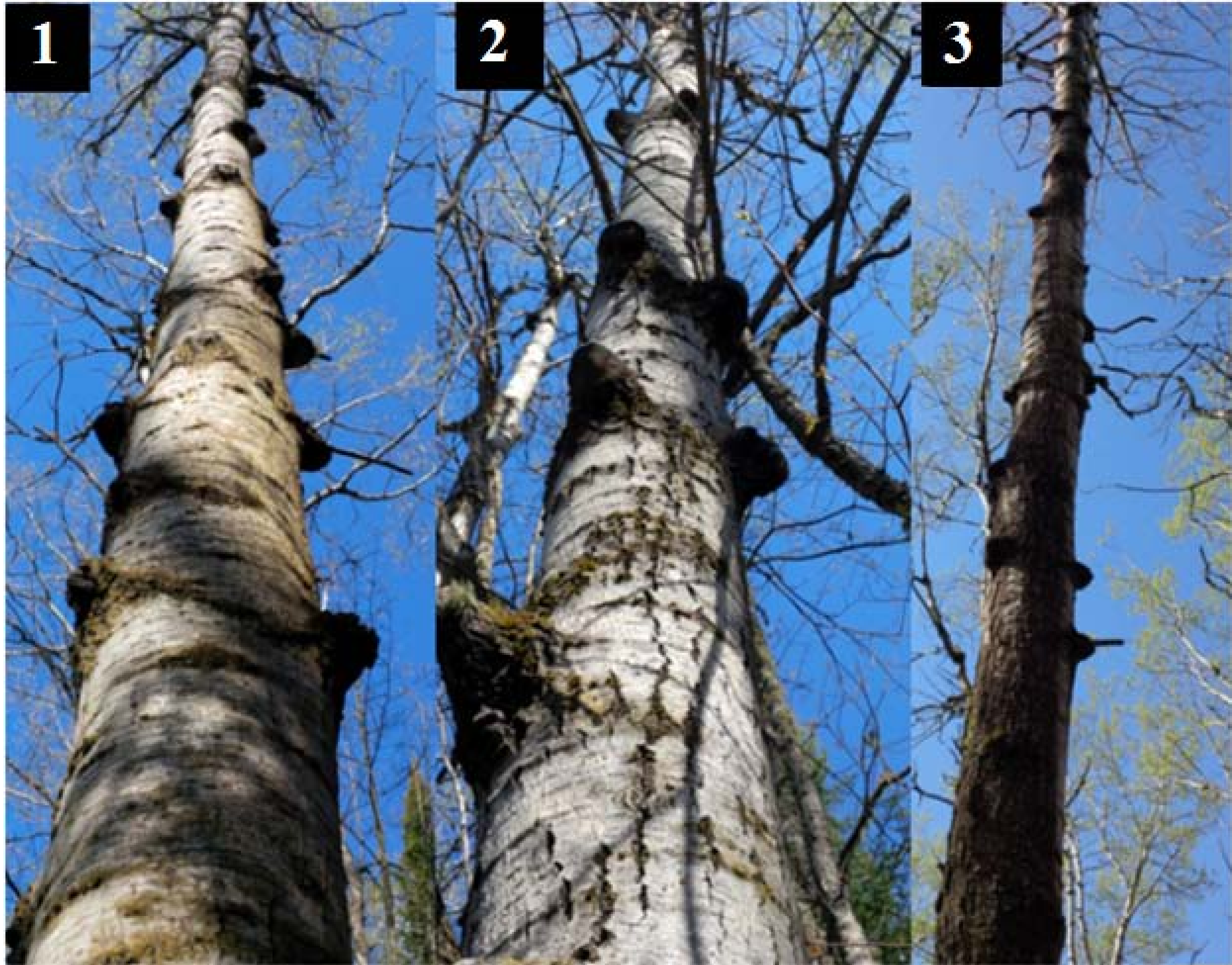

Figure 3.3 Distribution of basidiomata on trees 1, 2, and 3 before harvesting. Tree four had a reduced amount of basidiomata in comparison to the other three trees.

Table 3.1 Number of cookies collected from harvested trembling aspen, diameter at breast height (DBH), age, height of tree up to the base of the crown, and the height from ground level that measurements started.

Tree	Estimated Age (years)	DBH (cm)	Number of Cookies	Actual Height to Base of Crown (m)	Height of Stump (cm)
1	42	31	23	11.97	47
2	41	33	22	11.9	90
3	49	38	28	15.2	120
4	41	33	22	11.6	60

Isolations from Wood

When isolations were ready to be done, the cookies were thawed overnight. A scalpel blade (#21 blade) was sterilized using 95% ethanol and flamed, before it was used to remove a layer of wood that was originally cut with the chainsaw. This removal was done to prevent contamination, and a sample was taken aseptically from the area beneath. The pieces of wood were taken from incipient decaying sapwood because the heartwood was extremely degraded. The pieces were placed onto modified 2% malt extract agar (2% MEA) (20 g malt extract, 15 g agar, 1.0 g yeast extract, 1.0 L distilled water; 300 mg penicillin and 30 mg streptomycin were added after autoclaving). Once *P.tremulae* began to colonize the agar it was transferred onto a clean plate of 2% MEA. If the *P. tremulae* was not removed quickly enough (within a week or two of it starting to colonize the plate) it would often get overwhelmed by other fungi that were co-infecting the wood.

Somatic Incompatibility Studies

Once each isolate of *P. tremulae* had grown out sufficiently, it was paired against every other isolate from the same tree to observe the interactions in order to allow for the differentiation of genets (Fig. 3.4). The isolates were taken using a 7 mm plug and placed 2 cm from the other plug on a Petri dish containing carrot agar (CA) (Mallett and Myrholm 1995). The pairings were then incubated for 5-6 weeks in the dark at 20°C. The reaction zone that formed between isolates of differing genets would be a brownish black line (Fig. 3.4 B).

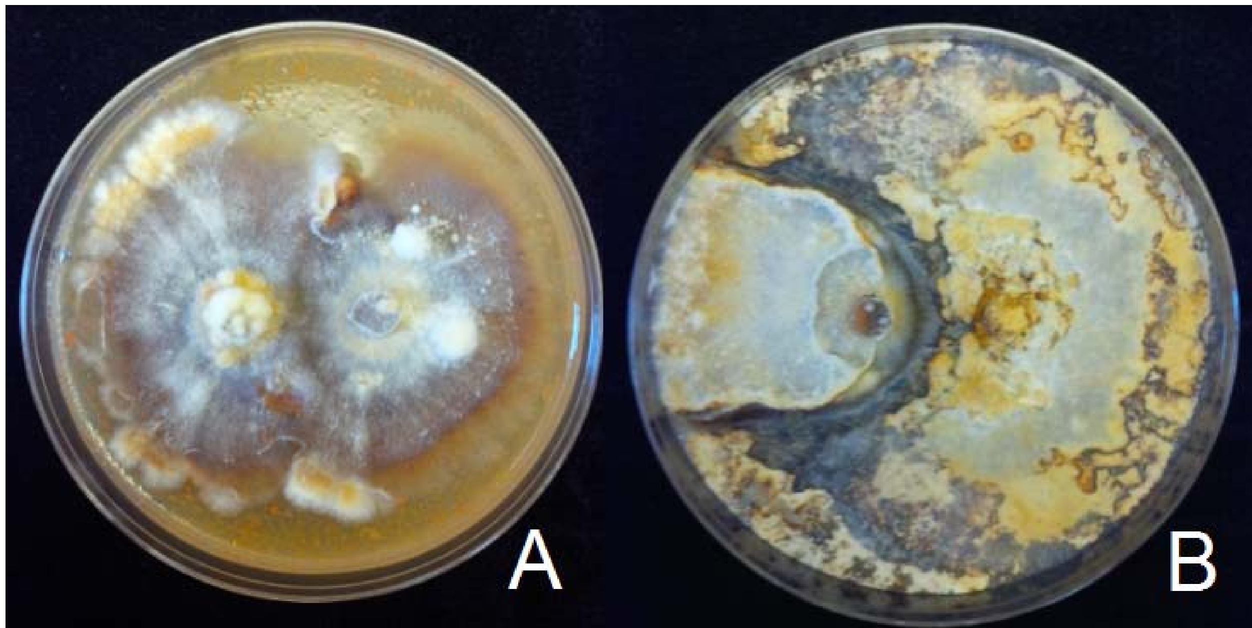

Figure 3.4 Somatic pairing reactions: genetically similar mycelium (A) and genetically different mycelium (B).

Results

Genet Distribution

Thirty three isolates of *Phellinus tremulae* were obtained out of 285 samples taken from tree 1, 16 isolates out of 294 samples from tree 3, and 7 isolates out of 41 samples taken from tree 4. Tree 2 yielded no successful isolations of *P. tremulae* as it was severely decayed by other fungi. Somatic incompatibility studies showed that tree 1 had two genets. The first one occurred from 4.0 to 11.5 m in height above the stump while the second genet intersected at 9.0 m (Fig. 3.5). Similarly, tree 3 had two genets, the first genet occurred from 0.5 to 3.5 m in height above the stump while the second genet was from 4.0 to 10.0 m in height (Fig. 3.6). The final tree, tree 4 had a single genet infecting it and it occurred from 1.5 to 10.5 m in height above the stump (Fig. 3.7). Samples were only taken from cookies of the trunk of the tree, extending from the stump to where the crown began to start. No isolations were made from branches.

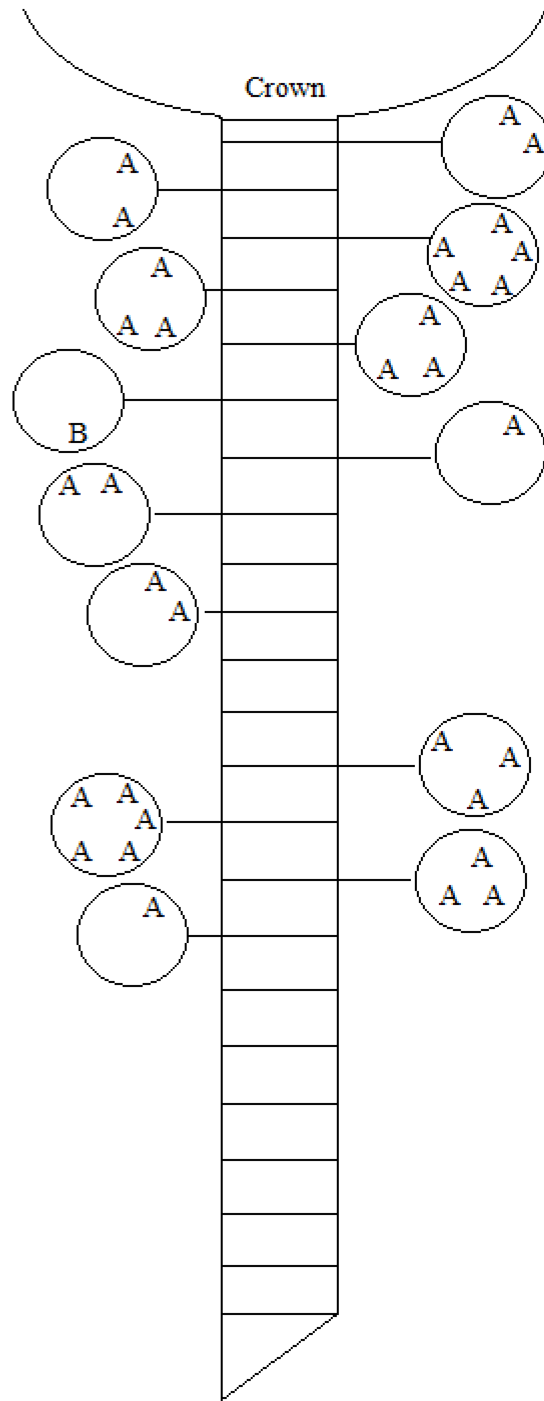

Figure 3.5 Distribution and orientation of *P. tremulae* genets within tree 1. Somatically compatible genets are identified by having the same letter. Each disc is indicated by bars and are 50 cm apart.

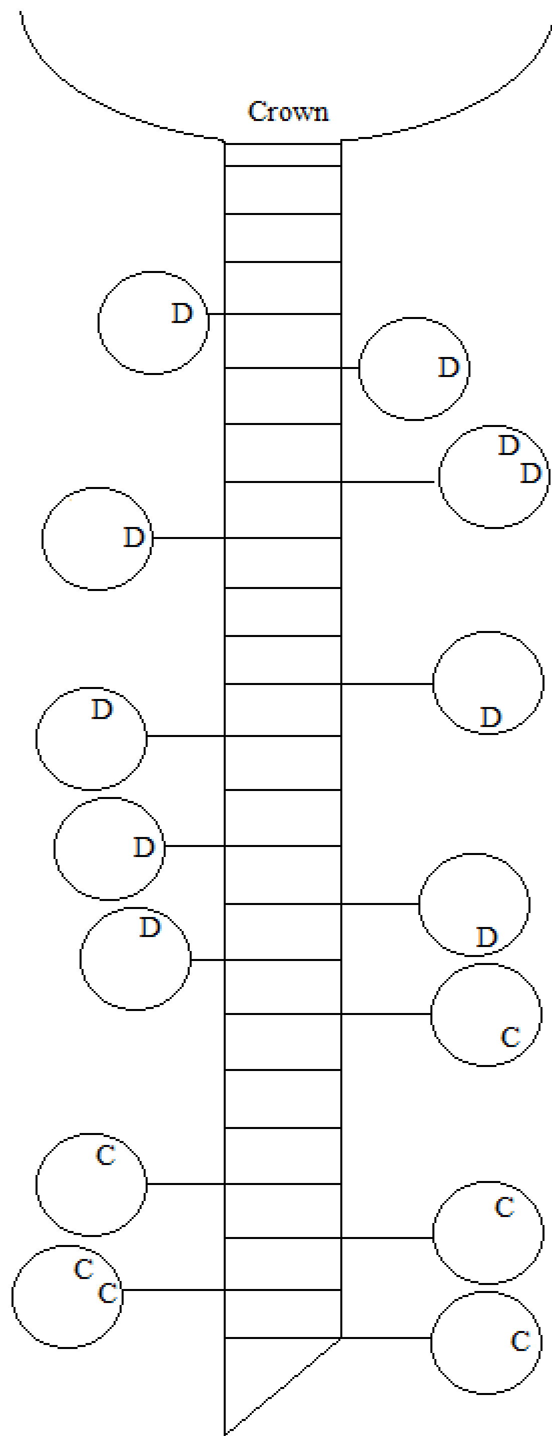

Figure 3.6 Distribution and orientation of *P. tremulae* genets within tree 3. Somatically compatible genets are identified by having the same letter. Each disc is indicated by bars and are 50 cm apart.

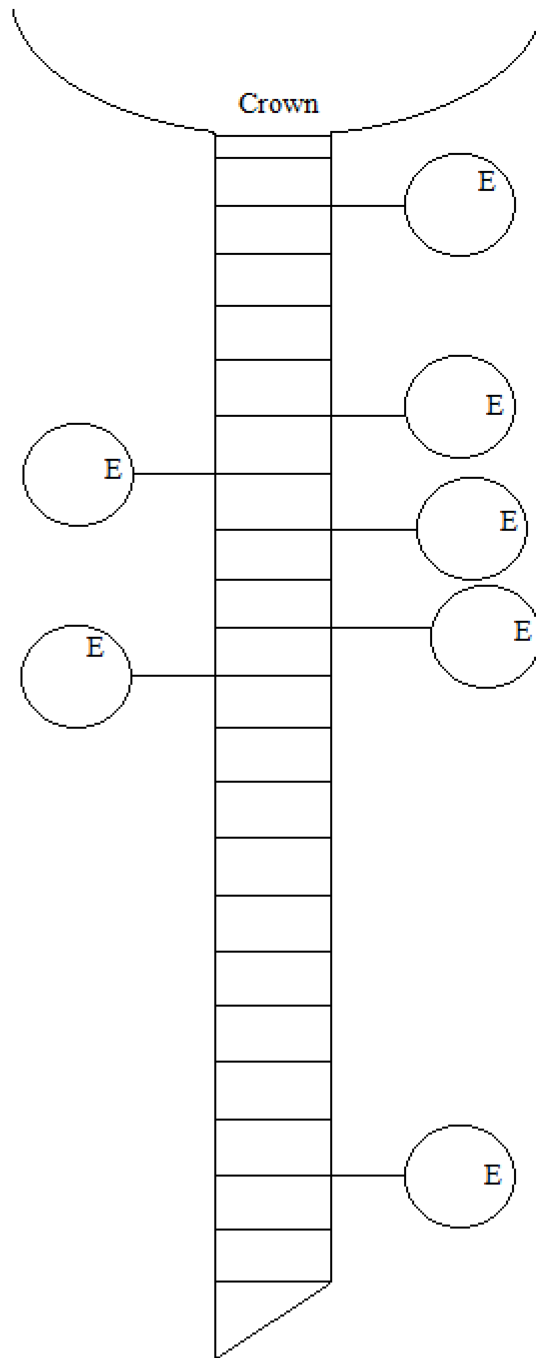

Figure 3.7 Distribution and orientation of *P. tremulae* genets within tree 4. Somatically compatible genets are identified by having the same letter. Each disc is indicated by bars and are 50 cm apart.

Discussion

Studies on the occurrence and distribution of genets of decay fungi in stems of living trees provide an opportunity for better understanding the complexity of fungal infection and colonization of host trees. In this present study, each of the four trembling aspen examined had a different story to tell. Tree 4 only had a single genet infecting it (Fig. 3.7). It was observed that tree 4 had (i) a reduced number of basidiomata compared to trees 1, 2, and, 3 and (ii) was in substantially better (sound wood) condition compared to the previous trees. Wikstrom and Unestam (1976) and Hiorth (1965) found a single column of decay throughout the host with no marked zone lines (which would have appeared if two genetically different strains of *P. tremulae* had met) suggesting only one genet was present in their represented studies.

Tree 2 was considerably more decayed than trees 1 and 3 and *P. tremulae* isolations failed, as other decay fungi and other common moulds were isolated. This could suggest that succession of fungi was occurring based upon a competition strategy theory first proposed by the British plant ecologist J.P. Grime (1977) for plants but quickly adopted by mycologists for fungi (Dix and Webster 1995). The theory refers to fungi that have selective niche strategies when parasitizing/colonizing a host. The first fungi to parasitize a host utilize the stress tolerant strategy (S) where the environments are harsher than dead wood reducing the amount of saprophytes that can compete. These fungi have distinct physiological adaptations such as slow spore germination, growth and reproduction rates. However, these fungi lack combative abilities and cannot compete with other fungi once the host dies. Ruderal strategy (R) fungi are primary colonizers of recently dead trees that have physiological adaptations that make them successful such as rapid spore germination, growth, and fruiting body production. They are ephemeral and cannot compete with C-strategy fungi. Combative strategy (C) fungi are secondary colonizers

that take over territories from primary colonizers by having better enzymatic capabilities compared to R-strategy fungi and can degrade more complex carbon compounds, and are able to last longer than R-strategy fungi (Dix and Webster 1995). *Phellinus tremulae* would be an example of a S-strategy fungus that would outcompeted by C-strategy fungi.

Trees 1 and 3 each had two different genets of *P. tremulae* occupying the stems (Fig. 3.5 and 3.6). Tree 1 had a single genet throughout its entire trunk except where a small second genet intersected the larger genet at 9.0 m. It is likely that the second genet entered the tree later via a broken or dead branch and used the branch as a channel to move to the host's heartwood. Work done by Holmer *et al.* (1994) shows that *P. tremulae* does in fact exist in small twigs and they hypothesized that, through the small branch or twigs *P. tremulae* could infect the heartwood of the host. The infection of the small twigs is extremely advantageous for a fungus that relies solely on spore dispersal for its spread (Holmer*et al.* 1994). The genets in tree 3 are separated and both occupy a large portion of the host stem. It is likely that wounds on the branches or wounds that occurred because of branches that have broken off lead to seperate prime infection points for *P. tremulae* spores. However, there does seem to be a point where once the *P. tremulae* has sufficiently decayed the trembling aspen host, the host starts to decline allowing for other opportunistic fungi to enter and eventual kill of the tree.

Evidently, from this present study and others previously mentioned, it is possible for one to multiple genets of *P. tremulae* to exist in a single host. Wounds on twigs and wounds caused by branches breaking along the side of the trunk are also important for genetically different basidiospores of *P. tremulae* to enter, germinate and start to decay already infected trembling aspen. Holmer *et al.* (1994) has discussed in detail that decay fungi may lie dormant within its ideal host until conditions become favourable for the fungus to attack, as long as the pathogen is

able to penetrate the cambium before it can become established. *Phellinus pini* (Thore: Fr.) Pilát is an example of one such fungus that resides in twig stubs of eastern white pine (*Pinus strobus* L.) until the area which the fungus occupies turns into heartwood allowing the fungus to establish itself (Haddow 1938; Sinclair *et al.* 1987).

For future studies, the population size should be increased and a larger variation of trees that are in various stages of the decay process examined in order to understand how *P.tremulae* enters its host and forms genetically distinct populations. It would also be important to get trembling aspen that vary in age to observe how genets grow as the host ages, and see if older hosts have fewer, or more genets. The sampling of attached branches would also be useful in determining how wounded branches can act as channels for the decay fungi to infect its targeted host. Molecular techniques such as single-strand conformational polymorphism PCR or microsatellites could be used to examine the genetic differences among *P. tremulae* populations but culturing isolations and pairing them on CA remains a time and cost effective way to observe genetic differences.

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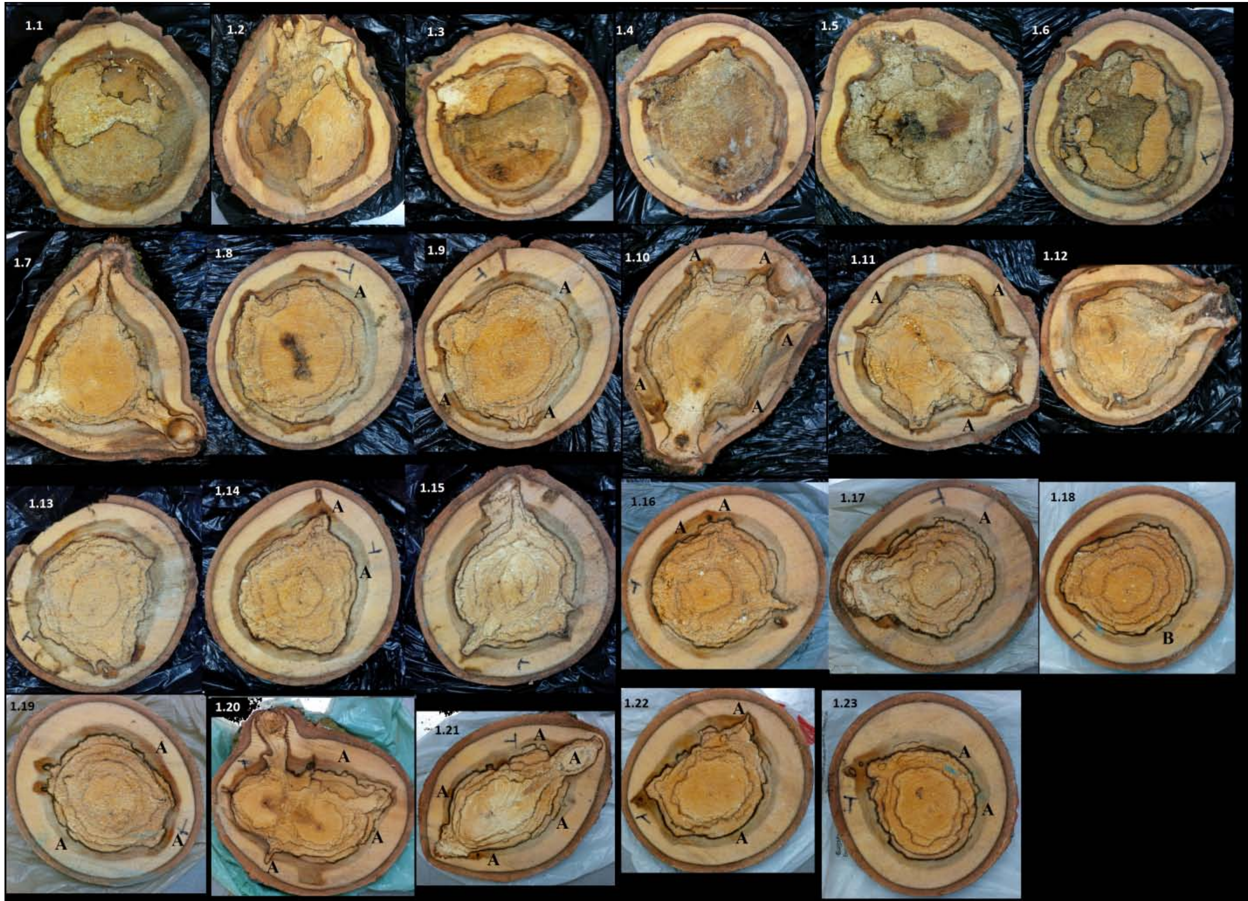
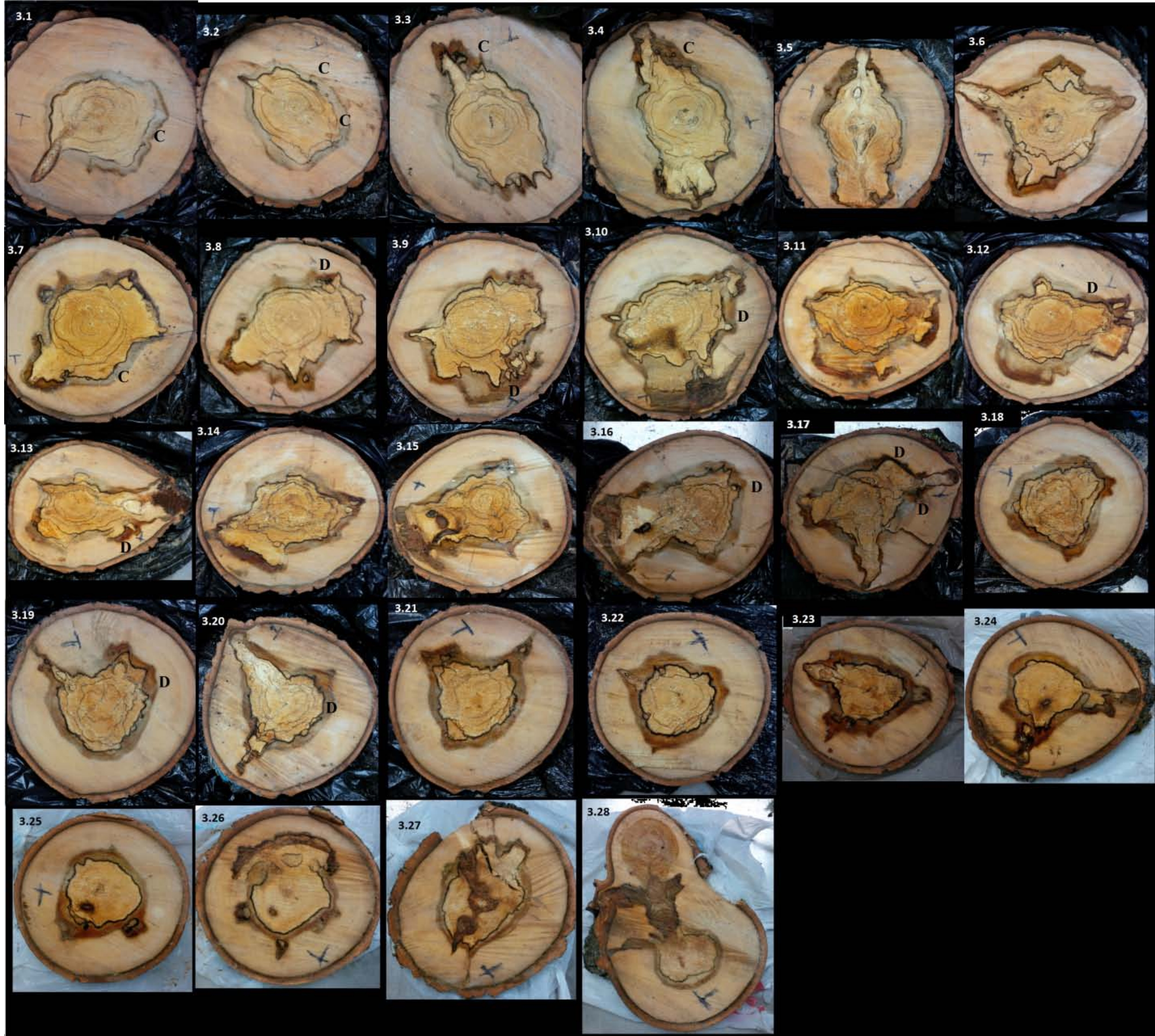
APPENDIX I

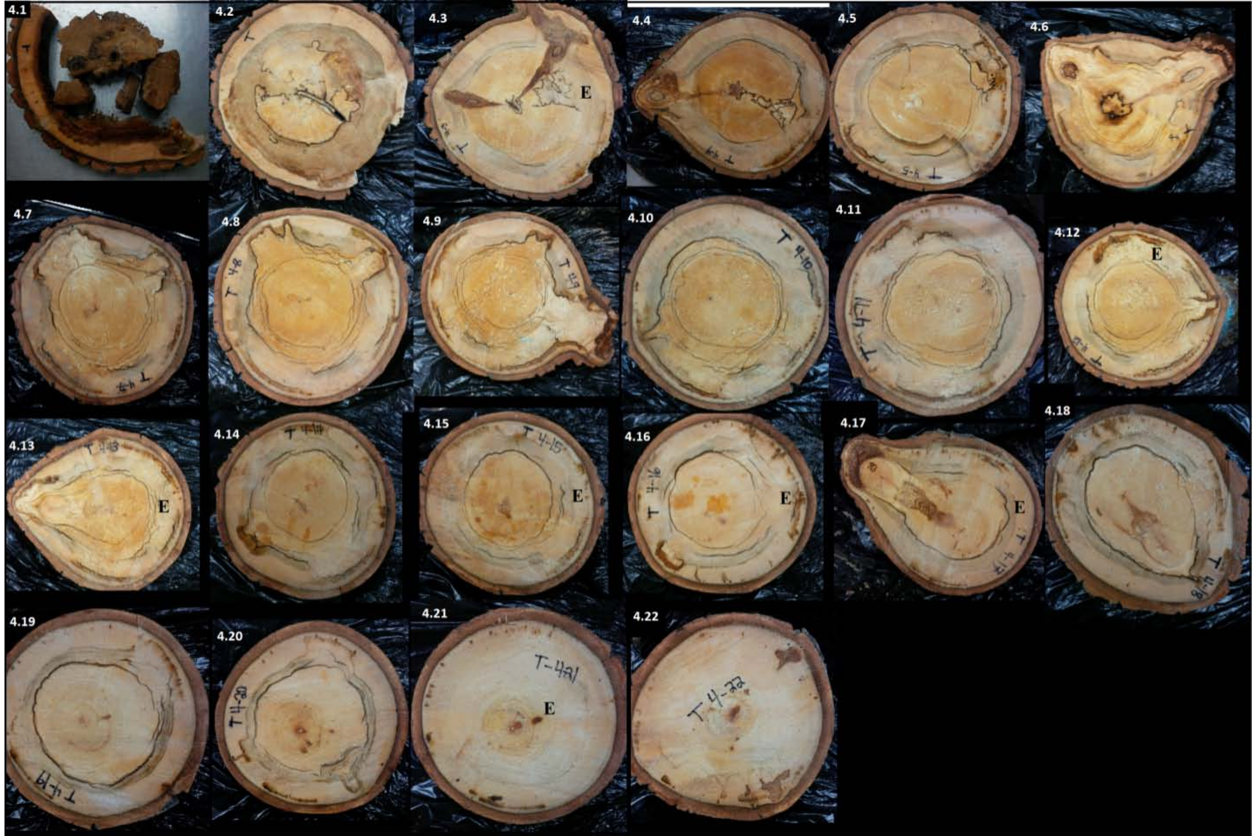
IMAGES OF WOOD COOKIES USED FOR ISOLATION OF PHELLINUS TREMULAE



Tree 1: Cookies 1 to 23. The numbers correspond to the order they were cut from the stem. The lettering on the surface of the wood cookie represent their respected genet from that tree.



Tree 3: Cookies 1 to 28. The numbers correspond to the order they were cut from the stem. The lettering on the surface of the wood cookie represent their respected genet from that tree.



Tree 4: Cookies 1 to 22. The numbers correspond to the order they were cut from the stem. The lettering on the surface of the wood cookie represent their respected genet from that tree.