Variations in jack pine (*Pinus banksiana*) monoterpene composition and subsequent effects on pheromone production by mountain pine beetle (*Dendroctonus ponderosae*)

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Abstract

The secondary compounds of pines (*Pinus)* can strongly affect the physiology, ecology and behaviour of bark beetles (Coleoptera: Curculionidae, Scolytinae) that feed on host sub-cortical tissues. Jack pine (*Pinus banksiana)* has a wide distribution range in North America and thus variations in its secondary compounds, particularly monoterpenes, could affect the host expansion of mountain pine beetle (*Dendroctonus ponderosae*), which has recently attacked jack pine as a novel host and expanded its range into the boreal forest. I analyzed variations in monoterpene composition of jack pine foliage and phloem from natural and provenance stands representing populations from Alberta to the Atlantic coast. Additionally, the effects of variations in phloem monoterpene composition on pheromone production by mountain pine beetle were analyzed.

Throughout its range, jack pine foliage monoterpenes were classified into three chemotypes characterized by high proportions of the monoterpenes α-pinene, β-pinene, or limonene. Expression of these chemotypes was controlled by both genetic and environmental factors and individual monoterpenes were correlated with climatic variables differently. Conversely, phloem monoterpenes were classified into groups characterized by high amounts of the monoterpenes (+)-α-pinene, 3-carene or no notably high individual compound and beetle aggregation and anti-aggregation pheromone production varied with these groups. Furthermore, pheromone production also varied between provinces, with the most aggregation pheromone produced in trees from Manitoba and Quebec. These results indicate that pheromone production by *D. ponderosae* will vary with host chemistry but remain a viable and important aspect of its survival and persistence in the boreal forest.

Preface

This document presents two studies (Chapters 2 and 3) intended for publication and represent collaborative work led by Dr Nadir Erbilgin of the University of Alberta. I was responsible for data collection and analyses, literature review and manuscript composition and writing throughout this document. Dr Erbigin was involved with concept formation and manuscript composition throughout the work. Additionally, Ahmed Najar of the University of Alberta was responsible for developing chemical analyses described in Chapters 2 and 3. For Chapter 2, Drs Jean Bousquet and Julie Godbout of Université Laval carried out field work and provided samples and manuscript edits. All research presented in this thesis was conducted in accordance with all applicable laws and rules set forth by provincial and federal governments and the U of A and all necessary permits were in hand when the research was conducted.

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Chapter 1

Thesis Introduction

1.1 Herbivorous insects and host monoterpenes

Herbivorous insects have adapted to obtain nutrients from all types of plant tissues through innumerable feeding mechanisms, but plant hosts do not merely suffer passively; rather, they wage chemical warfare against herbivorous insects by producing toxic phytochemicals to actively defend themselves, thereby greatly affecting herbivore survival, physiology and behaviour (Keeling and Bohlmann 2006; Howe and Jander 2008; Mithofer and Boland 2012; Raffa et al. 2013). Because of these chemically-mediated plant-insect interactions, an herbivorous insect's adaptations to a plant's secondary chemistry is of the utmost importance to the insect's sustained success (Jermy 1984). Conversely, the host plant must also have sufficiently adapted to chemically defend against attacking herbivores (Jermy 1984; Mithofer and Boland 2012). Under invasive circumstances, however, herbivorous insects may attack host plants which have not developed sufficient chemical adaptations to effectively fend off attacks, as they have not shared an evolutionary history with the insects (Futuyma 2008; Kausrud et al. 2012). While such attacks on these naïve hosts may not always be successful, an insect's host shift can provide expansive novel habitats of poorly adapted hosts that are more easily exploited than the hosts with which the insects have evolved (Mooney and Cleland 2001; Walther et al. 2009). One recent instance of such a host and range shift is exemplified by the mountain pine beetle (*Dendroctonus ponderosae*) (Coleoptera: Curculionidae, Scolytinae).

1.2 Mountain pine beetle and host chemistry

Dendroctonus ponderosae is indigenous to western North America and, within Canada, it has been historically restricted to British Columbia's interior due to physiological constraints, though it has recently invaded higher latitudes and new territories east of the Rocky Mountains (Bentz et al. 2010). Its primary hosts are lodgepole pine (*Pinus contorta)* and ponderosa pine (*Pinus ponderosa*) though it has spread to the novel host jack pine (*Pinus banksiana*) in Alberta (Cullingham et al. 2011). This host shift is of particular concern for boreal forest ecological sustainability, as jack pine is distributed from Alberta to the Atlantic coast in a 4,000 km corridor between western and eastern pine species. Differences in susceptibility between *D. ponderosae*'s historical and novel hosts are unknown, though lodgepole pine has had a long evolutionary history alongside the beetle, potentially allowing for defensive adaptations, including chemical defenses, whereas jack pine is a naïve host (Cullingham et al. 2011) and may not have such adaptations to the beetle. Not only does the role of host secondary chemistry generally affect herbivorous insect host shifts (Jermy 1984), but host monoterpenes play a direct role in *D. ponderosae* biology and ecology, warranting further investigation into this chemically mediated host-insect interactions in novel habitats (Raffa et al. 2005).

Monoterpenes are a class of phytochemicals involved in many types of host-insect interactions (Phillips and Croteau 1999; Raffa et al. 2005, 2013; Moore et al. 2014). They are a central aspect of a host's chemical defenses and are maintained in a tree's defensive resins, which are toxic to many insects including bark beetles (Raffa et al. 1985, 2005; Cates et al. 1987; Phillips and Croteau 1999; Franceschi et al. 2005; Keeling and

Bohlmann 2006). However, bark beetles can also identify and evaluate suitable hosts for colonization based on monoterpene composition, thereby reducing energy and risks associated with host finding (Miller and Borden 2003; Fettig et al. 2004; Keeling and Bohlmann 2006). Additionally, some bark beetles can exploit host monoterpenes and use them as direct precursors to their pheromone components demonstrated by their biosynthesis of verbenol, verbenone, and verbenene from the host monoterpene, α-pinene (reviewed by Blomquist et al. 2010). This synthesis is especially important to *D. ponderosae* as *trans*-verbenol and verbenone are major components of its aggregation and anti-aggregation pheromones, respectively (Blomquist et al. 2010).

Female *D. ponderosae* initiate attacks on host trees and convert the host monoterpene α-pinene to *trans*-verbenol, which is critical for the attraction and aggregation of male and female beetles and subsequent successful host colonization and reproduction (Pitman et al. 1968; Safranyik et al. 2010). Male *D. ponderosae* produce a second aggregation pheromone, *exo*-brevicomin, *de novo* which, along with specific host monoterpenes, further attracts conspecifics to overwhelm tree defenses (Pureswaran et al. 2000; Borden et al. 2008). To avoid intraspecific competition, *D. ponderosae* terminates its aggregation when an optimal density (60-90 attacks m^{-2}) is reached by producing the anti-aggregation pheromones frontalin and verbenone (Raffa and Berryman 1983a; Berryman et al. 1985; Pureswaran and Borden 2003). Verbenone is produced by both sexes through an auto-oxidation of the host monoterpene α-pinene (Pureswaran and Borden 2003).

In addition to the link between host monoterpenes and *D. ponderosae* pheromone production, the beetle is affected by monoterpenes in numerous ways. For example, many monoterpenes, such as myrcene and terpinolene, can act as synergists with *D. ponderosae*

pheromones to increase beetle attraction over pheromones alone (Pitman 1971; Billings et al. 1976; Miller and Borden 2000; Borden et al. 2008). Conversely, monoterpenes can also be harmful to *D. ponderosae,* and if persisting at high concentrations, many monoterpenes, including α-pinene, terpinolene, myrcene are toxic to the beetle (Reid and Purcell 2011). Similarly, 3-carene and limonene have been found to be ovicidal and more toxic to the beetle than other monoterpenes (Raffa and Berryman 1983a; Reid and Purcell 2011). Finally, host monoterpenes can impede growth of *D. ponderosae* obligate associated fungi, which provide nutrients to the beetle and help it overwhelm host defenses (Raffa and Berryman 1983b; Bleiker and Six 2007).

1.3 Thesis aims

Due to *D. ponderosae*'s recent host shift into jack pine forests and the importance of monoterpenes to its biology and ecology, this thesis investigates variations in monoterpene composition of jack pine across Canada and how such variations affect pheromone production by *D. ponderosae.* The second chapter will focus on whether jack pine exhibits different chemotypes based on monoterpene proportions throughout the boreal forest. Additionally, it will assess how jack pine's monoterpene composition varies with climatic factors and relate these results to chemotype frequency. Finally, the chapter will explore enantiomeric ratios of major chiral monoterpenes in jack pine and, where variation exists, establish phenotypes based on enantiomeric composition. Because monoterpene expression is affected by both genetics and environment (Baradat and Yazdani 1988; Ott et al. 2011), I hypothesize that jack pine monoterpene composition will vary considerably throughout the boreal forest as it is genetically diverse (Godbout et al. 2005, 2010) with an expansive range. Different ratios of dominant

monoterpenes in individual trees will lead to chemotype classification and certain monoterpenes will be under strong genetic control, while others will be more closely correlated to environmental variables (Baradat and Yazdani 1988; Ott et al. 2011).

In the third chapter, I will focus on how monoterpene composition of jack pine affects pheromone production by *D. ponderosae*. I will rear beetles in jack pine trees sampled from five provinces (Manitoba, Ontario, Quebec, New Brunswick and Nova Scotia) east of *D. ponderosae*'s current range limit (Alberta). I will link pheromone production to monoterpene composition of host phloem to determine whether differences in jack pine chemistry affect pheromone production by *D. ponderosae*. I hypothesize that there will be considerable variations in monoterpene composition of jack pine phloem across the sampled range and that such variations will in turn affect pheromone production by *D. ponderosae*. Because α-pinene is a direct precursor to the beetle pheromones *trans*-verbenol and verbenone, I expect α-pinene content will be positively correlated to these two pheromones (Erbilgin et al. 2014).

Chapter 2

Survey of foliar monoterpenes across the range of jack pine reveal three widespread chemotypes: implications to host expansion of invasive mountain pine beetle

2.1 Introduction

In pine trees (genus *Pinus*), monoterpenes are a prominent class of phytochemicals that play a significant role in tree-insect interactions (Phillips and Croteau, 1999; Franceschi et al*.,* 2005; Raffa et al., 2005, 2013; Moore et al., 2014). Generally, monoterpenes are a central aspect of pines' constitutive and inducible defences and are an essential component of pine defensive resins that are toxic to many herbivorous insects, including subcortically-feeding bark beetles (Coleoptera: Curculionidae, Scolytinae) (Raffa et al*.,* 1985, 2005; Cates et al., 1987; Phillips and Croteau, 1999; Keeling and Bohlmann, 2006). The influence of pine monoterpenes on bark beetles is critical as about 500 species of these sub-cortical herbivorous insects feed on pine trees, including many tree species of significant ecological and economic importance (Wood, 1982a; Bentz et al., 2010; Safranyik et al*.,* 2010; Raffa et al., 2013). Moreover, various aspects of beetle biology, such as dispersal, host selection and colonization, physiology and behaviour are strongly influenced by host monoterpenes (e.g. Raffa et al., 2005, 2013; Seybold et al., 2006).

There are numerous means by which monoterpene composition of host trees mediates and influences bark beetle-host interactions. However, the relationship between monoterpenes and bark beetles is not simple or one sided, as, despite their toxic properties, many monoterpenes can attract or otherwise benefit bark beetles (Ch*é*nier and Philog*è*ne, 1989; Erbilgin and Raffa, 2000a, b; Seybold et al*.,* 2006; Blomquist et al*.,* 2010). For example, the most abundant monoterpenes of pines, such as α-pinene, β-pinene, myrcene, limonene, camphene, and 3-carene can all act as attractants for pine engraver beetles (*Ips grandicollis*) (Werner, 1972; Ch*é*nier and

Philogène, 1989; Erbilgin and Raffa, 2000a, b). Furthermore, host plant volatiles can attract bark beetle predators or mediate predator attraction to bark beetle pheromones (e.g. Erbilgin and Raffa, 2001).

In addition to directly attracting beetles, certain host monoterpenes can act as precursors to pheromone components of some bark beetle species, best exemplified by biosynthesis of the pheromone constituents verbenol, verbenone, and verbenene from host derived α-pinene (reviewed by Blomquist et al., 2010). This specific metabolic process is of particular importance to the mountain pine beetle (*Dendroctonus ponderosae*) as this pathway is the production means for the primary aggregation pheromone of the female beetles (Blomquist et al., 2010). Upon initial infestation, female *D. ponderosae* hydroxylate the host monoterpene α-pinene into their primary aggregation pheromone, *trans*-verbenol. This compound is essential for attraction of male and female beetles and successful aggregation and thus successful reproduction (Safranyik et al., 2010). Following their arrival, male *D. ponderosae* produce another aggregation pheromone, *exo*-brevicomin, which is synthesized *de novo* by epoxidation and cyclization of its precursor long-chain fatty acids and acts synergistically with *trans*-verbenol to attract enough conspecifics to overwhelm tree defences (Pureswaran et al., 2000). When the beetles approach their optimum colonization density on host trees, males produce the anti-aggregation pheromone frontalin from monoterpenoid or long-chain fatty acid precursors to maintain the most favorable beetle density. Finally, the anti-aggregation pheromone verbenone is thought to be produced through an auto-oxidation of the host monoterpene α-pinene by both sexes. This close relationship between host secondary chemistry and *D. ponderosae* is of particular interest because host secondary chemistry can affect herbivorous insects' host shifts (Jermy, 1984), which *D. ponderosae* has recently experienced (Erbilgin et al., 2014).

First confirmed in 2011, *D. ponderosae* has expanded its range from lodgepole pine (*Pinus contorta*)-dominated forests into jack pine (*Pinus banksiana*)-dominated stands in the boreal forest in western Canada (Alberta), indicating a range and host shift (Cullingham et al., 2011). This is an ecological and economic concern, as jack pine is distributed through the boreal forest from Alberta to the Atlantic coast, representing a 4,000 km corridor between western and eastern pine species. There are numerous hypotheses addressing how various factors can affect an herbivorous insect's host shift, but the importance of the host's secondary chemistry to the insect is significant (Jermy, 1984; Feeny, 1991; Becerra, 1997; Murphy and Feeny, 2006; Erbilgin et al., 2014).

The complex relationship between host chemistry and *D. ponderosae* affects beetle physiology and ecology and illustrates the importance of understanding the phytochemical landscape of the beetles' novel host, jack pine. Considerable variation in other conifer monoterpene profiles has resulted in the classification of multiple intraspecific chemical phenotypes, or chemotypes. For example, lodgepole pine monoterpenes were shown to persist in three common chemotypes defined by different proportions of β-phellandrene and β-pinene as well as five rare chemotypes (Forrest, 1981). Furthermore, induced monoterpenes in lodgepole pine varied between trees sampled in northern and southern British Columbia, indicating chemically disparate populations (Clark et al., 2014). Similarly, monoterpene profiles of ponderosa pine (*P. ponderosae*), another host of *D. ponderosae*, can be classified into three discrete chemotypes based on 3-carene, α-pinene and β-pinene proportions (Latta et al., 2003; Davis and Hofstetter, 2012). Additionally, Scots pine (*P. sylvestris*) monoterpene profiles are categorized into chemotypes based on the presence of 3-carene, with subsequent variation in concentrations of α-pinene, β-pinene, and camphene (Thoss et al., 2007). Such variations in pine

monoterpene chemistry can influence bark beetle activities, including maternal gallery excavation, fecundity, survivorship, fitness, and pheromone production (Boone et al., 2011; Lusebrink et al. 2011; Reid and Purcell, 2011; Davis and Hofstetter, 2012; Erbilgin et al., 2014). Additionally, pine chemotypes affect growth performance of obligate fungal symbionts of bark beetles (Davis and Hofstetter, 2012).

However, causes underlying variation of pine monoterpene chemistry are not entirely understood and effects of genetic and environmental factors on monoterpene composition can be variable. For example, in Scots pine, individual monoterpenes appear to have different levels of broad sense heritability, as 3 carene, myrcene, limonene and terpinolene tend to be primarily genetically controlled, whereas α-pinene and β-pinene depend more on environmental factors (Baradat and Yazdani, 1988). Similarly, in lodgepole pine inoculated with fungus *Grosmannia clavigera* associated with *D. ponderosae*, environment affects the induction of certain monoterpenes while showing no effect on others, thereby suggesting genetic control of certain, but not all, monoterpenes (Ott et al., 2011). The varied influences that genetic and environmental factors have on monoterpene composition and the diverse and extensive means by which host chemistry affects bark beetles illustrate the importance of exploring the phytochemical landscape of jack pine. Furthermore, defining chemotypes which may variably affect the ecology and survival of *D. ponderosae* in jack pine forests could provide foresight into the beetle's continued eastward spread.

Due to importance of monoterpenes in bark beetle biology and ecology (reviewed by Raffa et al. 2005), in our investigations we focused on monoterpene composition of jack pine in both natural and provenance stands. Our study aims to first determine whether jack pine exhibits different chemotypes based on overall monoterpene proportions throughout the boreal forest.

Second, we determine how jack pine's monoterpene composition varies with climatic factors and how such variation relates to chemotype frequency. Finally, we investigate whether enantiomeric ratios of major chiral monoterpenes vary among different populations of jack pine throughout the boreal forest and establish whether such variation can be classified into different phenotypes based on enantiomeric composition.

2.2 Experimental

2.2.1 Sampling

All needle samples had been previously used for genetic diversity analysis at the DNA level in Godbout et al. (2005, 2010). Needles were collected during the active growth period from a total of 601 jack pine trees across the north eastern range of jack pine in Canada and the United States. Of these, 231 trees were from natural stands, representing 25 locations, with 6-10 trees per location. The remaining 369 trees were collected from four provenance trials in Petawawa (Ontario), Ste-Christine-d'Auvergne and Fontbrune (Quebec), and Dubee Settlement (New Brunswick), representing 38 provenance locations with 6-10 trees sampled per provenance. Trees were 28-37 years old. After collection, samples were stored at −25°C until they were packed in dry ice and shipped from Laval University to the University of Alberta, at which time they were stored at −40°C.

2.2.2 Tissue extracts

Needle tissue was ground in liquid nitrogen and 100 mg of the tissue were transferred to a 1.5 ml microcentrifuge tube where samples were extracted twice with 0.5 ml methyl tert-butyl ether solvent with 0.002% tridecane as an internal standard. After adding solvent, samples were

vortexed at 3,000 rpm for 30 s, sonicated for 10 min and centrifuged at 13,000 rpm and 0°C for 15 min. Extracts were transferred into amber GC vials and stored at −40°C until analysis.

2.2.3 GC-MS analysis

Monoterpenes extracted were analyzed using similar methods reported in Erbilgin et al. (2014). Briefly, extracts (1 µl) were analyzed using a GC-MS (7890A/5062C, Agilent Tech, Santa Clara, CA, USA) equipped with a chiral column (HP Innowax-20B column (ID 0.25 mm, length 30 m); Agilent Tech) with helium as the carrier gas flow set to 1.1 ml min^{-1}. Each analysis began at an initial temperature of 75°C for 15 min, followed by an increase in 5°C per min until 230°C was reached. Peaks were identified using the following standards: Borneol, pulegone, α-terpinene, γ-terpinene, α-terpineol, 3-carene, terpinolene, α- and β-thujone, (-)-α-pinene, (+)-α-pinene, (-)-β-pinene, (+)-β-pinene, (-)-limonene, (+)-limonene, (-)-camphene, (+)-camphene, sabinene hydrate, myrcene, p-cymene , *cis*-ocimene (SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (Glidco Inc., Jacksonville, FL, USA). Chemical purity of all these compounds was higher than 99%. Compounds were identified by comparing retention times and mass spectra to those of the standard chemicals. Quantity of chemicals was calculated using response curves generated from analyses of a dilution sequence of known quantities of standards. Calibration with these standards allowed for analysis of quantitative differences on monoterpene concentrations among samples. The amount of monoterpenes extracted per wet weight of needle (µg mg^{-1}) was reported.

2.2.4 Statistical analyses

Given initial analyses that suggested monoterpene composition is at least partially influenced by environmental conditions, all statistical analyses treated samples from natural and provenance stands separately. Direct comparisons using both stand types were not done unless stand type did not affect the variable in question. All statistical analyses on monoterpene concentration and proportion data was performed in R statistical program version 2.15.0 using the ecodist (Goslee and Urban, 2007), mvpart (Therneau et al., 2013), vegan (Oksanen et al., 2013) and pvclust (Suzuki and Shimodaira, 2006) packages. Proportions were determined by dividing the concentration of an individual monoterpene (ug mg^{-1}) by the sum of all monoterpene concentrations.

Hierarchical cluster analyses were performed separately on samples from natural and provenance stands to establish chemotypes based on overall similarities in monoterpene proportions of individual trees. The hierarchical cluster analysis uses a distance matrix to cluster samples in a hierarchical structure based on overall similarity, moving from broad to specific similarities. The Bray-Curtis distance measure was used to generate a distance matrix. Cluster analyses were each subjected to a bootstrap re-sampling analysis to generate approximately unbiased *p*-values with each cluster of interest. The cluster analysis was trimmed to the three broadest groups to be used as chemotypes, as these represented broad similarities between trees. In turn, these chemotypes were used as discrete, manipulated variables to determine variance explained by each and bar plots of the standardized data were generated to visualize monoterpenes driving these divisions. Additionally, a similar cluster analysis was trimmed to two groups and used to classify trees into different trends observed in α-pinene enantiomer composition.

Non-metric multidimensional scaling (NMDS) was used to visualize the relationship between chemotypes and monoterpene proportions as well as climatic variables and monoterpene concentrations. Climatic variables considered were mean annual precipitation, degree-days of 0°C and continentality (temperature difference between warmest and coldest months). Values were attained through the software package ClimateNA (v4.85) and represent monthly data over a 30 year period (1961-1990) generated by the Parameter Regression of Independent Slopes Model (PRISM) (Hamann et al., 2013). The Bray-Curtis distance measure was used to create a dissimilarity matrix from which an NMDS ranks the distances between samples. These distances were then represented in a two-dimensional configuration minimizing stress, which is a metric of how well the configuration plots similar points close together. Two vectors with angles that are less than 90° represent positively correlated variables while angles greater than 90° represent negative correlations and vector length corresponds to the strength of the variable.

Linear regression analyses were performed to determine Pearson's correlation coefficient (r) values between climatic variables and monoterpene concentrations. Correlations were considered strong if $r > 0.7$, moderate if $0.7 > r > 0.5$ and weak if $r < 0.5$. Additionally, for trees sampled from natural stands, correlations between climatic variables and the proportion of chemotypes occurring at individual sites were determined.

In order to test how the two enantiomers of α-pinene were distributed among trees, a permutational multivariate analysis of variance (per MANOVA) test was used to determine differences between observed α-pinene phenotype groups. Groups were defined by a hierarchical cluster analysis. A per MANOVA test assesses the ratio of distances between points within groups and across groups. For this, the Bray-Curtis distance measure was used. The class

variable (phenotype group) is permutated multiple times to establish a distribution of the test statistic, thereby eliminating any assumptions about normality. Finally, the test statistic is compared to the newly generated probability distribution to determine a p-value.

2.3 Results

2.3.1 Jack pine chemotypes

To define overall monoterpene chemotypes, we had an *a priori* focus on the monoterpenes (–) and (+) α-pinene, (–) and (+) β-pinene, (–) and (+) limonene, 3-carene, myrcene and terpinolene because of their biological and ecological relevance to bark beetles as well as their prominence in jack pine (>95% of total monoterpenes) (Raffa et al., 2005; Colgan and Erbilgin, 2011; Lusebrink et al. 2011, 2013; Erbilgin and Colgan 2012; Clark et al., 2014; Erbilgin et al., 2014). Additionally, we found these compounds defined overall monoterpene composition trends in our data well. The two enantiomers of each β-pinene and limonene were grouped together because they were very closely correlated ($r > 0.99$) and maintained at a constant ratio. However, the (–):(+)α-pinene ratio varied and exhibited different trends throughout jack pine's range so the α-pinene enantiomers were examined as individual compounds. Because our data suggests that monoterpene concentrations are correlated with climatic variables, evaluations of chemotypes were done separately in natural and provenance stands. Additionally, minor variations between stand types were observed and analyzing data by stand types allowed us to discern genetic vs. environmental effects on monoterpene composition. The complete monoterpene profiles of trees were reported in Supplementary Table A.

A hierarchical cluster analysis of monoterpene proportions in jack pines from natural stands established three broad groups of trees based on overall similarities of monoterpene proportions (Fig. 1a). These three groups were best defined by relatively high proportions of β-pinene (108 trees), α-pinene (100 trees), and limonene (23 trees) and indicate three distinct chemotypes (Table 1). Despite variations in the (–):(+) α-pinene ratio, high proportions of both enantiomers were closely associated with the same chemotype, leading it to be classified as simply α-pinene chemotype. These chemotypes explained a total of 58.6% of the observed variance in monoterpene proportions. The division between the β-pinene chemotype and the other two chemotypes was the most distinct and explained 40.1% of monoterpene proportion variance, while the remaining division between the α-pinene and limonene chemotypes explained 18.5% of variance. An NMDS of monoterpene proportions from natural stands showed that individual trees grouped by the three chemotypes were closely associated with their respective defining monoterpene vectors (Fig. 2a).

Furthermore, a hierarchical cluster analysis of trees sampled from provenance stands maintained these chemotypes (Fig. 1b) and an NMDS showed similar chemotype divisions to that of trees sampled from natural stands (Fig. 2b). This cluster analysis was also compared to genetic information known about these specific samples from Godbout et al. (2005, 2010). However, current monoterpenes showed no correlations to phylogeographic history, genetic lineages, provenance location, provenance climate, phenology or ontogeny. Rather, the broadest divisions of monoterpene proportions from provenance stands aligned with the monoterpene proportions of the chemotypes observed in natural stands. Once again, the β-pinene chemotype was widely separated from the other chemotypes while the α-pinene and limonene chemotypes were distributed more closely to each other, which is in accordance with what was observed in

the hierarchical cluster analysis. However, in provenance stands, the relative numbers of trees in each group changed, as the α-pinene chemotype consisted of the most trees, with 180, while the β-pinene chemotype had 153 trees and limonene chemotype included 37 trees.

Considering trees from natural stands, all three chemotypes occurred across the sampled natural range, frequently all persisting within the same site while no site was exclusively any single chemotype (Fig. 3). Interestingly, the β-pinene and the α-pinene chemotypes were present in 96 % and the limonene chemotype was present in 56% of sites. Additionally, climatic variables were correlated to chemotype proportion at individual sites (Table 2) and therefore chemotypes of trees from provenance stands could not be plotted on a map, as all were grown in the same environment which would have a homogenizing effect on chemotype. For example, the proportion of the α-pinene chemotype was negatively correlated with mean annual precipitation, but was positively correlated with degree-days above 0°C (Table 2). The proportion of the β-pinene chemotype was negatively correlated with continentality and degree-days above 0°C, but was positively correlated with mean annual precipitation (Table 2).

2.3.2 Individual monoterpene variation

Climate variables affected concentrations of individual monoterpenes differently. In natural stands, both enantiomers of β-pinene and α-pinene showed significant correlations with all climate variables (Table 3). Myrcene, 3-carene, terpinolene and both enantiomers of limonene were not correlated to any climate variables, with the exception of a weak correlation between 3-carene and continentality (Table 3). Of any correlation between climate factors and monoterpene concentrations, mean annual precipitation had the strongest positive correlation with β-pinene and α-pinene, whereas continentality was most strongly negatively correlated with the same two

monoterpenes (Table 3). In provenance stands, there were no significant correlations between monoterpene concentrations and climate variables from provenance origin with the exception of a weak correlation between mean annual precipitation and terpinolene and myrcene (Supplementary Table 1B).

Because environmental factors affected individual monoterpene concentrations differently, monoterpene proportions of the total profile were not constant and thus it was difficult to elucidate overall trends between monoterpene proportions and climate. For example, β-pinene and α-pinene concentrations increased with increasing mean annual precipitation, while limonene concentrations remained unaffected, meaning that limonene's proportion of the total monoterpene profile decreased. Generally, though, climatic variables were less strongly correlated to monoterpene proportions than to concentrations. Finally, in both natural and provenance stands, (–) and (+)-α-pinene and myrcene proportions were positively correlated to each other, but were negatively correlated to β-pinene, limonene and 3-carene (Fig. 2).

2.3.3 Enantiomeric composition

Analyses of the chiral monoterpenes α-pinene, β-pinene and limonene showed that enantiomeric composition trends varied between individual compounds. While (–):(+)-β-pinene and (–):(+)-limonene remained at constant ratios in all sampled trees across jack pine's range, (–):(+) α-pinene exhibited two separate trends, thereby delineating two distinct phenotypes (Fig. 4). The separation of these two distinctive α-pinene phenotypes was supported by a hierarchical cluster analysis which divided trees into two broad groups (group 1 and group 2) depending on (–):(+) α-pinene ratios (Supplementary Fig. 1). These groups corresponded to the two distinct trends observed in (–):(+)-α-pinene ratio (Fig. 4). The enantiomeric composition patterns

observed for α-pinene, β-pinene and limonene were maintained in natural and provenance stands, though stand types were analyzed separately.

In natural stands, α-pinene phenotype group 1 consisted of 128 trees and had a mean (–):(+) ratio of 1.11 (± 0.22) (Fig. 5). Group 2 consisted of 103 trees and had a mean (–):(+)-α-pinene ratio of 3.48 (± 2.77) (Fig. 5). In provenance stands, group 1 consisted of 249 trees and had a mean (–):(+) α-pinene ratio of 1.09 (± 0.29) and group 2 included 120 trees with a mean (–):(+) α-pinene ratio of 3.11 (± 1.61) (Fig. 5). A perMANOVA test showed that groups 1 and 2 vary significantly ($F_{1,25.5,}$=983, P=<0.001) while there wasn't any difference between natural and provenance stands ($F_{1,0.04}$=1.36, P=0.24).

Due to the importance of α-pinene to pheromone production by *D. ponderosae*, we also evaluated the proportion of trees exhibiting each of the α-pinene phenotypes from all natural stands and provenance origins (Fig. 6). Although both groups occur throughout jack pine's range, it appears that jack pine is dominated by lower (–):(+)-α-pinene ratios (group 1).

2.4 Discussion

2.4.1 Jack pine chemotypes

We defined three distinct chemotypes in jack pine most notably characterized by relatively high proportions of three monoterpenoid compounds, α-pinene, β-pinene and limonene. These three chemotypes were maintained in both natural and provenance stands and can co-occur in the same locality. This indicates a strong genetic influence on chemotype formation in jack pine, as monoterpene proportions did not simply conform to their environment, but rather persisted as a chemically heterogeneous forest, supporting earlier conclusions that pine monoterpenes are in

part genetically controlled (Hanover, 1966, 1971; Forrest 1981; Davis and Hofstetter, 2012; Clark et al. 2014).

However, despite an apparent genetic influence on chemotype formation, chemotypes were not correlated to jack pine's phylogeographic history or historical genetic lineages as defined in Godbout et al. (2005, 2010). Through evaluation of maternally inherited (i.e., seed-dispersal) mitochondrial DNA minisatellite markers, Godbout et al. (2010) defined five jack pine genetic lineages and a genetically distinct population isolated on Canada's east coast, likely arising after the last glacial maximum about 21,000 cal BP. Nonetheless, these genetic groups apparently have no or minimal effect on monoterpene expression, as we consistently observed no monoterpene composition patterns corresponding to these genetic lineages. A possible explanation for the lack of correlations between genetic lineages and monoterpene composition is that paternally inherited (i.e., pollen-dispersal) chloroplast DNA has a relatively uniform distribution across jack pine's range (Godbout et al., 2010). Therefore, jack pine's widely dispersed pollen may be genetically homogenizing, thus potentially eliminating phenotypic differences in monoterpene expression correlated to historic genetic lineages. Conversely, genetic influences on chemotypes and monoterpene composition may be due to adaptations or factors unrelated to the phylogeographic and genetic factors considered in this study.

Moreover, in addition to evidence suggesting a genetic influence on jack pine chemotypes, we detected weak to moderate correlations between chemotype distribution and some climate variables, thereby demonstrating that chemotype formation is influenced by both genetic and environmental factors. This may explain why chemotype divisions were less pronounced in provenance stands, as these trees would be subjected to the same environment which would have somewhat of a homogenizing effect on monoterpene proportions. Additionally, it should be

noted that limitations to our study, including tree ages, may obfuscate correlations between monoterpenes and other factors, such as genetic lineages, though neither ontogeny nor phenology explained monoterpene proportions in the current study.

The observed heterogeneous monoterpene chemotype distribution also demonstrates that there is no single homogenizing selective pressure favouring one chemotype over the others as chemical polymorphism in plants is critical for reciprocal natural selection between plants and herbivorous insects (Raffa and Berryman, 1987; Becerra et al., 2009; Iason et al. 2011; Davis and Hofstetter, 2012; Moore et al., 2014). Because monoterpenes are primarily involved in conifer defences against biotic agents (Phillips and Croteau, 1999; Wallin and Raffa, 1999; 2001; Raffa et al., 2005, 2013; Erbilgin et al. 2006; Colgan and Erbilgin, 2011), there would be strong selection for one chemotype if it was superior or favourable in defending against attacking agents under all circumstances. Alternatively, each chemotype may provide better defenses against specific attackers, as monoterpene effects can vary between attacking guilds (Wallin and Raffa, 2001; Raffa et al., 2005).

2.4.2 Climate, monoterpenes, and chemotypes

In the current study, jack pine oleoresin shows substantial variation in its monoterpene concentrations across its range. Although the exact mechanism of such variation is not clear, we demonstrated that some climatic factors, such as continentality and mean annual precipitation, are correlated with certain monoterpene concentrations in natural stands and that individual monoterpenes responded differently to different climatic variables.

While all climatic variables were significantly correlated with concentrations of both enantiomers of α-pinene and β-pinene, concentrations of (–) and (+)-limonene, terpinolene, and

myrcene were not at all related to climate. The monoterpene concentrations not correlated to climate were also not correlated to jack pine's distribution, indicating environment has no effect on them. Finally, 3-carene concentration varied somewhat with environment, as it was significantly correlated to continentality and longitude, signifying that it changes across jack pine's distribution in part due to environmental variables that are not measured in the current study. In general, these findings demonstrate that individual monoterpenes in jack pine are controlled by different mechanisms and are variably influenced by genetic and/or environmental factors. Some monoterpenes, specifically α- and β-pinene, display phenotypic plasticity and change with climate, while others, such as limonene, terpinolene, and myrcene do not change with environment and are therefore likely under genetic control. Overall, these results are in agreement with previous studies which have reported that limonene, myrcene and 3-carene are strongly heritable while α-pinene and β-pinene are more dependent on environmental factors (Baradat and Yazdani, 1988).

Interestingly, mean annual precipitation, associated with favourable growing conditions, was positively correlated with monoterpene concentrations. Conversely, continentality, associated with more harsh abiotic conditions, was negatively correlated with monoterpene concentrations. This is similar to previous findings that have indicated overall pine secondary metabolite levels were higher in environments with reduced climate related abiotic stress, such as drought (Wallis et al*.,* 2011). Not only would such climates decrease abiotic limitations to a tree's physiology, but they would also increase pest development, thereby increasing selective pressure on trees to produce greater defence compounds, such as monoterpenes (Wallis et al*.,* 2010, 2011).

The observed environmental influence on only some monoterpene concentrations and lack of influence on others show that both environment and genetics affect jack pine chemotypes. This was supported by the positive correlations between the proportion of β-pinene chemotype trees at individual sites and climate variables that were also positively correlated to β-pinene concentration, such as mean annual precipitation. Because β-pinene concentration was affected by climate more strongly than any other monoterpene, the proportion of trees of its chemotype is also most closely associated with the same climate variables in the same directions. In contrast, the proportion of α-pinene chemotype trees at individual sites exhibited the opposite directional relationship to climate variables than α-pinene concentrations. This demonstrates that, because β-pinene concentrations were more strongly correlated to climate variables than were α-pinene concentrations, changes in the proportion of trees of the β-pinene chemotype came at the expense of trees of the α-pinene chemotype. Finally, despite no correlation with climate, the limonene chemotype persisted in the same sites with the other chemotypes, showing that climate did not equally influence the variation of all chemotypes, thereby indicating a genetic effect on chemotype.

2.4.3 Enantiomeric composition

In our jack pine trees, enantiomeric ratios of β-pinene and limonene were canalised and did not vary across jack pine's range or between natural and provenance stands. However, (–):(+) α-pinene ratios showed two distinct trends qualifying two phenotypes (group 1 and group 2). Both groups had (–) and (+) α-pinene, but group 1 was less variable overall and had a lower (–):(+) α-pinene ratio than group 2. These enantiomeric phenotypes were maintained in both natural and provenance stands, were both prominent across jack pine's range, existed at the same sites and

showed no correlation to any climatic variables, thereby strongly demonstrating genetic control of the trait. These α-pinene groups may be important to bark beetles, including invasive *D. ponderosae* as bark beetle pheromone production pathways that depend on α-pinene can be enantioselective (Klimetzek and Francke, 1980; Gries et al*.,* 1990; Blomquist et al*.,* 2010; Erbilgin et al. 2014). This may, in turn, represent a genetic basis for the suitability of individual jack pines for bark beetle colonization.

2.4.4 Concluding remarks

Variations in jack pine's monoterpene composition are influenced by both genetic and environmental factors and this variability is expected to have cascading impacts on attacking herbivorous insects. While the impacts of our findings on bark beetles should be interpreted cautiously, as monoterpene composition varies between tissues of an individual tree (Latta et al., 2000; Erbilgin and Colgan, 2012) there is reason to believe that our defined jack pine chemotypes will have important implications for the biology and ecology of *D. ponderosae* in the jack pine boreal forest. The beetle may preferentially colonize α-pinene chemotype trees as α-pinene is a direct precursor of the female beetle's primary aggregation pheromone, *trans*-verbenol (Blomquist et al., 2010; Erbilgin et al., 2014). This pheromone is essential for attraction of mates and successful aggregation to overwhelm host tree defences and beetles produce more *trans*-verbenol in host trees that have higher α-pinene concentrations leading to increased *D. ponderosae* attraction (Pitman, 1968; Gries et al*.,* 1990; Pureswaran et al., 2000; Safranyik et al*.,* 2010; Erbilgin et al., 2014). Conversely, limonene is known to be particularly toxic to *D. ponderosae* and its associated fungi (Raffa and Berryman, 1983; Clark et al., 2014) and therefore

limonene chemotype trees may have detrimental effects on beetle colonization. However, because these chemotypes persist as heterogeneous stands, areas of increased susceptibility or resistance to *D. ponderosae* on a landscape scale are not expected. At this point, how jack pine's diverse phytochemical landscape will affect *D. ponderosae* 's continued eastward spread is difficult to predict, though it should be further studied, as it will likely be an important factor for the beetle's overall success in its novel host ecosystem. Additionally, as certain monoterpenes are correlated to climate variables while others are not, climate's effect on monoterpene composition could alter jack pine's susceptibility to numerous insect herbivores and pathogens, as monoterpenes affect attacking guilds in different ways (Raffa et al., 2005; Colgan and Erbilgin, 2010). This information should be considered in the context of a changing climate and large implications of the impact of climate change on plant monoterpene composition should not be overlooked.

Figures

Figure 2.1. Hierarchical cluster analyses of jack pine (*Pinus banksiana*) monoterpene proportions in (a) natural stands across eastern Canada and northeastern U.S. and (b) provenance stands representing jack pine populations from Alberta to the Atlantic coast. The three broadest divisions of each cluster analysis were used to define chemotypes. The percent values at the first two divisions of each cluster analysis represent the variance explained at those divisions. Approximately unbiased bootstrap values are indicated in red for each chemotype. The error is the remaining variance that is not explained by the first two divisions, the CV error is the cross validated error and SE is the standard error. The bar plot represents normalized monoterpene proportion data and the bars above the mid-line represent monoterpene proportions that are the bases for group divisions.

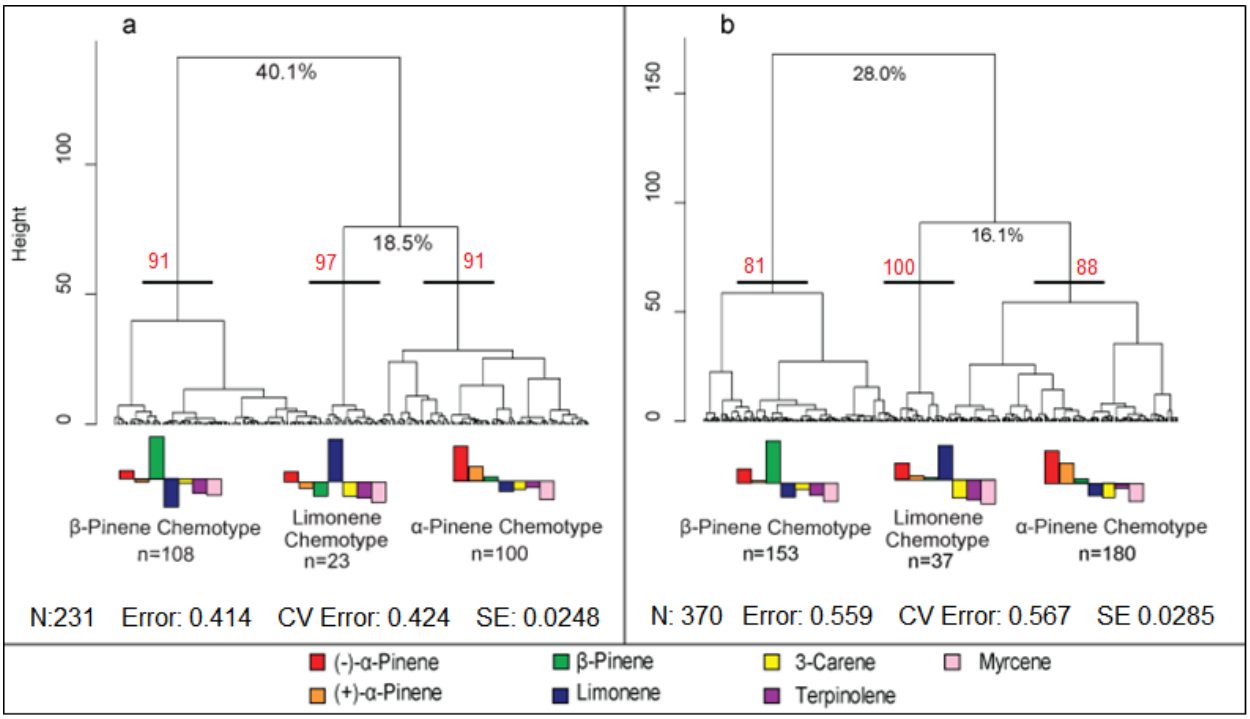


Figure 2.2. Non-metric multidimensional scaling (NMDS) plots of monoterpene proportions of jack pine (*Pinus banksiana*) in (a) natural stands and (b) provenance stands. Points are divided by chemotypes defined by a hierarchical cluster analyses performed on samples from each respective stand type. Vectors represent individual monoterpene proportions. Each point represents an individual tree.

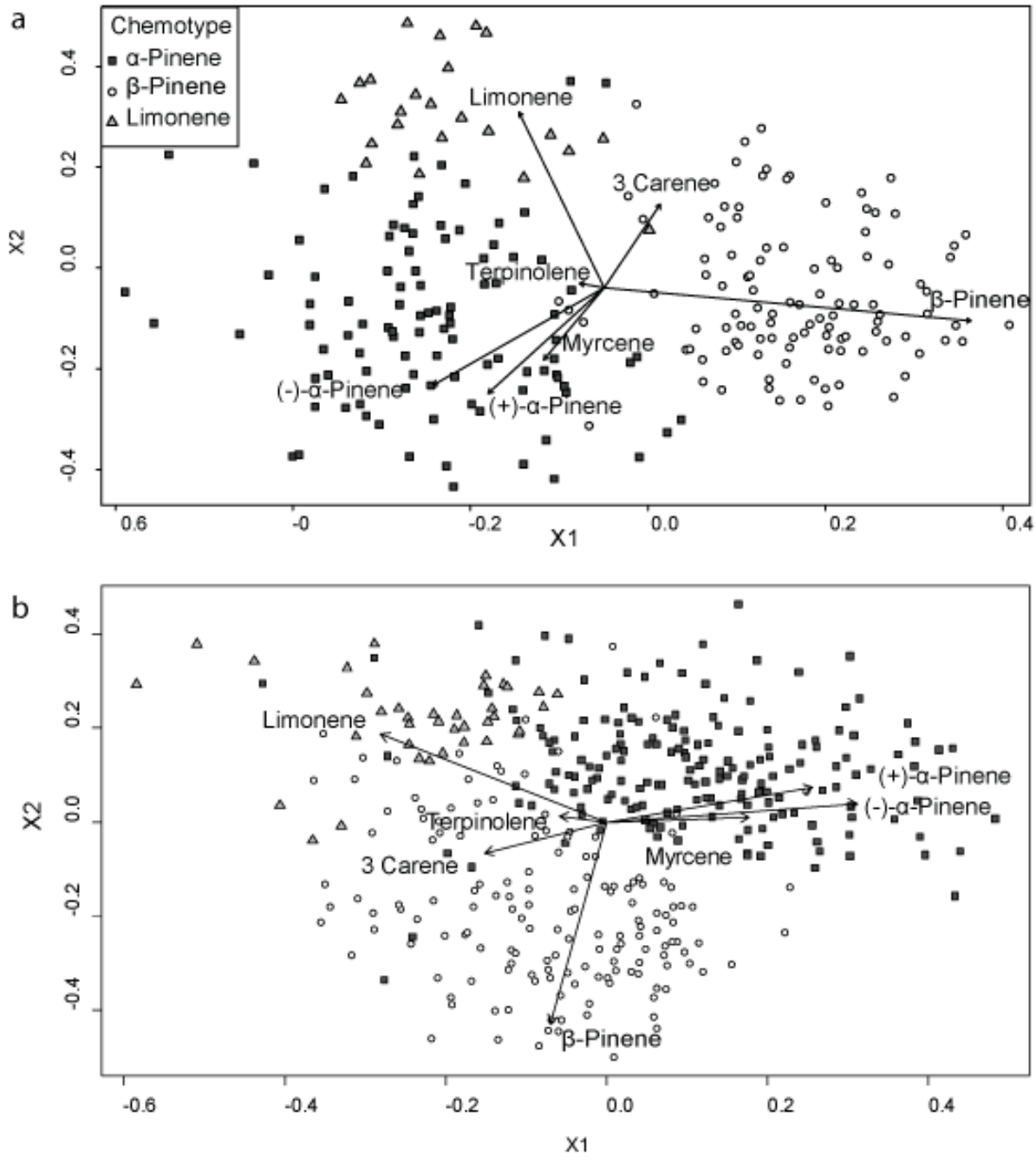


Figure 2.3: Jack pine (*Pinus banksiana)* natural stand locations in eastern Canada and northeastern US coloured proportionately by chemotype frequency. Shaded area of map represents jack pine's range.

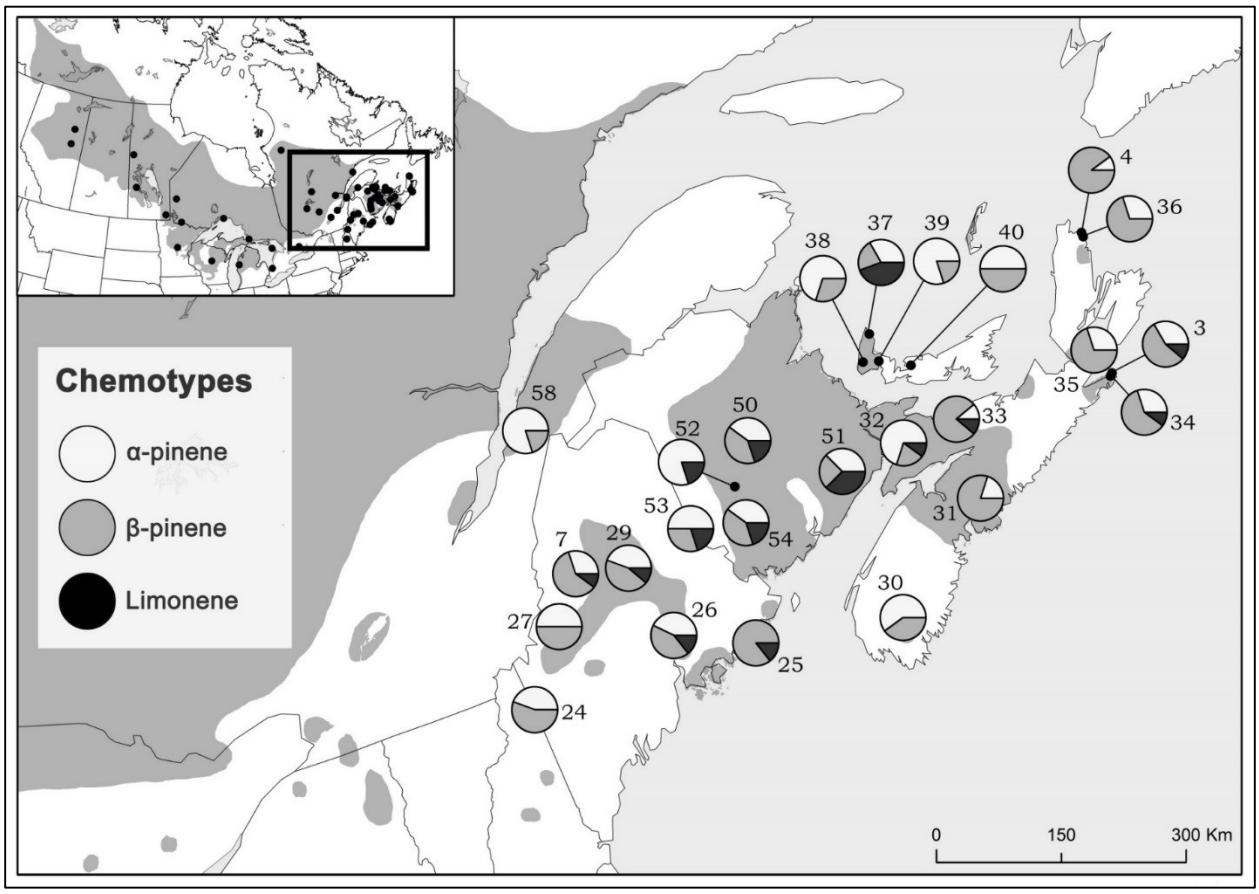


Figure 2.4: Concentrations of (a) (-) and (+) β-pinene (r=0.999) and (b) (-) and (+) limonene (r=0.999) in natural jack pine (*Pinus banksiana*) stands plotted against each other to illustrate the relative amount of each enantiomer. For concentrations of (c) (-) and (+) α-pinene, grey circles represent group 1 and inverted triangles represent group 2, both as defined by a hierarchical cluster analysis.

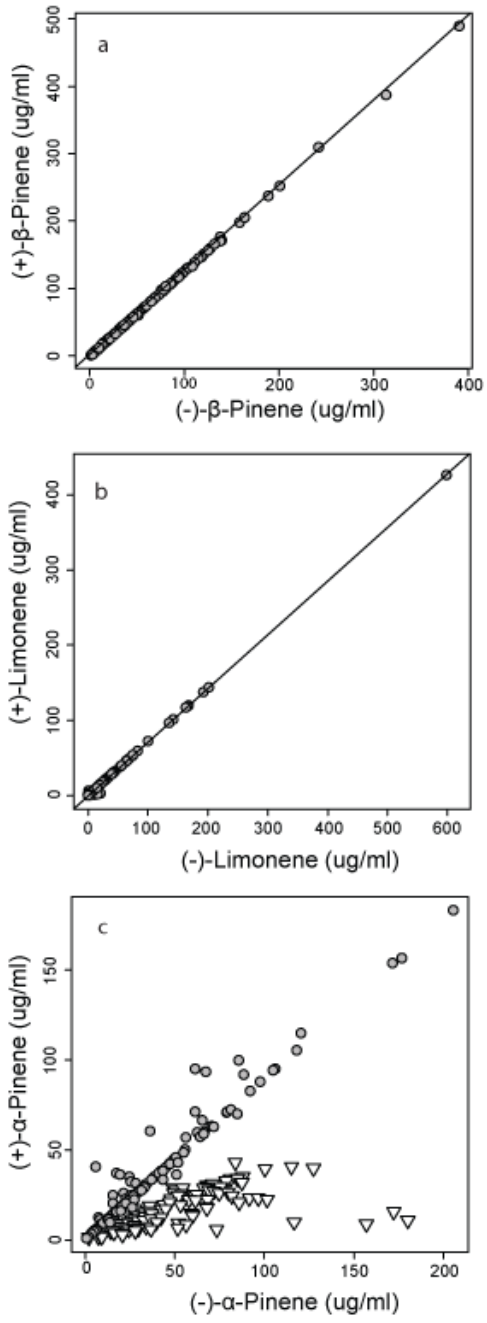


Figure 2.5: Box and whisker plots of jack pine (*Pinus banksiana*) α-pinene groups as defined by a hierarchical cluster analysis. Boxes represent data between the first and third quartiles while the whiskers represent the range of data excluding outliers, represented by dots. Letters represent statistically significant differences at α=0.05.

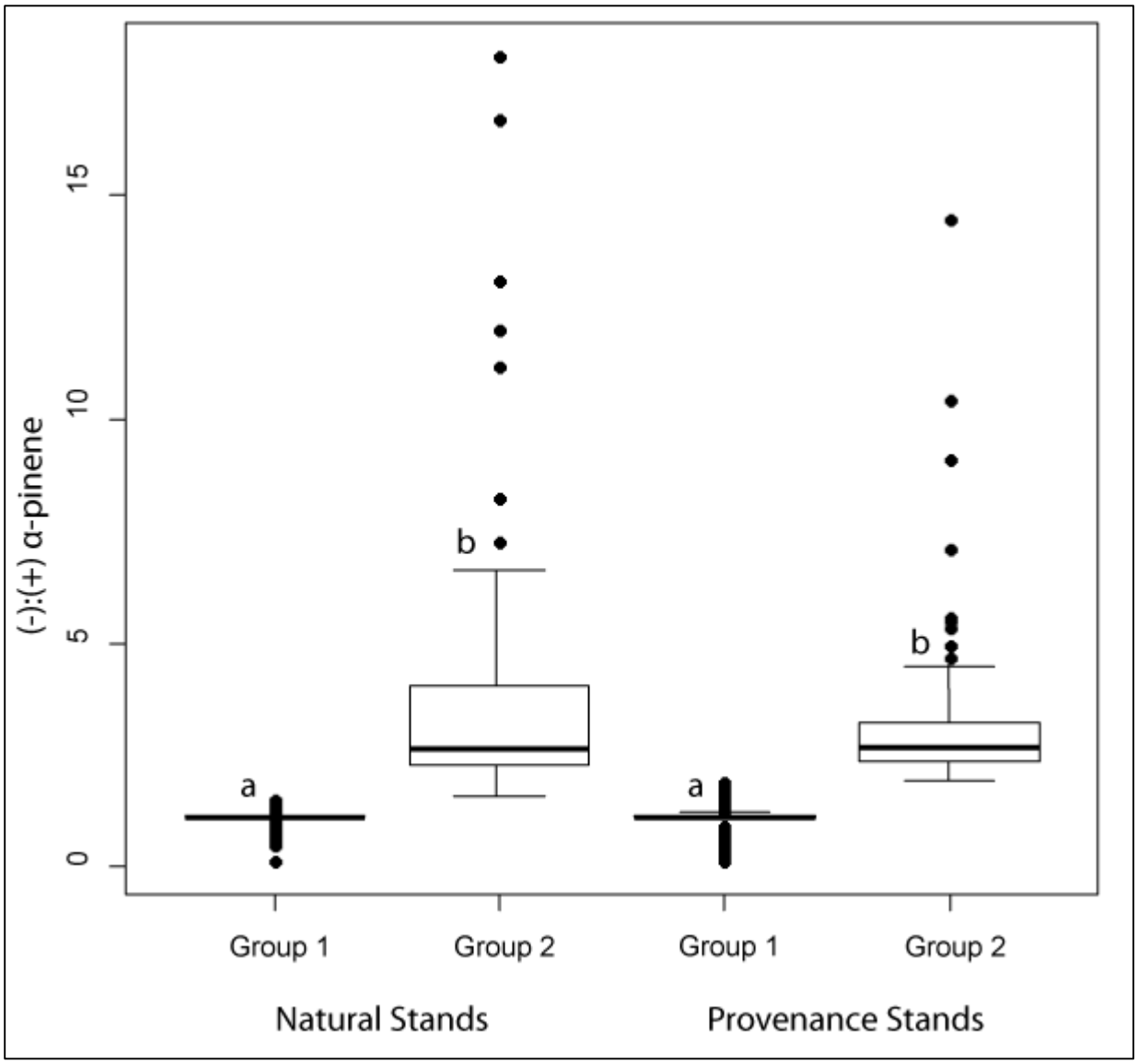


Figure 2.6: The proportion of trees exhibiting each of the α-pinene phenotypes from all natural stands and provenance origins. Group 1 has a mean (–):(+) α-pinene ratio of 1.10 (±0.25) and group 2 has a mean (–):(+) α-pinene ratio of 3.37 (±2.24). Shaded area of map represents jack pine's range.

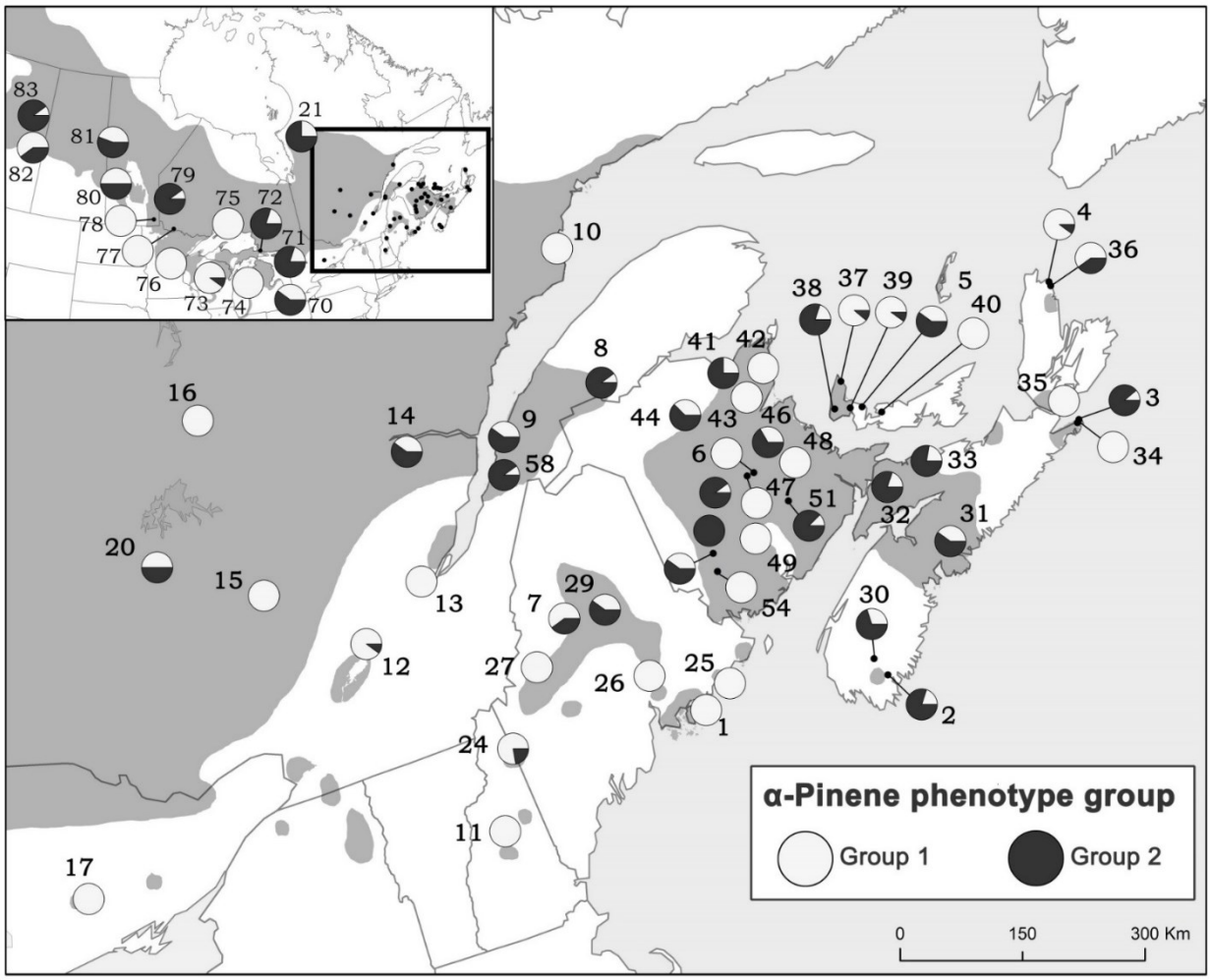


Table 2.1. Approximate defining properties of the three chemotypes derived from a hierarchical cluster analysis of jack pine (*Pinus banksiana*) trees in (a) natural stands in populations of eastern Canada and northeastern U.S.A., and (b) provenance stands representing jack pine populations from Alberta to the Atlantic coast.

Chemotypes	**Defining Monoterpene Trends (% in the total monoterpenes)**
Natural Stands	
α-Pinene	(-) and (+) α-Pinene > 20%, β-Pinene < 20%, Limonene < 20%
β-Pinene	β-Pinene > 24%, (-) and (+) α-Pinene < 18%, Limonene < 20%
Limonene	Limonene > 20%
Provenance Stands	
α-Pinene	(-) and (+) α-Pinene > 15%, β-Pinene < 15%, Limonene < 10%
β-Pinene	β-Pinene > 15%, (-) and (+) α-Pinene < 15%, Limonene < 20%
Limonene	Limonene > 15%, α-Pinene < 15%, β-Pinene < 15%,

Table 2.2. Pearson correlation coefficient values (*r*) between climatic variables and chemotype frequencies from natural stands of jack pine (*Pinus banksiana*) populations in Canada and northeastern U.S.A. TD=Continentality (Temperature Differance between warmest and coldest month), MAP=Mean Annual Precipitation, DD0=Degree-Days above 0°C.

Chemotypes	***r*-values for chemotype frequencies as a function of climate**			
	Longitude	TD	MAP	DD0
α-Pinene	-0.28	0.36	**-0.41***	**0.42***
β-Pinene	0.31	**-0.46***	**0.51***	**-0.44***
Limonene	-0.098	0.24	0.23	0.10

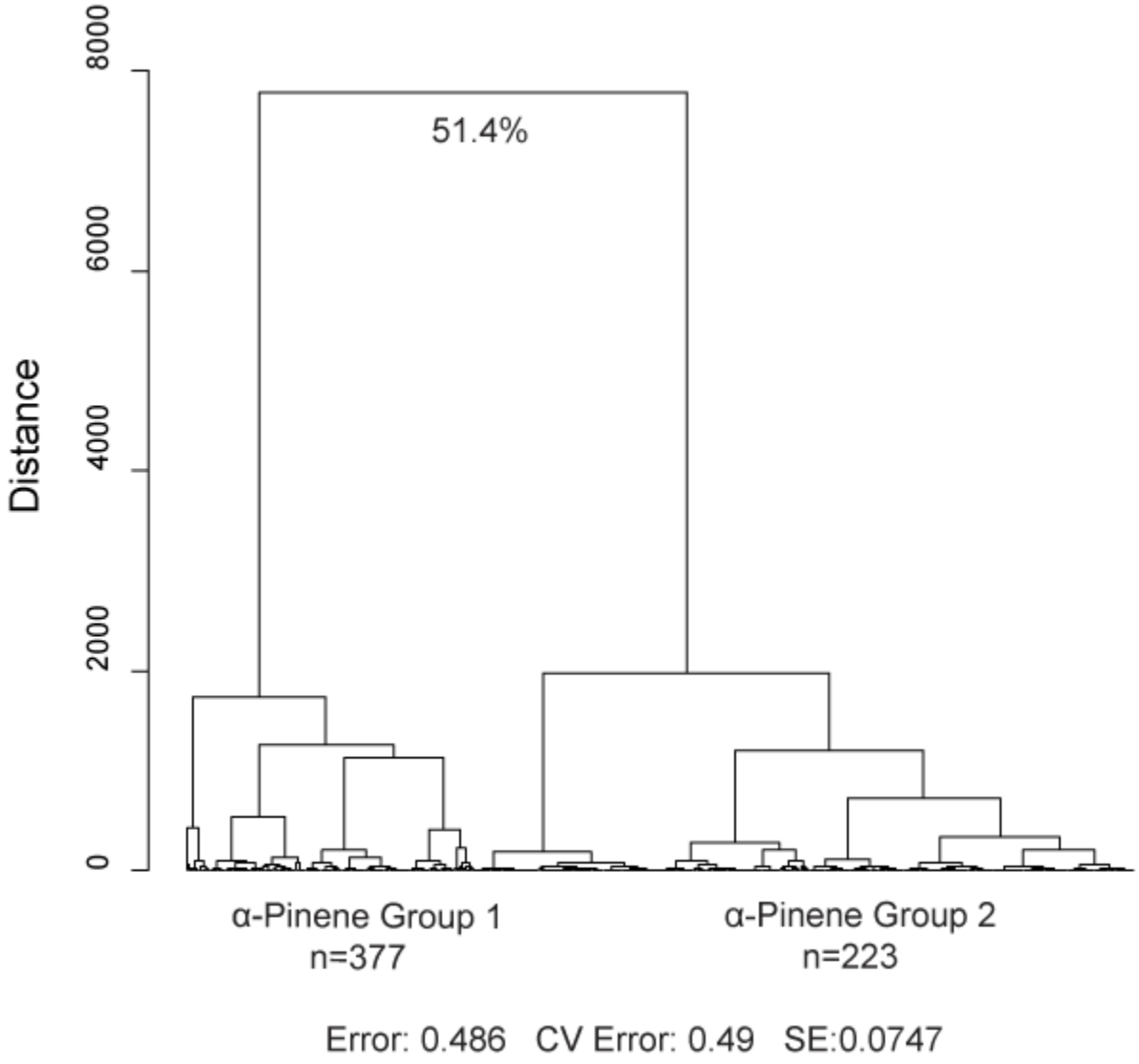
* indicates $P < 0.05$. Significant r-values are in bold.

Table 2.3. Pearson correlation coefficient values (*r*) between climatic variables and individual monoterpene concentrations in natural jack pine (*Pinus banksiana*) stands in eastern Canada and northeastern US. TD=Continentality (Temperature Differance between warmest and coldest month), MAP=Mean Annual Precipitation, DD0=Degree-Days above 0°C.

Monoterpenes	Longitude	TD	MAP	DD0
(-)-α-Pinene	**0.4422***	**-0.54901***	**0.741***	**-0.41917***
(+)-α-Pinene	**0.51***	**-0.56293***	**0.7***	**-0.45916***
(-)-β-Pinene	**0.6952***	**-0.74141***	**0.8197***	**-0.6544***
(+)-β-Pinene	**0.6785***	**-0.74031***	**0.8177***	**-0.65321***
(-)-Limonene	0.2411	-0.05026	0.0801	-0.07606
(+)-Limonene	0.2244	-0.04071	0.0669	-0.06793
3 Carene	**0.4573***	**-0.37397***	0.2556	-0.27614
Myrcene	0.247	-0.21444	0.3195	-0.1415
Terpinolene	0.1562	-0.07907	0.0767	-0.0766

* indicates $P < 0.05$. Significant r-values are in bolded.

Supplementary Figure 2.1: Hierarchical cluster analyses of (-) and (+) α-pinene enantiomers in jack pine (*Pinus banksiana*) from natural and provenance stands.



Supplementary Table 2.1 B. Pearson correlation coefficient values (r) between climatic variables and individual monoterpene concentrations in natural jack pine (Pinus banksiana) stands in eastern Canada and northeastern US. MAT=Mean Average Temperature, AWT=Average Winter Temperature, AST=Average Summer Temperature, TD=Continentality (Temperature Differance between warmest and coldest month), MAP=Mean Annual Precipitation, DD0=Degree-Days above 0°C.

	Longitude	**MAT**	**AWT**	**AST**	**TD**	**MAP**	**DD0**
(-)-α-Pinene	0.319	-0.0034	0.0171	0.00615	-0.008	0.223	-0.0109
(+)-α-Pinene	0.213	-0.0217	-0.0493	0.0653	0.078	0.155	0.0474
(-)-β-Pinene	-0.227	-0.204	-0.271	0.0525	0.258	-0.226	0.272
(+)-β-Pinene	-0.23	-0.235	-0.288	0.0281	0.259	-0.231	0.292
(-)-Limonene	0.152	-0.18	-0.214	0.0381	0.197	0.15	0.226
(+)-Limonene	0.121	-0.181	-0.237	0.0582	0.225	0.143	0.243
3 Carene	0.252	-0.127	-0.0154	-0.3798	-0.153	0.327	0.0535
Myrcene	0.349	-0.0974	0.0319	-0.2932	-0.173	0.367*	-0.00545
Terpinolene	0.237	0.0924	0.118	-0.0012	-0.105	0.443*	-0.106

Chapter 3

Pheromone production by an invasive bark beetle varies with the monoterpene composition of its naïve host

3.1 Introduction

Herbivorous insects have adapted to obtain nutrients from all types of plant tissues through innumerable feeding mechanisms, but plants do not merely suffer passively; rather, they wage chemical warfare against herbivorous insects by producing toxic or repelling phytochemicals to actively defend themselves, thereby affecting herbivore survival, physiology and behaviour (Keeling and Bohlmann 2006; Howe and Jander 2008; Winde and Wittstock 2011; Mithofer and Boland 2012; Raffa et al. 2013). However, complicating plant-insect interactions, insect herbivores have co-evolved to overcome and exploit these host chemical defenses through numerous means including sequestration and detoxification of certain phytochemicals (Holzinger et al. 1992; Hartmann 1999; Winde and Wittstock 2011). Additionally, herbivorous insects utilize phytochemicals as indicators of host suitability and direct precursors to biologically active compounds (Ehrlich and Raven 1964; *Chénier and Philogène* 1989; Conner et al., 1990; Coley and Barone 1996; Erbilgin and Raffa 2000a, b; Reddy and Guerrero 2004; Seybold et al. 2006; Blomquist et al. 2010). Moreover, considerable inter and intraspecific variations of host secondary chemistry can alter insect responses, leading to a complex and variable landscape-scale array of chemically mediated plant-insect interactions (Lill and Marquis 2001; Erbilgin and Colgan 2012; Moore et al. 2014).

In coniferous trees, monoterpenes are one class of phytochemicals that have important effects on interactions between plants and insect-herbivores (Phillips and Croteau 1999;

Franceschi et al. 2005; Raffa et al. 2005, 2013; Moore et al., 2014). Generally, monoterpenes are a central aspect of constitutive and inducible host defences and represent a significant component of a tree's defensive resins, which are toxic to many insects, including bark beetles (Coleoptera: Curculionidae, Scolytinae) (Raffa et al. 1985, 2005; Cates et al. 1987; Phillips and Croteau 1999; Franceschi et al. 2005; Keeling and Bohlmann 2006). However, bark beetles can identify susceptible hosts and evaluate a tree's suitability for colonization based on monoterpenoid kairomones, thereby decreasing energy expenditure and risks associated with colonizing an unsuitable host (Wood 1982b; Miller and Borden 2003; Fettig et al. 2004; Keeling and Bohlmann 2006; Seybold et al. 2006; Erbilgin et al. 2014). For example, pine engrave beetles, *Ips pini,* are more receptive to individual monoterpenes that they have already encountered and adjust their behaviour and responses to various volatiles based on conspecific density (Wallin and Raffa 2002b). Similarly, prominent pine monoterpenes, such as α-pinene, β-pinene, myrcene, limonene, camphene, and 3-carene can all act as attractants for *Ips grandicollis* (Werner 1972; *Chénier and Philogène* 1989; Erbilgin and Raffa 2000a, b). In addition to attracting insect herbivores, monoterpenes can also act as precursors to bark beetle pheromones. This is demonstrated by the biosynthesis of the bark beetle pheromone components verbenol, verbenone, and verbenene from the host monoterpene, α-pinene (reviewed by Blomquist et al. 2010). This biosynthetic process is particularly crucial to the mountain pine beetle (*Dendroctonus ponderosae*) (Blomquist et al. 2010).

Upon arrival to a new host, female *D. ponderosae*, the pioneer sex, hydroxylate the host monoterpene α-pinene to produce *trans*-verbenol, which attracts both male and female beetles and thus initiates host colonization (Safranyik et al. 2010). Following their arrival

and mating, male beetles produce *exo*-brevicomin *de novo* which acts in concert with *trans*-verbenol and specific host monoterpenes such as myrcene and terpinolene, to attract conspecifics to overwhelm tree defences (Pureswaran et al. 2000; Borden et al. 2008; Song et al. 2014). However, beetles terminate aggregation at an optimal density (60-90 attacks m^{-2}) to avoid intraspecific competition and overcrowding by producing the anti-aggregation pheromones frontalin and verbenone (Raffa and Berryman 1983a; Berryman et al. 1985; Pureswaran and Borden 2003). Males produce frontalin from monoterpenoid or long-chain fatty acid precursors while both sexes produce verbenone, likely through an auto-oxidation of the host monoterpene α-pinene (Pureswaran and Borden 2003).

Moreover, interactions between *D. ponderosae* and host monoterpenes go beyond pheromone production. For example, some host monoterpenes, including myrcene, terpinolene, and α-pinene, synergize with beetle pheromones to increase beetle attraction over pheromones alone (Miller and Borden 2000; Borden et al. 2008). While results regarding the strength of individual monoterpene synergism with beetle pheromones tend to vary, myrcene and terpinolene are generally implicated as the strongest synergists (Borden et al. 2008). Still, monoterpenes can be detrimental to beetles, as 3-carene and limonene have been found to be ovicidal and particularly toxic to the beetle (Raffa and Berryman 1983a, b; Reid and Purcell 2011; Clark et al. 2014). Additionally, if persisting at high concentrations, many monoterpenes, including α-pinene, terpinolene, myrcene and limonene, are toxic to *D. ponderosae* (Reid and Purcell 2011). Finally, host monoterpenes can affect *D. ponderosae* indirectly by impeding growth of its obligate associated fungi, which is important to the beetle for nutrition, development and survival in addition to helping overwhelm host tree defences (Raffa and Berryman 1983b; Bleiker and Six 2007).

These diverse roles that host monoterpenes play in beetle biology and ecology is important, as secondary chemistry of a host species can affect herbivorous insects' host expansion (Jermy 1984), which *D. ponderosae* has recently experienced (Erbilgin et al. 2014).

Dendroctonus ponderosae has expanded its range from lodgepole pine (*Pinus contorta*)-dominated forests to jack pine (*Pinus banksiana*)-dominated forests in the boreal forest in western Canada (Alberta), indicating a range and host expansion (Erbilgin et al. 2014). This is an ecological concern for future sustainability of jack pine boreal forests throughout Canada, as jack pine is distributed from Alberta to the Atlantic coast in a 4,000 km corridor between western and eastern pine species. Whether jack and lodgepole pine differ in susceptibility to *D. ponderosae* is unknown, though lodgepole pine's evolutionary history alongside the beetle may have selected for effective defences, including monoterpenes, whereas jack pine is a naïve host and its monoterpene constitution varies from that of lodgepole pine (Lusebrink et al. 2011; Goodsman et al. 2013; Clark et al. 2014). A recent analysis of needle monoterpenes of 63 jack pine populations throughout its native range in North America indicate that substantial differences exist in jack pine chemistry which may have differential effects on *D. ponderosae* (Taft et al. unpublished record). However, lack of further knowledge concerning how such variations in jack pine phytochemistry affects pheromone production by *D. ponderosae* represents a substantial unknown factor when predicting the beetle's continued eastward spread through the boreal forest.

In this study, we characterized variations in pheromone production by *D. ponderosae* reared in jack pine trees sampled from east of the beetle's current range limit (Alberta) in jack pine forest stands from Manitoba to Nova Scotia. We related variations in pheromone

production to the monoterpene composition of host phloem to determine whether differences in jack pine chemistry affect pheromone production by beetles. We hypothesize that considerable variations in jack pine monoterpene composition will affect pheromone production by *D. ponderosae*. Because α-pinene is a direct precursor to the beetle pheromones *trans*-verbenol and verbenone, we expect α-pinene content will be positively correlated to these two pheromones (Blomquist et al. 2010; Erbilgin et al. 2014).

3.2 Materials and methods

3.2.1 Sampling

In July 2014, we selected multiple jack pine trees in two sites in Manitoba (50°66.5'N, 96°50.4'W; 51°79.77'N, 100°61.6' W), one site in Ontario (48°85.1'N, 92°41.7'W), three sites in Quebec (49°19.8'N, 67°37.8'W; 49°43.2'N, 68°12.4'W; 49°34.9'N, 67°58.8'W), two sites in Nova Scotia (45°74.9'N, 64°09.6'W; 45°31.2'N, 61°04.5'W), and two sites in New Brunswick (45°98.6'N, 66°39.0'W; 47°08.7'N, 65°85.8'W) (Fig. 1). Based on our previous chemical analysis of 63 jack pine populations (Taft et al., unpublished record), these sites were chosen to best represent variations in jack pine monoterpene composition. At each site, healthy trees (20-30 cm diameter at 1.4 m) were felled and one bolt, about 35 cm long, was cut from each tree at 1.4 m from the its base. Open ends of bolts were immediately covered with paraffin wax to minimize moisture and secondary metabolite loss. Bolts were shipped to the University of Alberta in nylon duffle bags to prevent subsequent insect attacks.

3.2.2 Pheromone and volatile collection

Within one day of receiving the bolts, we bored two 5 mm holes (5 cm above the bottom of each bolt) through the bark on opposite sides using a cork borer (5 mm diameter) then introduced a female *D. ponderosa* into each hole and kept the beetles in place with half of a gel capsule. If female introduction was successful, i.e. boring dust was observed in the entrance hole, we introduced a single male beetle into each hole 24 h later. If the female introduction was not successful, i.e. female died at the entrance hole, the dead beetle was replaced with a new female until the female beetle entered the host. In addition, we sampled a 2x2 cm piece of phloem above each hole at the top of the bolt. Samples were stored at −40°C until chemical analysis. Live beetles were collected in flight intercept traps baited with the aggregation pheromones of *D. ponderosae* at the Weyerhaeuser lumber mill directly south east of Grande Prairie (Alberta) (N55°10.15', W118°47.41') and were introduced into bolts within seven days of trapping.

Pheromones emitted by beetles were collected as described in Erbilgin et al. (2014). Briefly, we placed a 15 ml Teflon funnel on each hole and placed a charcoal filter (Honeywell, Southborough, MA, USA) on the bark around the base of the funnels. A Teflon tube was used to attach each funnel to a vacuum pump (Cole-Parmer Canada Inc.). An adsorbent glass tube (Porapak Q (OD, 6 mm; length, 110 mm; adsorbent: front layer, 150 mg; back up layer, 75 mg; separated by glass wool), SKC Inc., Eighty Four, PA, USA) was inserted in the Teflon tube between the pump and the funnel. Thus, the vacuum pumps drew out air, including pheromones and plant volatile chemicals, from each introduction hole into the adsorbent tubes continuously for 4 h intervals at a constant flow rate (100 ml min^{-1}). Volatile collection was with a new adsorbent tube at 12, 24, 36, 48, 60, 84 and 108 h after female beetle introduction. After each collection, the adsorbent tubes were capped and stored at −40°C until extraction. All bolts were kept at room temperature for the duration of the experiment.

3.2.3 Chemical analysis

Volatiles trapped in the adsorbent tubes were extracted with 1 ml of dichloromethane with 0.002% tridecane as an internal standard, as described in Erbilgin et al. (2014). All extracts were transferred into amber Gas Chromatography vials and stored at −40°C until analysis. Extracts (1 µl) were analyzed using a Gas Chromatography coupled with a Mass Spectrometry (7890A/5062C, Agilent Tech., Santa Clara, CA, USA) equipped with a chiral column (HP Innowax-20B column (ID 0.25 mm, length 30 m); Agilent Tech.). Extracts of the adsorbent tubes were run with helium as the carrier gas at a flow set to 1.1 ml min^{-1}. Each analysis began at 75°C (held for 15 min), followed by an increase of 5°C per min to 90°C (held for 1 min), followed by an increase of 10°C per min to 155°C (held for 1 min), followed by an increase of 25°C per min to 230°C. To extract phloem monoterpenes, samples were ground in liquid nitrogen and 100 mg of ground tissue was extracted twice with 0.5 ml methyl tert-butyl ether solvent with 0.002% tridecane as an internal standard. Equipment and procedures were the same as outlined above, except, phloem extracts were run with hydrogen carrier gas and each analysis began at began at 75°C (held for 6.8 min), followed by an increase of 15°C per min to 130°C (held for 5 min) before an increase of 120°C per min to 235°C.

Since Erbilgin et al. (2014) already demonstrated that *D. ponderosae* can produce *exo*-brevicomin and frontalin in jack pine and there was no difference in production of both pheromone components between host species, we only focused on pheromones that are thought to be synthesized from host monoterpenes. Thus, only two pheromones, *trans*-verbenol and verbenone were identified and quantified using the following standards: (–)-*trans*-verbenol (enantiomeric composition, 82%(-)/18%(+)) and verbenone (>95% pure). We also identified and

quantified nine monoterpenes using the following standards: (-)-α-pinene, (+)-α-pinene, (-)-β-pinene, (+)-β-pinene, (-)-limonene, (+)-limonene, myrcene, 3-carene, terpinolene (SAFC Supply Solutions, St. Louis, MO, USA). Chemical purity of all these compounds was higher than 99%. Compounds were identified by comparing retention times and mass spectra to those of the standard chemicals. These nine monoterpenes were chosen as a focus *a priori* because of their prominence in jack pine chemistry, constituting upwards of 95% of total monoterpenes as well as driving overall variations in jack pine monoterpene trends (Lusebrink et al. 2011; Erbilgin and Colgan 2012; Clark et al. 2014; Taft et al. unpublished data). With the exception of β-pinene, all are ecologically or biologically important to *D. ponderosae* pheromone production, attraction or survival (Seybold et al. 2006; Erbilgin et al. 2014). Quantity of chemicals was calculated using response curves generated from analyses of a dilution sequence of known quantities of standards. Calibration with these standards allowed for analysis of quantitative differences on monoterpene concentrations among samples. The amount of pheromones per beetle pair (μg ml^{-1}) and volatile monoterpenes (μg ml^{-1}) emitted from each entrance hole were reported. Phloem monoterpene percentages were determined by dividing the concentration of an individual monoterpene by the sum of all quantified monoterpene concentrations.

3.2.4 Statistical analyses

All statistical analyses on monoterpene concentrations and proportion data was performed in R statistical program version 2.15.0 using the ecodist (Goslee and Urban 2007), mvpart (Therneau et al. 2013), vegan (Oksanen et al. 2013) and lmperm (Wheeler 2010) packages. Proportions were determined by dividing the concentration of an individual monoterpene by the sum of all nine monoterpene concentrations.

Regression analyses were performed to determine the coefficient of determination (R^2) between phloem and volatile monoterpenes and pheromones produced. Additionally, a hierarchical cluster analysis was performed on all phloem samples to establish groups of trees with similar phloem monoterpenes (Fig. 2). The hierarchical cluster analysis used the Bray-Curtis distance measure to divide samples into clusters in a hierarchical structure based on overall differences, moving from broad to narrow differences. The three broadest divisions were used to define monoterpene group, as these represented broad similarities between trees. These groups were then used as discrete variables to determine variance explained by each. Barplots of average monoterpenes were generated for each group to visualize which monoterpenes were driving group divisions.

Non-metric multidimensional scaling (NMDS) was used to visualize phloem monoterpene groups, monoterpene proportions and pheromone production. The NMDS ranked the distances between samples in a dissimilarity matrix produced by the Bray-Curtis distance measure. These distances were then represented in a two-dimensional plot, resulting in similar points being plotted closer together. In an NMDS, two vectors at an angle less than 90° to each other represent positively correlated variables while angles greater than 90° represent negative correlations. Vector length corresponds to variable strength.

A permutational ANOVA was used to determine whether pheromones produced varied significantly with phloem groups and collection locations. This test assesses the ratio of distances between points within groups and across groups using the Bray-Curtis distance measure. The discrete, manipulated variable (phloem group and collection location) is permutated repeatedly to create a distribution of the test statistic, thereby eliminating assumptions of normal distribution.

The test statistic is then compared to the generated probability distribution to determine a p-value.

3.3 Results

3.3.1 Phloem monoterpenes

Analysis of phloem monoterpene composition indicated that, overall, α-pinene (sum of (+) and (-)-α-pinene) was the dominant compound of quantified monoterpenes (36.5%-93.4%), as it was the most abundant in every sample except one, in which 3-carene was the most abundant (39.0%) (Table 1). Additionally, α-pinene constituted over 50% of total monoterpenes in every sample except two and made up over 75% of quantified monoterpenes in 18 of 32 samples. Between α-pinene enantiomers, (+)-α-pinene was more abundant in 27 of 32 trees and, as proportions of total monoterpenes, the two enantiomers were negatively correlated to each other ($r = -0.50$, $P<0.01$). Furthermore, as a proportion of total monoterpenes, (+)-α-pinene was not correlated to any other monoterpene. No individual monoterpene concentration or proportion was correlated with geographic distribution, thus patterns of overall monoterpene composition, as opposed to variation of individual monoterpenes in isolation, were required to further explain variations.

A hierarchical cluster analysis of phloem monoterpenes established three broad groups of trees based on overall similarities of monoterpene proportions (Fig. 2). These monoterpene groups explained 58.1% of variance and each was associated with characteristic proportions of different monoterpenes (Table 2). One monoterpene group was associated with high proportions of (+)-α-pinene (>58%) (15 trees) and is referred to as the (+)-α-pinene group. The second monoterpene group was best defined by high proportions of 3-carene (>29%) and terpinolene

(>1.2%) (4 trees) and is hence referred to as the 3-carene group. The final monoterpene group was less uniform and consisted of no monoterpene proportions persisting at a notably high level and is therefore referred to as the moderate group. An NMDS visualizes the monoterpene group associations with each other and their respective monoterpene vectors (Fig. 3). While there were no overall correlations between monoterpene groups and latitude, longitude or climate, some patterns were observed and the distribution of these groups is included on a map of all sample sites (Fig. 1). For example, all bolts collected from Ontario fell into the moderate group, whereas all bolts from Manitoba fell into the (+)-α-pinene group while, 3-carene group bolts were only found in Quebec and Nova Scotia.

3.3.2 Pheromones and volatiles

α-Pinene was the most abundant monoterpene, constituting at least 50% of total volatile monoterpenes collected from each tree bolt. Among pheromones emitted, (–)-*trans*-verbenol was consistently more abundant than verbenone and on average, verbenone concentration was 4.4% of *trans*-verbenol. (–)-*trans*-Verbenol production showed weak, but significant correlations to total α-pinene ($F_{1, 44}$=5.16, P=0.028, r^2=0.11), (+)-α-pinene ($F_{1, 44}$=4.41, P=0.042, r^2=0.09) and (-)-α-pinene ($F_{1, 44}$=3.86, P=0.056, r^2=0.08) emission concentrations as well as (+)-α-pinene proportions ($F_{1, 44}$=3.80, P=0.058, r^2=0.08). Verbenone production was not correlated to any monoterpene concentration or proportion. Frontalin and *exo*-brevicomin were detected in trace amounts but not analysed, as they are synthesized *de novo* and do not vary between historic and novel hosts (Erbilgin et al. 2014).

On average, (–)-*trans*-verbenol amounts produced by beetles varied significantly with the previously defined phloem monoterpene groups ($F_{2,43}$=11.06, P<0.001) (Fig. 4). Beetles reared

in bolts in the (+)-α-pinene group produced more (–)-*trans*-verbenol (31.63 *μg/ml)* than beetles reared in the 3-carene (5.035 *μg/ml)* and moderate (16.18 *μg/ml)* groups. Additionally, verbenone production also varied among monoterpene groups ($F_{2,43}$=2.79, P=0.056) and beetles reared in bolts in the (+)-α-pinene group produced more (1.27 *μg/ml)* than beetles reared in 3-carene group (0.49 *μg/ml*), though not statistically more than the moderate group (0.79 *μg/ml*). Pheromones produced by beetles in the 3-carene and moderate group bolts did not vary.

Additionally, *trans*-verbenol produced by beetles also varied with the province from where bolts were collected ($F_{4,41}$=9.38, P<0.01) (Fig. 6), though it should be noted that sites are not representative of provinces as a whole. Beetles produced more *trans*-verbenol when reared in bolts collected from Manitoba (31.27 ug/ml) and Quebec (33.25 ug/ml) than in bolts from Ontario (8.73 ug/ml) and New Brunswick (2.041 ug/ml). Also, beetles in bolts from Quebec produced more *trans*-verbenol than beetles in bolts from Nova Scotia (16.62 ug/ml). Verbenone production also varied by province ($F_{4,41}$=11.22, P<0.01) and beetles in Quebec bolts produced more verbenone (2.01 ug/ml) than beetles in bolts from any other province (Manitoba: 0.54 ug/ml, Ontario: 0.50 ug/ml, New Brunswick: 0.033 ug/ml, Nova Scotia: 0.70 ug/ml).

Furthermore, pheromone production between phloem groups showed different chronological trends (Fig. 5). While (–)-*trans*-verbenol production for each group peaked at 24 hrs after beetle introduction ((+)-α-pinene group=46.97 ug/ml, moderate group=22.94 *μg*/ml, 3-carene group=11.03 *μg*/ml), it decreased to zero at 84 hrs for the 3-carene group but never fell below 56.2% of the peak production in (+)-α-pinene group (minimum of 26.42 *μg*/ml at 84 hrs) or 46.3% of the peak production in the moderate group (minimum of 10.61 *μg*/ml at 108 hrs). Conversely, for each group, the highest amount of verbenone was measured at 108 hrs and the lowest at 12 hrs (Fig. 5 b).

Chronological trends of pheromone production also varied between provinces, with peak *trans*-verbenol production occurring at different times according to province of bolt collection (Fig. 7 a). For example, beetles reared in bolts from Quebec produced a maximum of 66.46 ug/ml of *trans*-verbenol 24 hrs after introduction which fell to a minimum of 10.03 ug/ml at 84 hrs. Conversely, *trans*-verbenol production by beetles reared in bolts from Manitoba trended upward for the entire experiment with the highest amount (55.65 ug/ml) produced at 108 hrs. Beetles in bolts from Nova Scotia produced the most *trans*-verbenol at 84 hrs, whereas beetles in bolts from Ontario produced the most *trans*-verbenol at 36 hrs. Finally, beetles in bolts from New Brunswick consistently produced the least *trans*-verbenol, with a peak at 24 hrs. Verbenone production in bolts from Quebec and Manitoba peaked at 108 hours, at 48 hrs in bolts from Nova Scotia and New Brunswick and at 36 hrs in bolts from Ontario (Fig 7. b).

3.4 Discussion

3.4.1 Monoterpene composition and beetle pheromones

We demonstrated that *D. ponderosae* can produce its primary aggregation pheromone, (–)-*trans*-verbenol, and anti-aggregation pheromone, verbenone, on jack pine bolts collected from central and eastern Canada, beyond *D. ponderosae*'s current range. This new information is in addition to previous demonstrations of beetles producing pheromones in jack pine forests in Alberta (Canada), where jack pine trees have been under attack by the beetle since at least 2011 (see references in Erbilgin et al. 2014). However, the suitability of individual trees for pheromone production by beetles varies and is not uniform throughout jack pine forests. We show that pheromone production is strongly affected by monoterpene chemistry of jack pine phloem, as the amount of (–)-*trans*-verbenol produced was correlated to concentrations of (+) and (-)-α-pinene

and higher proportion of (+)-α-pinene to (-)-α-pinene . This was expected, as α-pinene is a direct precursor to (–)-*trans*-verbenol and *D. ponderosae* has previously been shown to produce more (–)-*trans*-verbenol in its historical (Pitman et al. 1968; Gries et al. 1990; Blomquist et al. 2010) and novel (Erbilgin et al. 2014) hosts with higher amounts of α-pinene.

To further investigate the relationship between monoterpene composition of jack pine trees and pheromone production of *D. ponderosae,* we used a cluster analysis to group trees based on similarities of major monoterpenes, representing different sub-cortical chemical environments encountered by beetles. Two of the resulting groups were best characterized by high proportions of (+)-α-pinene or 3-carene, while the last group had a more moderate blend of all nine monoterpenes. Pheromone production varied significantly among the three monoterpene groups. Beetles associated with the (+)-α-pinene group produced 4.5 and 2.5 fold more (–)-*trans*-verbenol than beetles associated with the 3-Carene and moderate groups, respectively. These results suggest that jack pine trees with high (+)-α-pinene content in their phloem would experience increased beetle aggregation, which could improve successful colonization and mate finding in this novel host ecosystem (Pitman et al.1968; Pureswaran et al. 2000; Safranyik et al. 2010). Conversely, jack pine phloem high in 3-carene was least suitable for pheromone production and beetles in bolts of the 3-carene group consistently produce the least amount of pheromones (about 22% of the (–)-*trans*-verbenol produced on (+)-α-pinene group). The lower pheromone production in the 3-carene group may possibly be due to reduced α-pinene, resulting in lower amounts of (–)-*trans*-verbenol production. Alternatively, 3-carene is also particularly toxic to the beetle and may therefore interfere with its pheromone production, demonstrating a potential impediment to successful aggregation and colonization of these trees (Lusebrink et al. 2011; Clark et al. 2014).

In addition to overall variation in pheromone production among monoterpene groups, production patterns varied over time. For example, (–)-*trans*-verbenol production reached zero in the 3-carene group at 84 hrs after female beetle introduction, whereas beetles in the (+)-α-pinene and moderate groups produced (–)-*trans*-verbenol at considerable levels throughout the experiment and never reached zero. This continued (–)-*trans*-verbenol production contrasts a previous study in lodgepole pine in which (–)-*trans*-verbenol production by *D. ponderosae* nearly stopped when male and female beetles were paired (Pureswaran and Borden 2003). Therefore, pheromone production between novel and historical hosts, as well as within the novel host, is chronologically variable. While prolonged (–)-*trans*-verbenol production could result in greater aggregation, colonization and mating success, it could also result in overcrowding and increased intraspecific competition (Raffa and Berryman 1983a). Further studies should investigate how continuous pheromone production affects attraction of *D. ponderosae* in field and whether this will impact long-term sustainability of beetle populations in the novel host habitats.

Beetles reared in (+)-α-pinene group trees produced approximately 3 and 1.5 fold more verbenone than beetles associated with the 3-carene and moderate groups, respectively. However, in contrast to (–)-*trans*-verbenol, verbenone production patterns were chronologically more uniform across monoterpene groups, with a peak production at 108 hrs for each. The continued verbenone production on jack pine bolts could inhibit beetle colonization, as supplements of verbenone can reduce successful mass attacks on hosts also baited with aggregation pheromones (Huber and Borden 2001). Therefore, correlations between host monoterpene composition and *D. ponderosae* verbenone production should be investigated as a means to mediate beetle aggregation and colonization.

These complex relationships between *D. ponderosae* and monoterpene composition of jack pine should be considered with the continued eastward spread of beetles in jack pine boreal forests. While these chemical groups co-occur at individual sites and showed no correlations with jack pine distribution, there is initial evidence that certain jack pine forests may have higher proportions of certain chemical groups. For example, jack pine trees sampled in Ontario fell into the moderate group, whereas trees sampled from Manitoba fell into the (+)-α-pinene group while 3-carene group trees were only found in Quebec and Nova Scotia. These results suggest that there may be landscape scale trends of monoterpenoid group proportions in jack pine including geographic pockets with different dominant chemical groups across its natural range.

3.4.2 Pheromone variations across provinces

We investigated differences in the amounts of pheromones produced by *D. ponderosae* among five provinces as a convenient proxy for geographical distribution. We found that beetles produced their pheromones in bolts from all five provinces, indicating potential suitability of jack pine forests in these locations. However, there were considerable differences in the amount of pheromones produced by beetles among provinces. On average, beetles produced the most aggregation pheromone in jack pines from Manitoba and Quebec and the least in Ontario and New Brunswick, with an intermediate amount in Nova Scotia. Verbenone production also varied among provinces. Beetles in jack pine from Quebec produced the most verbenone, whereas verbenone production by beetles in the remaining provinces did not vary. Notably, these trends are not correlated with east-west distribution of jack pines. For example, bolts from Ontario represent a considerable drop off in *trans*-verbenol production compared to Manitoba to the west and Quebec to the east. Also, the data shows that trees highly suitable for production of

aggregation pheromones aren't necessarily suitable for production of anti-aggregation pheromones, as illustrated by jack pines from Manitoba.

Not only were there overall variations in pheromone production by province, but chronological pheromone production patterns also varied. Beetles in jack pine trees from Quebec exhibited an early peak in *trans*-verbenol production 24 hrs after female beetle introduction, before decreasing to a minimum at 84 hrs. This pattern sharply contrasts *trans*-verbenol production in jack pine bolts from Manitoba, which exhibited an upward trend that continued to the end of the experiment. Beetles on bolts from Nova Scotia produced *trans*-verbenol steadily throughout the experiment, and showed no indication of stopping 108 hrs after female beetle production. This increasing or maintained *trans*-verbenol production in bolts from Manitoba and Nova Scotia is unusual as generally *trans*-verbenol production nearly stops when female beetles are paired with males, as was the case in jack pine bolts from New Brunswick, Ontario and Alberta (Erbilgin et al. 2014) and lodgepole pine bolts from BC (Pureswaran and Borden 2003). Verbenone production chronology also varied between provinces, as its production in bolts from Quebec was highest and continued in an upward trend to the end of the experiment. Conversely, beetles in bolts from Nova Scotia and Manitoba produced verbenone at more moderate levels until the experiment's end, whereas verbenone production by beetles in bolts from New Brunswick and Ontario reached negligible levels by 60 and 108 hrs respectively. We currently do not know how these differences in aggregation and anti-aggregation pheromone production among provinces will affect *D. ponderosae*'s colonization sequences in novel jack pine habitats. One possibility is that delayed production of aggregation pheromones may decrease attraction of weak males that die early in dispersal, thereby attracting higher quality mates (Raffa and Berryman 1983a).

Variations in pheromone production between beetles reared on bolts from different provinces suggest that as the beetle continues its spread to eastern Canada it will encounter areas of variable suitability for pheromone production and therefore variable aggregation and colonization potential. It should be noted that jack pines from Manitoba seem particularly suited for the beetle, as it produced high amounts of *trans*-verbenol on these bolts. Additionally, all jack pine trees sampled from Manitoba were classified in the (+)-α-pinene group, the most favourable monoterpene composition for pheromone production by *D. ponderosae*. Furthermore, Manitoba's proximity to the current range of the beetle and its ability to disperse over 100 km a day in wind (Ainslie and Jackson 2011) should be considered by the province when addressing issues of beetle spread.

3.4.3 Enantiomeric composition and pheromones

Interestingly, our results indicate a closer connection between (+)-α-pinene and (–)-*trans*-verbenol production than (–)-α-pinene. This is supported by the exclusive correlation between (–)-*trans*-verbenol production and (+)-α-pinene proportions, as well as a stronger correlation with (+)-α-pinene concentrations than (–)-α-pinene. Furthermore, beetles associated with the (+)-α-pinene group, which were characterized by >51% (+)-α-pinene, produced more (–)-*trans*-verbenol than beetles associated with the moderate group, which tended to have higher (–)-α-pinene proportions. It has been proposed that (–)-*trans*-verbenol is synthesized through two enantioselective pathways, one specifically using (–)-α-pinene, which is responsible for the majority of the (–)-*trans*-verbenol pheromone component, and one using both enantiomers, primarily for monoterpene detoxification (Pierce et al. 1987; Blomquist et al. 2010). The proposed pathway is based on studies conducted on the beetle's historical hosts, such as

lodgepole pine, which has higher (–)-α-pinene content than jack pine (Clark et al. 2014; Erbilgin et al. 2014). Although enantiomeric ratios of α-pinene differ between lodgepole pine and jack pine, this does not seem to constrain (–)-*trans*-verbenol production by and attraction to *D. ponderosae* (Erbilgin et al. 2014). While other factors are likely at play, if pheromone production was strongly controlled by enantiomeric composition, changes in pheromones produced would be expected as the ratio between (–) and (+)-α-pinene changed. Alternatively, there may be a separate enantioselective pathway for each (+)- and (-)-α-pinene that function independently of each other to both produce (–)-*trans*-verbenol in an additive manner (Gries et al. 1990).

3.4.4 Concluding Remarks

We investigated the suitability of jack pine as a novel host to *D. ponderosae* and showed that the beetle can produce its primary aggregation pheromone, (–)-*trans*-verbenol, and anti-aggregation pheromone, verbenone, in jack pine trees from central and eastern Canada where jack pine is a primary forest species. Nevertheless, additional investigations are required to determine whether beetles can maintain pheromone-mediated signaller-receiver co-evolution and whether beetle attacks will be synchronized to successfully colonize naïve host trees. For example, even though we show that pheromone production varies considerably between beetle's historical and novel hosts, as well as within different populations of jack pine (Erbilgin et al. 2014), it is unknown whether pheromone production trends will show similar variations in jack pine forests as in its historical range (Birgersson et al. 1984; Grosman et al. 1997; Pureswaran et al. 2000, 2006, 2008). This variation is likely in part due to host chemistry, which appears to be highly variable across jack pine's natural range (Taft et al. unpublished data). Variations in pheromone production by *D. ponderosae* are critical to maintain stable populations, especially at the low

beetle density, in its historic habitats by controlling intra-specific behaviours, such as aggregation and mate finding (Pureswaran et al. 2008), and will likely retain a similar role in novel habitats. Finally, since pheromone production by *D. ponderosae* is linked to beetle fitness (Raffa and Berryman 1987; Raffa 2001), it may be implicated in beetles experiencing Allee Effects in invaded habitats (Liebhold and Tobin 2008; Erbilgin et al. 2014). Regardless, as *D. ponderosae* continues its eastward spread through jack pine forests, we expect its pheromone production to remain viable and an important to overcome Allee Effects in the novel habitats.

Figure 3.1 Locations of all sites from which jack pine (*Pinus banksiana*) bolts were sampled and the proportion of trees at each site classified into each monoterpenoid group. Natural ranges of jack pine and lodgepole pine (*P. contorta*) are indicated on the map. Province abbreviations in Canada are as follows: BC, British Columbia; AB, Alberta; MB, Manitoba; ON, Ontario; QC, Quebec; NB, New Brunswick; NS, Nova Scotia.

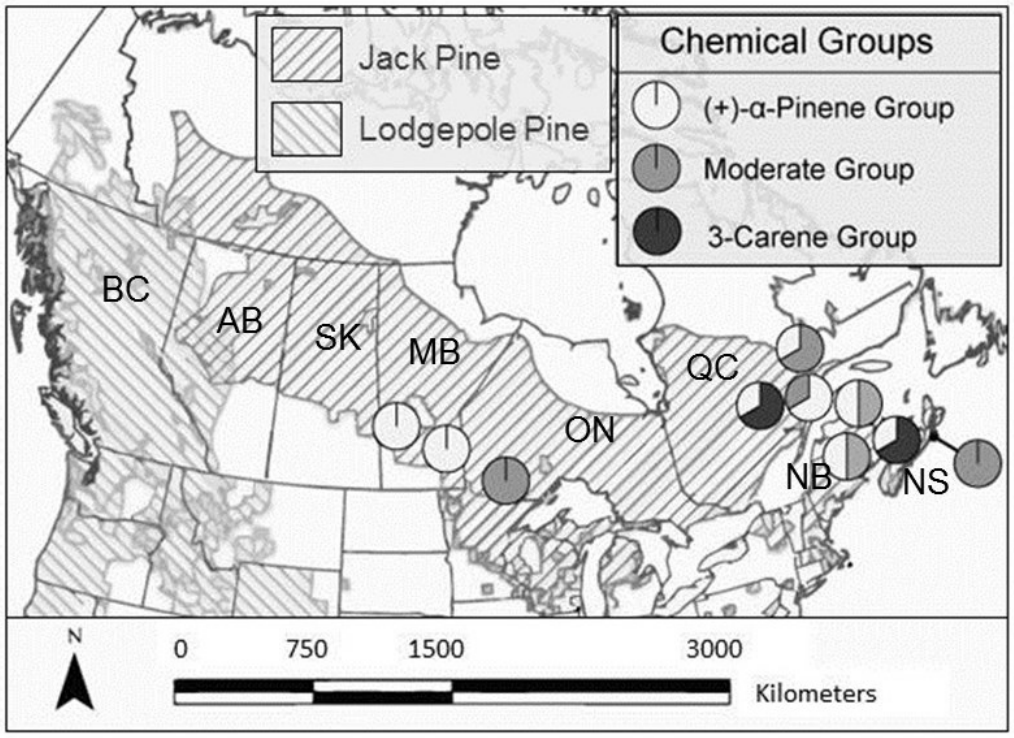


Figure 3.2. Hierarchical cluster analysis of phloem monoterpene proportions of jack pine (*Pinus banksiana*) found in bolts collected at sites in Manitoba, Ontario, Quebec, New Brunswick and Nova Scotia. The three broadest divisions of each cluster analysis were used to define phloem monoterpenoid groups. The percent values at the first two divisions represent the variance explained at those divisions. Coloured bars represent relative monoterpene proportions within each group and *n* represents the number of trees in each group. The cross-validated error was 0.497 and the standard error was 0.11.

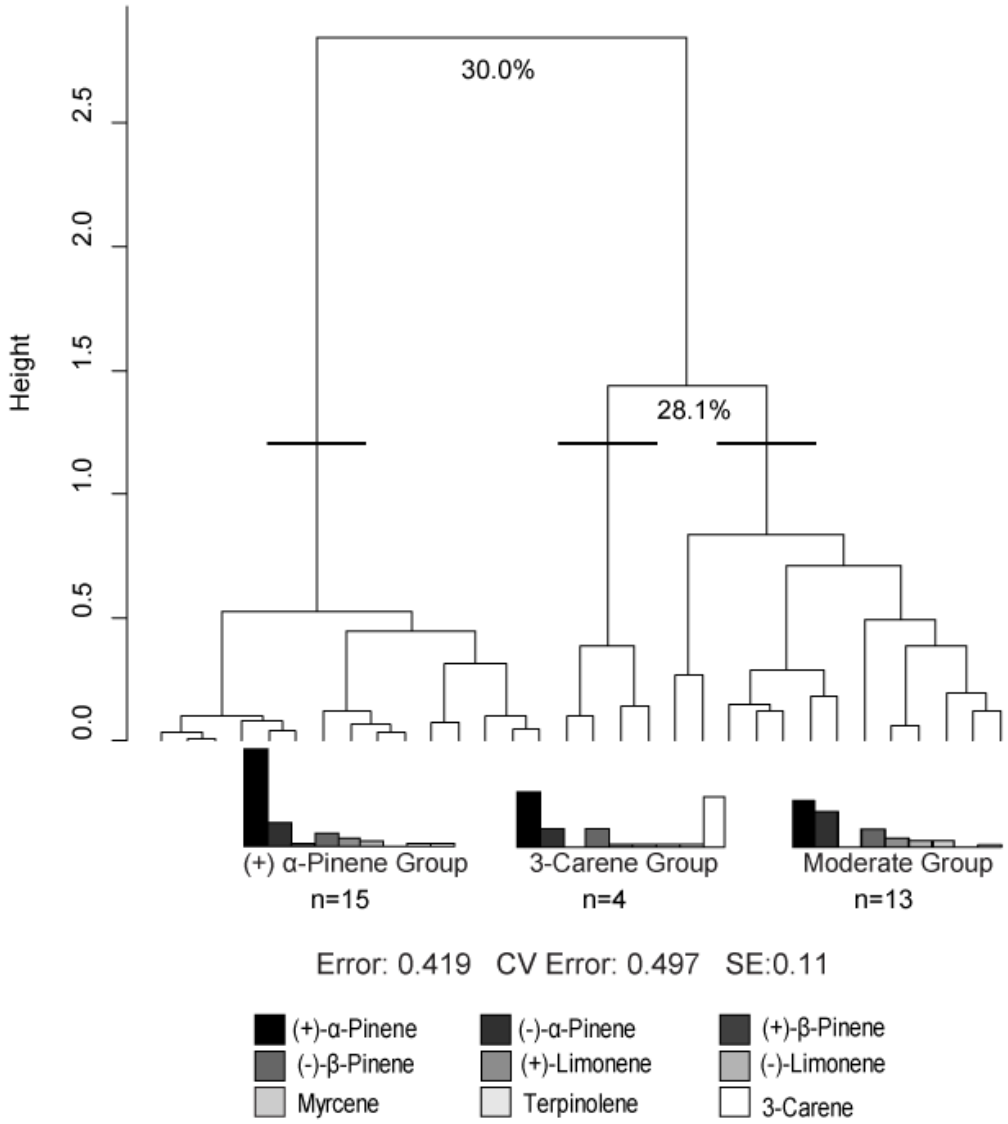


Figure 3.3. Non-metric multidimensional scaling plot of monoterpene proportions of jack pine (*Pinus banksiana*) divided by phloem monoterpenoid groups defined by a hierarchical cluster analysis. Black circles represent trees in the 3-carene group, red squares represent trees in the (+)-α-pinene group, and blue triangles represent tree in the moderate group. Solid vectors represent individual monoterpene proportions and dashed vectors represent pheromones produced by *Dendroctonus ponderosae*.

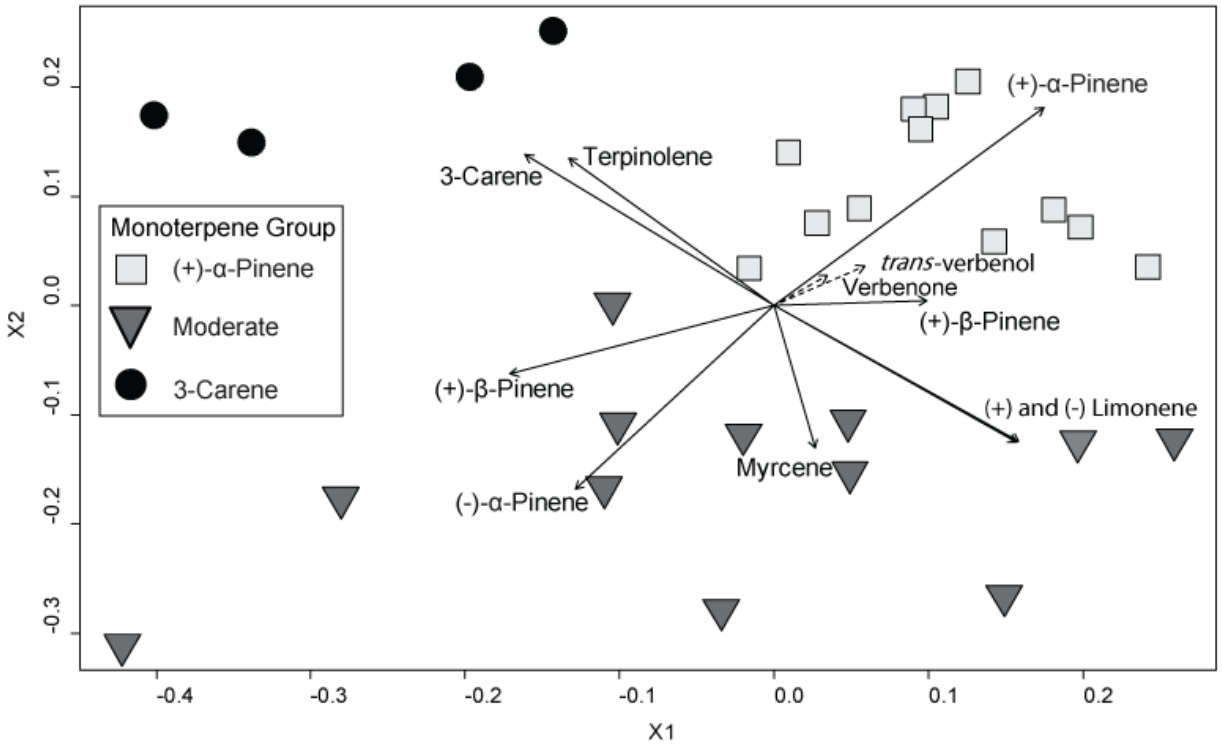


Figure 3.4. Mean (±SE) amounts (µg/ml) of (a) *trans*-verbenol and (b) verbenone released per pair of *Dendroctonus ponderosae* at an introduction point on jack pine (*Pinus banksiana*) bolts of each monoterpenoid group. Beetles were introduced into bolts in the laboratory and volatiles released from beetle entrance holes were monitored for the first 108 hrs following female beetle introduction. A male beetle was introduced to each point 24 hr after female beetle introduction Letters represent differences between groups at α=0.05 and error bars represent standard error.

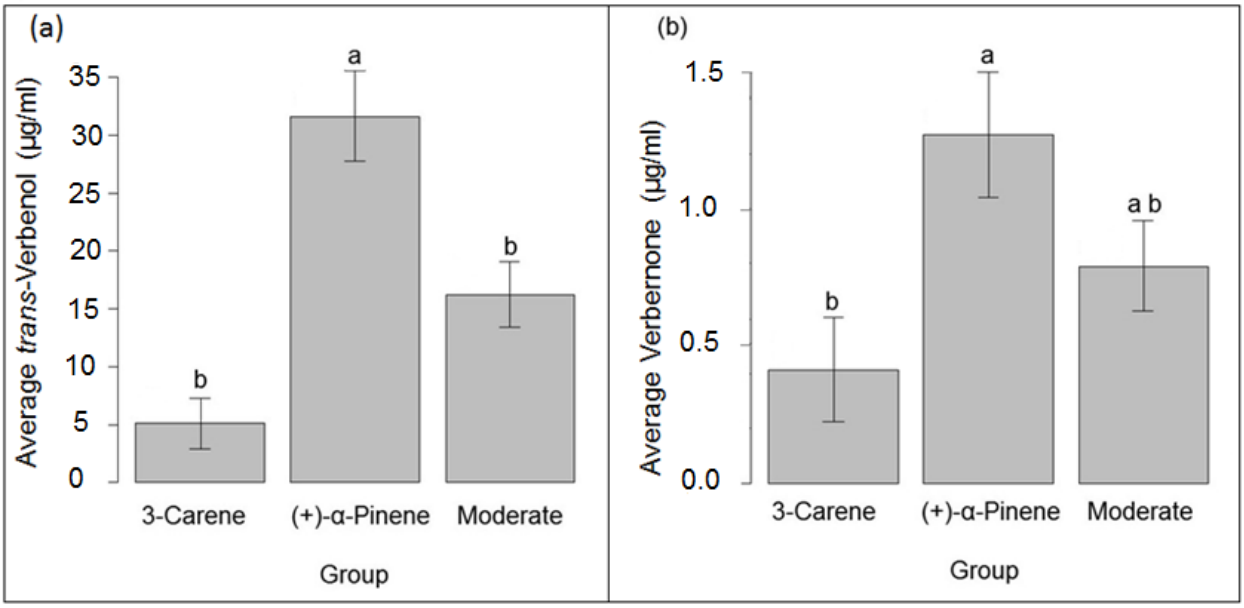


Figure 3.5. Mean amounts (µg/ml/4-hr) of (a) *trans*-verbenol and (b) verbenone released per pair of *Dendroctonus ponderosae* at introduction point on jack pine (*Pinus banksiana*) bolts in the three monoterpenoid groups. Beetles were introduced into bolts in the laboratory and volatiles released from beetle entrance holes were monitored for the first 108 hrs following female beetle introduction. A male beetle was introduced to each point 24 hr after female beetle introduction. Note different scales on axes.

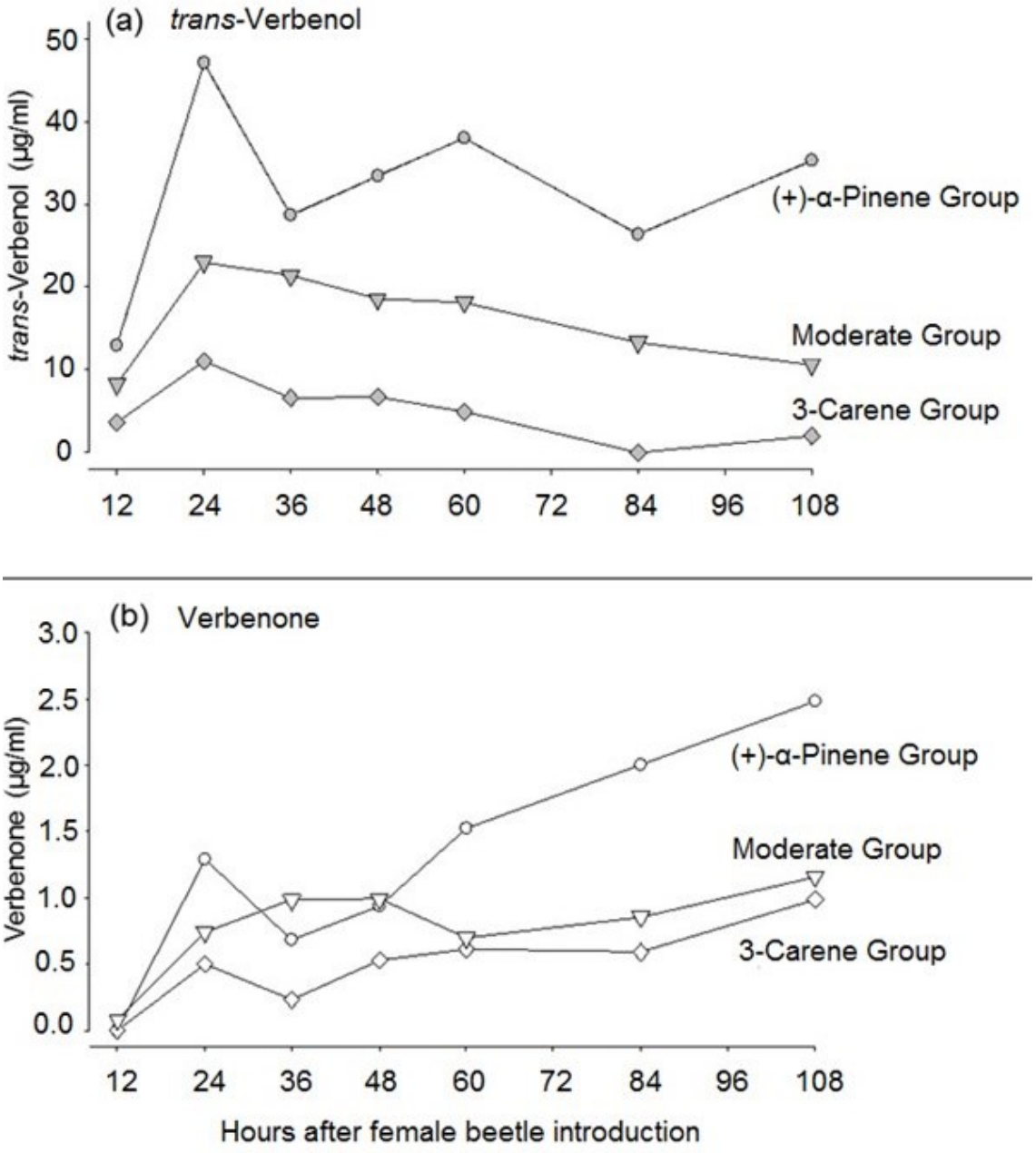


Figure 3.6. Mean (±SE) amounts (µg/ml) of (a) *trans*-verbenol and (b) verbenone released per pair of *Dendroctonus ponderosae* at introduction point on jack pine (*Pinus banksiana*) bolts from five provinces in Canada. Beetles were artificially inoculated into bolts in the laboratory and volatiles released from beetle entrance holes were monitored for the first 108 hrs following female beetle introduction. A male beetle was introduced to each point 24 hr after female beetle introduction. Letters represent differences between groups at α=0.05 and error bars represent standard error. Provincial abbreviations are as follows: MB=Manitoba, ON=Ontario, QC=Quebec, NS=Nova Scotia, NB=New Brunswick.

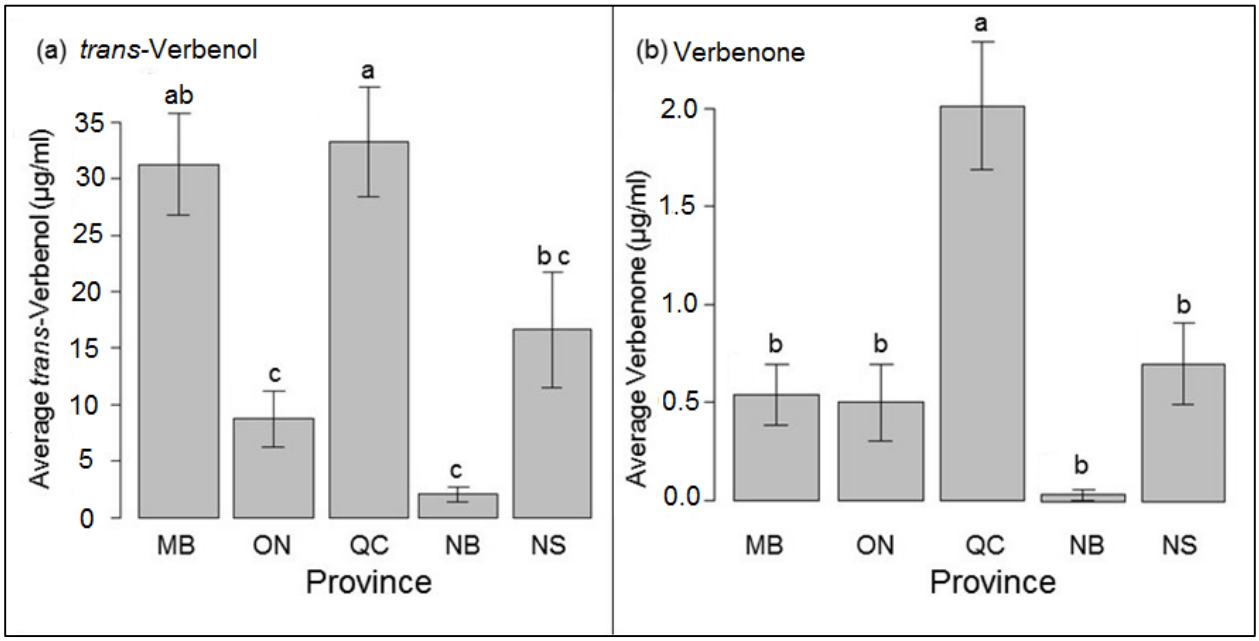


Figure 3.7. Mean amounts (µg/ml/4-hr) of (a) *trans*-verbenol and (b) verbenone released per pair of *Dendroctonus ponderosae* at introduction point on jack pine (*Pinus banksiana*) bolts collected from different provinces in Canada. Beetles were introduced into bolts in the laboratory and volatiles released from beetle entrance holes were monitored for the first 108 hrs following female beetle introduction. A male beetle was introduced to each point 24 hr after female beetle introduction. Note different scales on y-axes. Provincial abbreviations are as follows: MB=Manitoba, ON=Ontario, QC=Quebec, NS=Nova Scotia, NB=New Brunswick.

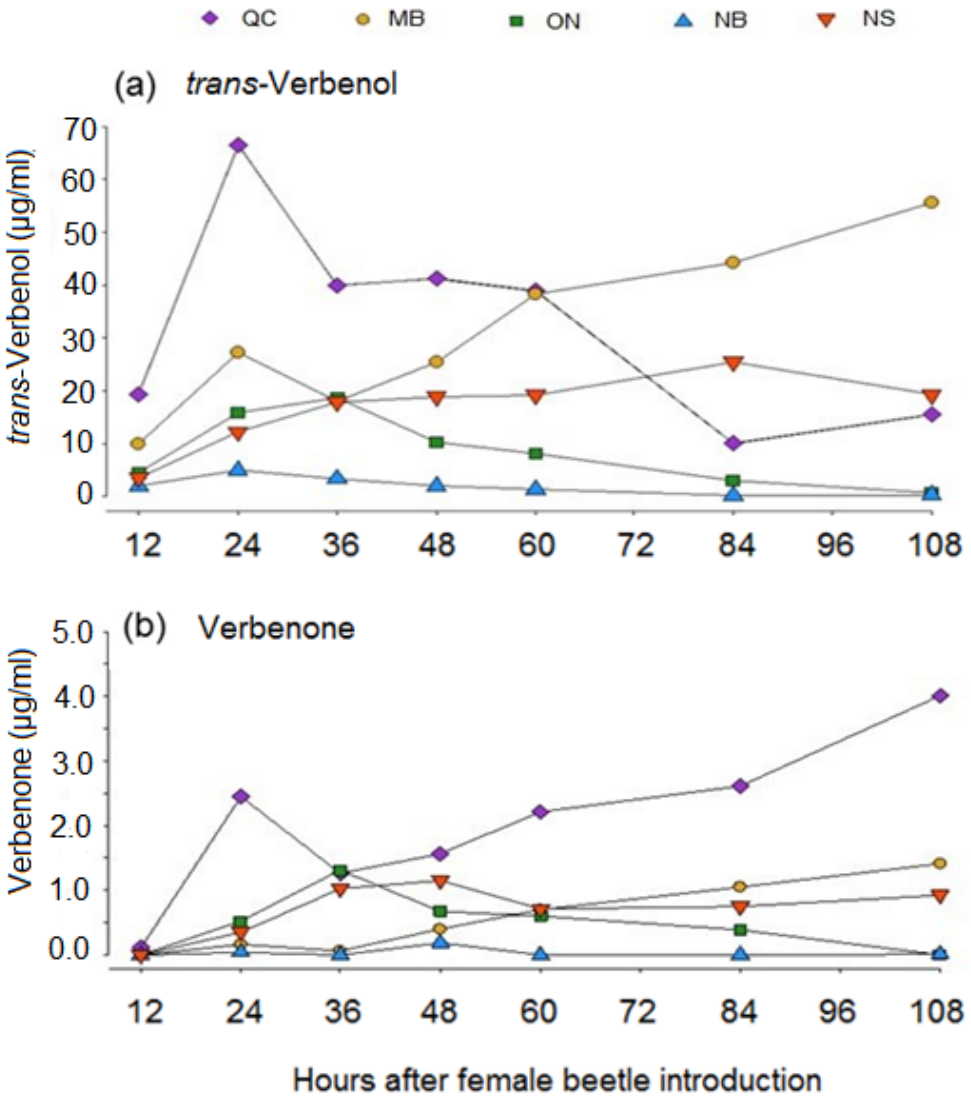


Table 3.1: Information concerning jack pine (*Pinus banksiana)* bolts, including collection sites, provinces, phloem monoterpene groups and percentages of individual monoterpenes out of total quantified monoterpenes. Site.Tree column indicates the number of trees harvested at each site. Province abbreviations are as follows: MB: Manitoba; ON: Ontario; QC: Quebec; NB: New Brunswick; NS: Nova Scotia. Monoterpene abbreviations are as follows: α-Pin: α-pinene; β-Pin: β-pinene; Lim: limonene; Myr: myrcene; Terp: terpinolene; 3-Car: 3-carene.

			Individual monoterpenes as a percent of total monoterpenes								
Site.Tree	Province	Phloem Group	(+)-α-Pin	(-)-α-Pin	(+)-β-Pin	(-)-β-Pin	(+)-Lim	(-)-Lim	Myr	Terp	3Car
1.1	MB	(+)-α-Pinene	71.65	19.65	0.18	3.27	4.06	1.04	0.00	0.16	0.00
1.2	MB	(+)-α-Pinene	64.06	24.66	0.35	9.61	0.83	0.50	0.00	0.00	0.00
1.3	MB	(+)-α-Pinene	78.40	14.96	0.57	4.21	0.43	0.67	0.00	0.15	0.60
2.1	MB	(+)-α-Pinene	62.82	13.10	0.76	21.17	0.91	0.40	0.00	0.12	0.71
2.2	MB	(+)-α-Pinene	75.02	17.87	0.68	4.64	0.67	0.38	0.00	0.09	0.65
2.3	MB	(+)-α-Pinene	61.41	6.58	0.53	7.56	13.63	9.68	0.00	0.12	0.49
3.1	QC	Moderate	38.31	42.55	0.82	10.30	4.17	2.95	0.00	0.10	0.80
3.2	QC	(+)-α-Pinene	74.51	14.04	0.74	9.27	0.26	0.33	0.00	0.12	0.72
3.3	QC	(+)-α-Pinene	67.05	12.29	0.53	4.11	8.99	6.39	0.00	0.08	0.56
4.1	QC	3-Carene	26.47	10.14	0.00	23.96	0.68	0.21	0.00	1.20	37.34
4.2	QC	3-Carene	50.25	10.11	0.00	6.89	0.32	0.08	0.00	1.58	30.79

4.3	QC	(+)-α-Pinene	66.30	23.99	0.68	5.38	2.34	0.37	0.00	0.12	0.82
5.1	QC	Moderate	43.88	45.42	0.00	1.30	0.13	0.22	0.00	0.07	8.98
5.2	QC	Moderate	46.96	37.50	0.63	3.77	6.09	4.31	0.00	0.03	0.70
5.3	QC	(+)-α-Pinene	65.75	10.01	0.46	5.42	10.36	7.39	0.00	0.06	0.56
6.1	ON	Moderate	28.01	33.89	0.45	35.08	2.57	0.00	0.00	0.00	0.00
6.2	ON	Moderate	44.21	32.03	0.38	9.76	0.50	0.32	12.79	0.00	0.00
6.3	ON	Moderate	10.99	54.26	0.44	33.39	0.46	0.34	0.00	0.12	0.00
7.1	NB	(+)-α-Pinene	66.46	11.16	0.33	15.73	3.72	2.57	0.00	0.04	0.00
7.2	NB	Moderate	43.17	11.58	0.16	13.71	18.29	12.99	0.00	0.11	0.00
7.3	NB	Moderate	42.43	13.48	0.39	3.69	4.67	3.31	31.60	0.06	0.37
7.4	NB	(+)-α-Pinene	63.69	15.94	0.19	5.75	8.40	5.91	0.00	0.12	0.00
8.1	NB	(+)-α-Pinene	73.62	16.16	0.29	8.11	1.43	0.38	0.00	0.00	0.00
8.2	NB	(+)-α-Pinene	58.30	28.16	0.26	11.58	0.99	0.51	0.00	0.20	0.00
8.3	NB	Moderate	42.38	19.44	0.11	21.42	9.64	6.89	0.00	0.11	0.00
8.4	NB	Moderate	40.35	24.76	0.20	11.13	11.64	8.26	3.64	0.03	0.00
9.1	NS	Moderate	48.45	26.00	0.37	23.44	0.49	0.35	0.00	0.09	0.80
9.2	NS	Moderate	30.95	32.43	0.18	5.91	10.31	7.31	12.53	0.07	0.30
9.3	NS	Moderate	44.17	7.70	0.43	10.63	21.39	15.20	0.00	0.08	0.42
10.1	NS	3-Carene	42.94	13.55	0.00	4.57	0.88	0.25	5.48	2.76	29.58
10.2	NS	(+)-α-Pinene	73.95	16.36	0.07	7.74	0.99	0.28	0.00	0.05	0.56
10.3	NS	3-Carene	28.92	17.83	0.00	12.21	0.43	0.26	0.00	1.34	39.01

Table 3.2: Approximate percentages of monoterpenes used to define phloem chemical groups for jack pine (*Pinus banksiana)* sampled from Canadian provinces of Manitoba, Ontario, Quebec, New Brunswick and Nova Scotia.

Chemical Groups	**Defining Monoterpene Trends (% of total quantified monoterpenes)**
3-Carene	3-Carene > 29%, Terpinolene > 1.2%
(+) α-Pinene	(+) α-Pinene > 58%
Moderate	No single monoterpene or enantiomer > 50%, 3-Carene < 9%, (+) α-Pinene < 49%

Chapter 4

Thesis Discussion

4.1 Major findings

This research shows that jack pine (*Pinus banksiana*) is a suitable host for mountain pine beetle (*Dendroctonus ponderosae)* beyond the beetle's current geographical distribution in western Canada. In addition, monoterpene composition of jack pine varies considerably throughout its range and these variations affect pheromone production by *D. ponderosae*, which will affect its survival and persistence throughout the boreal forest. This direct link between host monoterpenes and pheromone production necessitates an in depth examination of the effect that variations of jack pine monoterpene composition will have on *D. ponderosae* pheromone production.

4.2 Monoterpene variations in jack pine trees

The observed variations in monoterpene composition of jack pines show that it persists in chemically heterogeneous forests and individual trees in the same stands have different ratios of major monoterpenes. In Chapter 2, I found monoterpene composition of individual trees tended to be dominated by α-pinene, β-pinene or limonene, which led to the classification of three chemotypes based on these compounds. Furthermore, enantiomers of each chiral compound were closely correlated to each other. However, in Chapter 3, I found individual trees were dominated by either (+)-α-pinene or 3-carene or else no monoterepene persisted in notably high amounts. These differing trends in ratios of major monoterpenes are possibly explained by the different

tissues sampled (needles and phloem), as monoterpene composition varies between tissue types within a tree (Latta et al. 2000).

Moreover, our results show that jack pine monoterpene composition is under both genetic and environmental control. While monoterpenes did not simply conform to their environment, there were correlations between chemotype proportions and some climate variables. Additionally, individual monoterpenes responded differently to climate and some were correlated to climate variables while others were not. This information should be considered in the context of a changing climate and the impact of climate change on plant monoterpene composition, not necessarily with concern to *D. ponderosae,* but rather considering the larger implications of such a phenomenon, should not be overlooked.

4.2 Potential effects of monoterpene variations on mountain pine beetle

On a tree to tree basis, variable jack pine chemistry will render individual hosts more or less suitable to *D. ponderosae* colonization as the beetle is affected differently by different monoterpenes. While these effects are complex, *D. ponderosae* reared in trees with high α-pinene content produce more of its aggregation and anti-aggregation pheromones, increasing the hosts' susceptibility to colonization (Pureswaran et al., 2000; Pureswaran and Borden, 2003; Safranyik et al., 2010). Conversely, beetles in trees with high 3-carene content produce lower amounts of its aggregation and anti-aggregation pheromones. Finally, trees dominated by other monoterpenes, such as limonene, will likely present a more lethal environment to *D. ponderosae* and may represent more resistant hosts to beetle attacks (Raffa and Berryman, 1983; Reid and Purcell, 2011; Clark et al., 2014). Because of these variable effects that host chemistry has on pheromone production, factors affecting monoterpene composition of jack pine should be

considered when predicting *D. ponderosae*'s continued spread. Additionally, our results suggest that pheromone production by *D. ponderosae* varies considerably between jack pine and lodgepole pine hosts. This may affect its continued eastward spread, as the beetle's pheromones are critical to maintain stable populations in its historic habitats by controlling intra-specific behaviours, such as aggregation and mate finding (Pureswaran et al., 2008).

Furthermore, because jack pine's monoterpene composition is partially under genetic influence, trees with innate chemical resistance to *D. ponderosae* could be considered for forest management as sources for re-forestation. The range and host expansion of *D. ponderosae* may necessitate changes to the chemical landscape of its novel host, jack pine, in order to maintain an ecologically sustainable boreal forest. Jack pine has not had an evolutionary history alongside *D. ponderosae* to allow for natural selection of effective chemical defenses over millennia; therefore, the feasibility of artificially selecting trees with high levels of monoterpenes particularly toxic to *D. ponderosae,* such as limonene, for use in re-forestation projects should be investigated as a means of controlling the prolific pest. However, the natural heterogeneous chemical landscape of jack pine should also be considered, as chemical polymorphism in plants may provide better defenses against a wide range of attackers and individual monoterpenes effect attacking guilds variably (Wallin and Raffa, 2001; Raffa et al., 2005). Aiming to homogenize jack pine chemistry in order to control *D. ponderosae* could lead to increased susceptibility to other herbivores or pathogens on a landscape scale, but a controlled anthropogenic increase in the proportion of the relatively rare limonene (needle) and 3-carene (phloem) jack pine chemotypes may help inhibit *D. ponderosae* populations in novel habitats.

In conclusion, it is still difficult to predict how the varied chemical landscape of jack pine will affect *D. ponderosae*'s spread into the boreal forest, though the novel host's monoterpene

composition will certainly be an important factor for the beetle's overall success in this invaded territory. Because the observed jack pine chemotypes and monoterpene groups persist as heterogeneous stands, large areas of increased susceptibility or resistance to *D. ponderosae* on a landscape scale are not expected. However, I provide compelling evidence of *D. ponderosae'*s ability to colonize jack pine trees beyond its current range and as the beetle continues its eastward spread through the boreal forest, I expect its pheromone production to remain viable and an important aspect of its survival and persistence.

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