**Landscape Genetics of *Cronartium ribicola***

by

Simren Brar

B.Sc., The University of British Columbia, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Forestry)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2012

## Abstract

**White pine blister rust, *Cronartium ribicola* (Basidiomycota, Pucciniales), is a macrocyclic (5 different spore types) heteroecious (requires two hosts) rust that alternates on *Ribes spp*. It is an exotic pathogen in North America that causes high levels of mortality of pine in the subsection Strobus (white pines). To better understand the epidemiology of the pathogen, the population structure of white pine blister rust in North America was investigated. Thirty one single nucleotide polymorphism (SNP) markers have been developed to genotype 1341 individuals from 76 populations from across North America including samples from diverse landscapes. In western Canada, sampling was structured to contrast different landscapes and pine hosts. Distance-based and Bayesian likelihood methods indicated the presence of two major genetic clusters: 'eastern' and 'western' in North America, separated by the Great Plains that act as a barrier to gene flow. The eastern cluster had greater genetic diversity than the western cluster, which confirms that multiple introductions occurred in eastern North America in contrast to a single introduction in the west. Two populations, New Mexico and Minnesota were each found to form a separate cluster in some assignment analyses and the distance based analyses clearly placed them outside of the main clusters. Both of these populations displayed the hallmarks of population bottlenecks, i.e. low genetic diversity and/or inbreeding. The pathogen was discovered in New Mexico in the 1970's, almost a century later than the populations in the two major clusters. Although white pine blister rust has been present for longer in Minnesota, the population parameters strongly suggest a bottleneck and a barrier to gene flow between Minnesota and the populations within the eastern cluster. However, no landscape, host, or other patterns could be correlated with these clusters. A rare SNP was detected in Smithers, a population with high levels of inbreeding located at the northern most extent of the rust. Understanding the population structure will provide great knowledge of the rust for breeding programs and deployment of rust resistant pines.**

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## Acknowledgements

I would like to thank my research committee, for all their input and expertise. A special thank you to Dr. R. Hamelin for giving me the opportunity to work with him, and Dr. Y. El-Kassaby for all his encouragement and for being a great mentor.

I was fortunate to have many people help with collection without all their help this project would not have been possible, M. Cleary, E. Harvard, M. Murray, R. Reich, C. Smith, N. Stromberg-Jones, A. Woods, S. Zeglen, S. Beauseigle, C. Eisenhut, M. Gratecap, T. Kess, A. Mohan, A. Vialle, and B. Vickers, thank you for all your help with sampling. R. Russell and E. Chalmers for your help in the lab. C. Tsui, J. Klapste and B. Dhillon for all their help with analysis and technical work.

To M. Cleary, I am truly grateful for the opportunity to have worked with you. You exposed me to the world of forest pathology. It was an experience that allowed to me learn and grow and share in your excitement and passion for the field. T&J Qureshi thank you for sharing your expertise in the field, I cannot imagine better people to have worked with.

Finally to my family, I would not have come this far without their unconditional love and support. Mom and dad I am truly grateful for all your encouragement. To my grandfather who taught me the value of an education. J.Pirnak and D. Mulukutla for keeping me sane throughout the years.

*To my Papa ji, my inspiration.*

# 1 Introduction

## 1.1 Ecology

*Cronartium ribicola* J.C. Fisch is a basidiomycete fungus in the order Pucciniales (the rusts). This fungus is a pathogen that is responsible for white pine blister rust, a disease that affects pines in the subsection Strobus (white pines), resulting in branch and stem cankers that can be lethal. The pathogen was introduced in North America at the end of the 19$^{th}$ century and has since caused one of the most spectacular forest disease epidemics.

The pathogen was first reported in 1856 in western Russia. However, it was not known in Europe and Asia prior to the introduction of *Pinus strobus*, the eastern white pine, from North America (Hummer 2000). It is believed that the rust was introduced into North America on seedlings that had been grown in Europe and had been exposed and infected by the pathogen. The disease was first reported in eastern North America in 1906 on infected seedlings that had been imported from France, Germany and Holland (Mielke 1943); by 1911 the epidemic had spread and rust-infected nursery stock was found in New Hampshire, Vermont, Massachusetts, Connecticut, Pennsylvania, Indiana, Ohio, Ontario and Quebec indicating multiple introduction (Hummer 2000).

The situation was different in western North America where a single documented introduction of white pine blister rust was reported from imported seedlings from France in 1910 (Hummer 2000; Hunt 2009; Mielke 1943). Hunt (2009) calculated the spread in western North America to be 66 to 71 km/year. From the initial introduction in Vancouver in 1910 the pathogen spread east and south. By 1913 it was found in Washington and by 1918 in Oregon (Hunt 2009). From southern Oregon the rust entered California in 1930 but did not reach the Sierra Nevada until 1964 (Schwandt et al 2010). The first report of the rust in

Wyoming was in 1978, and in 1998 it was found in Colorado on limber pine; it is believed that this introduction originated from Wyoming (Johnson and Jacobi 2000). White pine blister rust was first reported in isolated populations in New Mexico in 1990 on southwestern pine (Hawksworth 1990), in South Dakota on Limber pine in 1992 (Lundquist et al. 1992), in North Dakota in 1993 (Draper and Walla 1993), and in Arizona in 2003 (Frank et al. 2008). It was first discovered in Alberta on Limber pine in 1952 (Schwandt et al 2010).

White pine blister rust infects all native white pine species including Western White Pine (*Pinus monticola* D. Don), Whitebark Pine (*P. albicaulis* Engelm), Eastern White Pine (*P. strobus* L.), limber pine (*P. flexilis* James), sugar pine (*P. lambertina* Doug), southwestern white pine (*P. flexilis* James var. *reflexa* Engelm. syn. *P. strobiiformis* Engelm.), Rocky Mountain bristlecone pine (*P. aristata* Engelm), foxtail pine (*P. balfouriana* Grev. & Balf.) and Swiss stone pine (*P. cembra* L) which is not a native pine to North America but is infected by this pathogen. The only species that has not been yet infected is Great Basin bristlecone pine (*P. longaeva* D.K. Bailey) (Schwandt et al 2010,Tomback and Achuff 2010). However in inoculation studies, Great Basin bristlecone pine was found to be susceptible (Hoff et al 1980).

Since all North American white pine species are susceptible, the distribution of the pathogen tends to follow the distribution of the pine host. White pines cover a wide geographical, elevational, and landscape distribution and at least one species of pine is found in most conifer forests (Geils et al 2010, Schwandt et al 2010,Tomback and Achuff 2010). This results in a wide distribution of white pine blister rust.

## 1.2 Life Cycle

White pine blister rust is a heteroecious macrocyclic rust, which means that it requires an alternate host to complete its life cycle and produces five distinct spore types. Infection of the pine host occurs after germination of a single meiosis-derived haploid monokaryotic basidiospore (Fig. 1). Following germination under conducive temperature and pH conditions, fungal hypha enter needles thorough stomata (Hansen and Patton 1975). The first symptoms of the pathogen are yellow spots on needles that are caused by the pathogen damaging the chlorophyll and unmasking carotene in the mesophyll tissue of the needle (Kinloch 1992). After entering the pine needle, mycelium continues to grow down the needle into the phloem and spreads to the branches and then the main stem of the tree when the branches are close to the main stem.

On the outer bark of the infected branch or stem, the hyphae form the spermogonia in late summer, where the haploid spermatia are produced. The spermatia are produced in sweet nectar that attracts insects, in a process akin to pollination, insects carry the spermatia to other cankers where they are deposited into spermogonia and germinate to fuse with the receptive hypha (Hunt 1984). This results in the dikaryotization of the mycelium. At least one year following this, fruiting structures called aecia produce dikaryotic aeciospores. These spores can infect the telial host, usually a member of the Grossulariacea (the currant family) but also the Orobanchaceae family (Macdonald et al 2006). In the spring aeciospores enter the telial host through the stomata and produce dikaryotic mycelium, from which uredia are produced (Patton and Spear 1989). The urediniospores are produced on the uredia and these spores can re-infect the telial host. This represents the inoculation build-up phase of the life cycle. Before winter, the uredia transition into thick-walled telia, long hair-like projections

found on the abaxial surface of *Ribes* leaves. Teliospores are not disseminated but are the site where karyogamy occurs between the two nuclei, followed by meiosis. This results in the production of 4 haploid basidiospores per cell that are infectious on the aecial host. Genetically, sexual reproduction takes place over the two hosts, with dikaryotization occurring on the pine host, and karyogamy and meiosis on the telial host. The uredospores represent the asexual reproduction phase.

## 1.3 Epidemiology

### *1.3.1 Climatic Conditions*

Understanding the epidemiology of white pine blister rust is important for restoring white pine in forests. Key factors affecting host infection are climatic variables such as temperature and humidity which can differ across landscapes or microclimatic sites. These can be used to determine the likelihood of rust infection and to assess the rust hazard. Relative humidity is usually considered to be a predictive factor in determining rust infection, but temperature also plays a very important role in white pine blister rust epidemiology. All spore stages require a minimum temperature and relative humidity for dispersal and germination (Van Arsdel 2006).

Epidemiological parameters conducive to white pine blister rust epidemics have been studied most extensively in the U.S. Lake States. In forest openings the temperature regimes vary more than in the closed canopy. In large openings the temperature is much warmer during the day, reaching 35°C but is cooler at night, dropping below 5°C; in comparison, the temperature under the forest canopy is cooler during the daytime and warmer at night (Van Arsdel 1962). This creates extreme conditions in forest openings that are not favorable for

rust infections; accordingly, it was observed that *Ribes* generally do not have rust infection in large openings (Van Arsdel 1972). However in smaller openings infection occurs at greater rates since a more even cool and wet microclimate is created by the opening edges which favours rust infection (Van Arsdel 1972).

One of the most important environmental factors for rust infection is humidity. High levels of humidity are required for spores to germinate and penetrate the leaves or needles. At a fine microclimatic scale, leaf temperature can be higher or lower than the air, causing dew formation. The dew allows for spores to adhere to the surface and provides conditions that are conducive to spore germination. This provides an explanation for the understory white pines generally being more infected than the mature pines (Van Arsdel 1972).

### *1.3.2 Spore Dispersal*

The success in the rapid and extensive spread of white pine blister rust can be attributed in large part to the dispersal of its spores. Dispersal distance varies among the spore types, some being restricted to more localized spread while others are able to travel long distances. The description of spore dispersal pattern can be complex, but generally, the relationship between distance from the source and spore density is an inversed J-shaped with a long tail. This means that more spores will fall close to the source, but some spores can be carried over long distances (Blenis et al 1993).

Two important factors that play a key role in the epidemiology of white pine blister rust are the spore resistance to environmental stress and the wind currents. Some spores are sensitive to desiccation and UV light and are disseminated at night. Close to lakes, breezes are formed because of the difference in water and land temperatures. This wind current is

able to carry spores over water bodies, resulting in infections of trees at distances of several kilometers from the source where they are released (Arsdel 1972). Forest edges also create conditions that can result in spores being released and disseminated over some distance. The air current around forest edges flow from the ground close to edges into openings then up into the crown of trees and then back down into the opening (Arsdel 1972). In an experiment, aspen trees were planted around pines, and the pines in the middle of the field had 50 times more infection than those around the edge (Arsdel 1972).

Dispersal potential is dependent on the characteristics of the different spore types. One spore type, the teliospore, is not dispersed at all. These spores still play an important role since they are the site where karyogamy and meiosis take place, producing basidiospores. Spermatia are dependent on insects for dispersal. Insects attracted to the nectar-like drops forage from spermogonia to spermogonia on other sporulating cankers and carry spermatia thereby effecting spermatization (Hamelin et al 2005). Most of the insects involved in this process are small diptera and have limited ability to fly long distances. This leads to localized spread.

The other spores are dispersed medium to long distance. Urediniospores are wind dispersed asexual spores that can generate multiple sporulation and infection cycles within a season, resulting in local increases of inoculum. Viable urediniospores have been found 15 – 275m away from the source, but infection was found to decline as distance increased (Zambino 2010). Moisture, although important for rust infection, could also restrict movement by causing the spores to clump together (Zambino 2010). Aeciospores and basidiospores are most likely to be involved in medium to long distances dispersal; these spores can be carried by wind or in humid air masses of different densities and can be

transported at varying distances depending on direction and strength of air movement (Van Arsdel 2006). Aeciospores have a thick cell wall and are pigmented, providing them with protection against desiccation and damage from UV light. These spores are believe to travel up to hundreds of kilometers from the source (Frank 2008, Van Arsdel et al 2006) and could be involved in long distance dispersal. Basidiospores are more delicate spores that are prone to desiccation and UV light damage and are commonly dispersed at night (Van Arsdel 1972). These spores have been shown to travel up to 1.6 km in air masses (Van Arsdel et al. 2006; Zambino 2010).

## 1.4 Alternate Host

Rusts in the genus Cronartium are heteroecious and therefore require alternation between two hosts to complete their life cycles. The telial hosts of *C. ribicola* play a crucial role in the epidemiology and management of white pine blister rust. The main telial hosts belong to the Grossulariaceae and include plants in the genus *Ribes*. There are many species in this genus and the center of diversity of *Ribes spp* is believed to be North America (Hummer 2010). When *Ribes* are in close proximity to pines or other *Ribes*, they can play a role in the rust epidemiology. However, other factors are relevant, such as their susceptibility to local races of rust, the timing of sporulation, and the ability of *Ribes* to shed their leaves before the next spore stage (Zambino 2010). Proximity between the two hosts is an important factor for spore dispersal and spread of the pathogen, as incidence of rust infection declines with distance from the source (Van Arsdel 1961, Zambino 2010).

*Ribes* species vary in susceptibility to white pine blister rust; species can exhibit monogenic resistance or multigenic resistance (Zambino 2010, Van Arsdel 2006; Zambino and Macdonald 2004). Also, resistant *Ribes* have been developed and are commercially available. However, races of *C. ribicola* that can overcome resistance in the *Ribes* cultivars have been reported (Anderson and French 1955).

Efforts to manage and control the spread of white pine blister rust have been focused on *Ribes* being the only alternate host for this pathogen. In a laboratory test, *C. ribicola* was shown to infect *Castilleja miniata* Dougl. Inoculation with aeciospores produced lesions with telia but no uredinia (Hiratsuka and Maruyama 1976) but this had not been observed in nature. In 2004 in Idaho, leaf lesions with telia similar to those produced by *C. ribicola* were observed on four different species of *Orobanchaceae*: *Pedicularis racemosa* Dougl., *P. bracteosa* Benth., *C. miniata* and *Castilleja rhexifolia* Rydb. (Macdonald et al 2006, Mulvey 2011). These findings have serious implications on the epidemiology of this pathogen; more alternate hosts could support an increase of inoculum as well as aid in spreading the rust to new or less infected sites.

## 1.5 Management Strategies

Controlling and managing white pine blister has proven to be a difficult task, though many attempts have been made since the 1900's. Management has gone through different phases as a result of changing environment and management objectives. The first management phase was the eradication of *Ribes* followed by chemical control and silviculture practices; the current phase is genetic resistance and tolerance in the pine (Geils et al. 2010 King et al. 2010, Zeglan 2010). The eradication of *Ribes* was deployed as a

method to stop the spread of white pine blister rust and was based on the knowledge that the alternate host of white pine blister rust was necessary to complete the life cycle. Removing it was believed to result in reducing the spread of the rust. Eradication involved the removal of *Ribes* by uprooting the plants in pine stands as well as in surrounding areas to create a *Ribes*–free barrier around the pine trees (Martin 1944). It was recommended that all *Ribes* within 180-275 meters of pine hosts be eradicated (Zambino 2010). Although this effort lasted for decades, it was an ineffective approach. Among the reasons for this failure are the following: 1) finding and uprooting all *Ribes* plants proved extremely difficult and plants could re-establish rapidly; 2) the distance for the barrier around the pine stand was established arbitrarily and was not wide enough; 3) the timing of the removal was often after the spores had already been released from the *Ribes* to the pine hosts, therefore the most important part of the disease cycle was not interrupted (Martin 1944). This approach to eradication was also very labour intensive and expensive because sites needed to be revisited frequently to ensure *Ribes* had not re-established in the stands (Zambino 2010). Local eradication was somewhat successful in eastern white pine stands around plantations or nurseries in targeted areas. However, in western white pine stands this proved much more difficult because of the difficult terrains and abundance of alternate hosts; *Ribes* could not be decreased to a level that would have impact on rust infection (Liebhold et al 1995).

Following the failure of *Ribes* eradication to generate satisfactory control of white pine blister rust, focus shifted to chemical control as a potential solution. Two chemicals, cycloheximide and phytoactin, were shown to kill the pathogen within cankers, but treating individual cankers was impractical (Maloy 1997). It was discovered that cycloheximide when mixed with fuel oil and sprayed within the bottom 6 feet of the tree would be translocated up

the main stem. This practice became operational in Idaho forests, but it was eventually found to be ineffective, with little effect on the epidemic (Maloy 1997). Purple mold, *Tuberculina maxima* Rostr., was discovered on many white pine blister rust cankers producing spermatia and aecia and was believed to act as a biological control by supressing sporulation (Maloy 1997, Zeglan 2010). It has not been successful at controlling white pine blister rust because it colonizes older cankers and often dies before it is able to overrun the rust (Zeglan 2010).

Thinning and pruning are common management strategies against white pine blister rust. Delayed thinning of a white pine stand once it has reached the age of 25 allows trees to self-prune. This results in shading out *Ribes*, which are shade-intolerant. This could result in reduced infection. In addition, infection is easy to identify in 25 year-old trees and can be thinned out of the stand. Pruning is the most common silvicultural management strategy for white pine blister rust. Pruning infected branches, especially on lower branches, can increase the survival of the trees that are already infected. Hunt (1982) reported that 75% of cankers found on 12-30 year old western white pine in British Columbia were below a height of 2.5m. Pruning can also alter the microclimate and reduce some infections. White pine blister rust favors cool moist conditions for infection. Therefore, removing some branches could increase the amount of light and air circulation, thereby making conditions less conducive for infection (Zeglan 2010).

### *1.5.1 Hazard Rating*

Integrating information of the epidemiology (climate, proximity to host, spore dispersal) of white pine blister rust has allowed for hazard zones to be mapped. Hazard zones can be used to determine areas of high infection probability and to develop zone specific management

strategies (Van Arsdel 2006). Zone boundaries are designated based on the distribution of pine and *Ribes* with consideration of the regional climate to reflect the distribution and abundance of infection of the rust on the pine. These zones are dynamic and need to be reassessed at regular interval, as pine and *Ribes* populations can change (Van Arsdel et al 2006).

Hazard ratings have been developed for many areas in North America. In Quebec, hazard zones were based on temperature, humidity, altitude, and topography; these factors reflected the probability of infection by the rust. The province was separated into four zones; in zone 1 average summer temperatures are above 20°C and the elevation is low with a flat landscape. In Zone 2 temperatures occasionally reach 20°C, elevation is up to 300 m with some elevation changes. In zone 3 temperatures above 20°C are never encountered and the topography varies, with 300-400 m mountain ranges; finally, in zone 4 the topography is variable with some mountains higher than 400 meters. Management recommendations for high hazard sites are to avoid planting in depressions, in kettles, or in sites where *Ribes* are abundant (Lavallée 1974; 1986).

Similar zones have been establishes in the Lake States (Wisconsin, Minnesota and Michigan), where four zones have been delineated based on elevation and proximity to a lake accompanied by management recommendations for each zone. In zone 1 lowland south and lowland central region has an elevation below 300 M above sea level; there is no management required in this zone because losses due to rust are below five percent. Zone 2, the moderate south central midlands has a moderate hazard with elevations between 300 and 400 m. Recommended management strategies are *Ribes* eradication and avoidance of planting pines in areas where cool pockets of air pool at night. Zone 3, the northern zone, has

an elevation less than 500 m and it is recommended that all *Ribes* be eradicated. In addition, a 30 m buffer zone without pine planting is recommended. Zone 4 in the northern highland and northern lake shore, rust conditions are very favorable and long distance dispersal can occur. Management strategies include *Ribes* eradication with a buffer zone, and planting of only rust resistant trees. In addition, an overstory of broadleaf trees can be used to reduce inoculum dispersal (Van Arsdel 1961).

British Columbia has been given a low hazard rating based on canker incidence and growth (Hunt 1983). High levels of white pine blister rust mortality are observed in British Columbia but the low hazard rating is based on a comparison with canker incidence in Idaho. The greatest incidence of cankers in BC was found between sites on slopes and flats; sites on the slopes were subject to high degree of rust infection due to wind currents causing cankers higher in the canopy that could lead to stem infection (Hunt 1983). Generally the low level of variation in canker incidence among sites negates the need to delineate zones in British Columbia (Hunt 1983).

## 1.6 Resistance Mechanisms

White pine blister rust is an introduced pathogen in North America and has not co-evolved with its host; however there is low level some natural resistance found among pine trees (Kinloch 1992). of natural resistance was found in most white pine species for which screening was conducted (Kinloch 1991). This naturally-occurring resistance has been used in breeding programs in North America with the aim to incorporate resistance into white pines for deployment as a management option. There are three main types of resistance found in white pines. The first is ontogentic resistance which is associated with tree age; older trees

are less susceptible to primary needle infection. The second is partial resistance, whereby trees are infected but to a lesser degree than susceptible trees; trees can tolerate infection because of features such as slow canker growth, early needle shed and bark reactions. The last is Major gene resistance (MGR), which follows the classical gene-for-gene model.

### *1.1.6 Major Gene for Resistance (MGR)*

Major gene resistance (MGR) is a resistance mechanism that elicits a hypersensitive reaction (HR) and is controlled by a dominant gene (R gene). A HR reaction occurs when the cells surrounding the intruding fungal hyphae undergo a type of programmed cell-death resulting in necrosis. Since rusts are obligate parasites, this results in fungal death and prevents any further spread of the pathogen. R genes are believed to follow the classical gene-for-gene relationship with the R gene in the pine recognizing an avirulence (avr) gene in the rust (Flor 1971). R genes are generally controlled by a single locus; but different alleles at a single R gene locus can recognize different avr alleles in the pathogen (Kinloch 1996).

### *1.6.2 Cr1*

A MGR, called Cr1, was first discovered in the sugar pine, *P. lambertiana*, at the Happy Camp site in California, where among infected sugar pines, some exhibited resistance (Kinloch 1970, Kinloch 1977). Cr1 is found at low frequencies and does not follow any climatic trends but does increase along a latitudinal cline from northern to southern Sierra Nevada (Kinloch 1992). The Cr1 resistance was characterized by conducting segregation studies. The Cr1 gene showed a 1:1 Mendelian segregation, thereby confirming that it is

controlled by a single gene, which made this gene amenable to rapid genetic improvement. Random amplified polymorphic DNA (RAPD) markers were used to map the gene for resistance in Sugar pine. Ten loci were linked to the gene and segregation data was obtained from five families (Devey et al 1995).

### *1.6.3 Cr2*

A major gene for resistance has also been found in *P. monticola*. The Cr2 gene was first found in a naturally regenerated stand in Champion Mine, Oregon (Kinloch et al 1999). It was detected because the surrounding trees had been attacked and killed by white pine blister rust and only a small non-infected population remained (Kinloch 2003). Cr2 also exhibits a hypersensitive reaction, however altered Mendelian ratios were observed in some resistant families suggesting the penetrance of Cr2 is influenced by the genetic background (i.e. the combination of parents that are crossed). Certain parents had a greater influence on the variation of penetrance than others (Kinloch et al 1999). Cr2 is also rare and found at detectable levels in the Sierra Nevada and Central Cascades but is undetectable further north of the Cascades, Rocky Mountains and Coast Mountains of North America (Kinloch et al 2003). Seedlings with the Cr2 gene were confirmed to be present in British Columbia and the original seed source was from Oregon (Hunt et al 2004). Resistance has been identified in most pine species that have been evaluated. A resistance gene, Cr3, has been identified in southwestern pine, and HR reactions have been observed in limber pine (Kinloch and Dupper 2002; Vogler et al. 2006).

In addition to the genetic characterization of resistance, there have been extensive molecular analyses of the host-pathogen interactions. Resistance genes have been observed in

most pines that have been screened for resistance. Studies have been conducted to identify the pathogenesis-related proteins in the rust during host-pathogen interactions. Monoclonal antibody analysis of susceptible and resistant sugar pines determined that 90% of susceptible seedlings had fungal antigens compared to only one of the resistant seedlings. This demonstrated the potential use of monoclonal antibodies as probes for screening resistant seedlings (Ekramoddoullah and Tan 1998). Proteins identified to belong the PR 10 family of pathogenesis-related proteins were up-regulated in white pines when infected by the rust (Ekramoddoullah et al 1999). A previously described protein called *Cro r* I was also found in a significantly higher amount in both western white pine and sugar pine, but unlike other proteins that are thought to be involved with pathogenesis-related reaction in trees, this protein is excreted by the rust (Ekramoddoullah et al 1998). However, there are still many unanswered questions and the proteins that are involved in resistance have not yet been identified.

### *1.6.4 Races of Rust*

One of the challenges facing the deployment of MGR is the rapid evolution of rust races that can overcome the resistance. Indeed, theoretically a change at a single locus in the rust can be sufficient to avoid the host resistant gene recognition. This pattern can be even more pronounced in resistant trees than in resistant crops given the long crop rotation of trees.

One or more virulent race of *C. ribicola* have been reported that can overcome Cr1 on sugar pine. Trees that have shown resistance for 14 years have become infected with the rust (Kinloch 1981). This race carries avCr1 and is believed to be present at a low

frequency. However, it appears that the avCr1 does not spread outside of the Happy Camp valley where it was originally described. It has been speculated that environmental factors could prevent its widespread distribution. It is also possible that the avCr1 gene carries some slight selective disadvantage that prevents its increase outside of the range where Cr1 originates (Kinloch and Dupper 1987).

Cr2 resistance in *P. monticola* has also been overcome by *C. ribicola*. At the Champion Mine site, P. *monticola* seedlings that carried Cr2 have become infected with *C. ribicola*. The ability of the pathogen to infect trees could be due to the evolution of *C. ribicola* races that can overcome the HR response (Kinloch et al 2004). Seeds from trees carrying the Cr2 gene were planted in southern BC. After 15 years there are no reports of infection and it appears that the Cr2 gene is stable in this area even though high mortality occurs on susceptible plants (Hunt 2004).

There is some specificity among rust populations. The population at Champion Mine was able to overcome resistance in western white pine (Cr2) but not in sugar pine (Cr1) (Kinloch 1999). It was determined there was one or more different races of white pine blister rust because new infections on previously resistant trees were observed (Kinloch 1980).

The impact of deploying resistant genetic material could also have an impact on the rust population, as measured by DNA-based markers. Significant differences were found in population genetic parameters measured using amplified fragment length polymorphism (AFLP) markers between natural stands and plantations Cr genes (Richardson et al 2008). The lowest diversity was found at Happy Camp where sugar pines carry the Cr1 gene. The highest diversity was in a western white pine plantation in Idaho where trees were selected for partial resistance. The low diversity at the Happy Camp site suggests that the selection for

the rust race carrying the vcr1 gene is causing a genetic bottleneck, possibly because of a selective sweep (Richardson et al 2008 and 2010).

### *1.6.5 Quantitative Resistance*

One of the strategies to avoid the evolution of pathogen races that can overcome resistance is the development and use of quantitative or partial resistance. The breeding program for *P. monticola* resistance in British Columbia has put a strong emphasis on this idea. This has resulted in the deployment of trees that comprise both MGR and partial resistance. This strategy has been beneficial so far because R gene resistance seems to remain effective 15 years after deployment and ontogenic resistance (the onset of phenological or physical resistance, related to tree maturity) has since set in and it appears that these pines will be protected against the rust (King et al 2010).

Breeding programs were developed to capture traits such as early needle shed and slow canker growth. The first breeding program was started in Idaho based on crosses of White pine conducted by Bingham (1983), looking for trees that showed phenotypic resistance in the form of early needle shed. In these trees, infected needles were shed upon infection preventing the rust from reaching the stem of the tree. However there are some limitations of this resistance reaction. In British Columbia the same resistance results obtained in Idaho could not be reproduced (Hunt 1998). This may be due to the potentially longer growing season with cool weather in spring and early summer allowing the basidiospores to infect pines for a longer period of time (Hunt et al 2000). On the British Columbia coast, basidiospore infection can occur as early as May but usually occurs around June whereas in the interior of BC infection can occur as early as June but typically occurs in

July (Hunt 2007). If basidiospore infection is occurring earlier in the season it may be too early for early-needle shed to occur. The Idaho resistant trees are successful in the interior of BC which can be attributed to a shorter growing season. There is also less rust infection of pines in high elevation stands compared to low elevation stands again this is related to growing season (Hunt 2004, Hunt 2005).

Slow Canker growth and reduced needle spotting are other quantitative traits used to assess resistance in trees. Slow canker growth is considered to be more of a tolerance mechanism than resistance because trees show symptoms but are able to survive with damage (King et al 2010). Both slow canker growth and reduced needle spotting have been used to screen seedlings for resistance as seedlings that exhibit one or both of these characteristics have been shown to be less susceptible than those seedlings lacking these traits (Hoff et al 1980, Meagher and Hunt 1996). However, slow canker was found to be a better predictor than reduced infection spotting in determining resistance (Hunt 1997, Hunt 2002). Slow canker growth and needle spotting traits were used to assess the effects of growing season and tree age on resistance to white pine blister rust. A shorter growing season and older trees were related to resistance showing reduced needle infection and slow cankering (Hunt 2005).

Biosynthetic studies on protein secretion in the bark of trees that were considered to have the slow canker growth trait were studied. It was determined that trees that exhibited slow canker growth had a greater number of proteins compared to susceptible trees (Davidson 1997). There were certain proteins that were more abundant in resistant tree and others that were only found in either the resistant or susceptible trees (Davidson 1997). Many of the proteins found in susceptible trees were found in lesioned areas of the tree. This is

common in incompatible host-pathogen interactions, which suggests that the resistant trees may lack certain proteins necessary for infection or that these proteins are suppressed in resistant trees (Davidson 1997).

## 1.7 Population Genetics

Population genetic approaches provide a valuable set of tools to better understand the evolution, migration, and epidemiology of pathogens and to develop more durable resistance approaches (Hamelin 2006; McDonald and Linde 2002).

Rust fungi have different spore types that possess different dispersal characteristics. In addition, fungi have a mixed mating system, with sexual and asexual cycles being produced at different times during the life cycle. Spore dispersal is an important factor that could have an impact on the genetic structure of white pine blister rust. The rust genetic structure can be greatly influenced by which type of spores is disseminated; aeciospores are the result of plasmogamy and dikaryotization and basidiospores are the direct result of meiosis. Dissemination and infection by these spores are expected to increase genetic diversity. By contrast, urediniospores are asexual spores and could result clonal propagation of individuals. Long distance dispersal of any of these spores would lead to high gene flow between populations, preventing genetic drift and maintaining homogeneous populations (Hamelin 1995). This results in individual cankers being mosaics of outcrossed dikaryotic individuals (Hamelin 2005, Kinloch 1997). The lack of genetic differentiation at different scales, for example between Newfoundland and Ontario, or from BC to California, suggests that gene flow is homogenizing the populations (Hamelin et al 1995). However, it is not clear which spore types are responsible for such long distance dispersal. The presence of the

signature of recombination among populations suggests that asexual spores could play a lesser role in long distance spread (Hamelin et al 2005).

White pine blister rust was independently introduced into eastern and western Canada, which has contributed to the genetic structure of rust in Canada. Historical records (Hummer 2000) show that multiple introductions have occurred in eastern North America from Europe, but a single introduction has been reported in western North America. In support of these historical records, a higher genetic diversity was reported in eastern North America than in western North America. Many of the random amplified polymorphic DNA (RAPD) markers that were heterozygous in eastern *C. ribicola* populations were fixed in the western populations (Hamelin et al 2000). These genetic differences are believed to be maintained by a barrier to gene flow between the two populations. It is hypothesized that the large area in the US Great Plains and the Canadian Prairies where there are no 5-needle pine hosts and no naturally-occurring *Ribes* spp is preventing the rust from migrating between populations (Hamelin et al 2000, Joly et al not published). These findings were supported with the use of single nucleotide polymorphisms (SNP) markers (Joly et al not published). A different result was obtained by Kinloch et al (1998). Using isozymes, random amplified polymorphism DNA (RAPDs) and restriction fragment length polymorphisms (RFLPs) it was determined that there were low levels of genetic diversity in Western North America and there was great genetic variation in populations geographically closer together compared to geographically distant populations (Kinloch 1998). But eastern populations (from North Carolina and Virginia) clustered with western populations. There are differences in these studies that could explain this result. The Kinloch study used *C. ribicola* cultures, which resulted in low sample sizes in some populations, notably the eastern populations where

sample sizes of n=9 and n=11 were reported. Secondly, the Kinloch study sampled only two eastern populations in southern US. By contrast, the Hamelin study conducted direct genotyping of samples, allowing larger sample sizes and more populations to be included. The low genetic diversity observed in *C. ribicola* may be due to the long-distance dispersal of urediniospores starting a new infection. These spores are asexually propagated which could cause a population bottleneck (Hamelin 1995, Kinloch 1998).

Comparing natural stands and plantations in North eastern Canada, it was determined that there is little differentiation between the two, indicating that either gene flow is occurring or there is a common ancestor (Hamelin et al 1995). At a finer scale, unspermatized canker showed a uniform genotype, indicating each canker is caused by a single basidiospore infection, and once a single genotype is established it may be able to exclude others (Hamelin 1998). Within a single canker, a large proportion of diversity can be collected from multiple aecia and 90% of the diversity can be obtained from collecting multiple aecia from cankers in a single site. The amount of diversity decreases to 10% when collecting aecia from different geographical sites giving more evidence that gene flow is occurring (Hamelin 1996).

## 1.8 Single Nucleotide Polymorphism

Single nucleotide polymorphisms (SNP) are usually biallelic markers that show a single nucleotide change in a DNA sequence. For a base pair change to be considered a SNP, the less frequent allele must have a frequency of 1% or greater (Vignal 2002). There are two ways to construct SNP data bases. The first is genome wide screening, where the entire genome is sequenced. This method does not require PCR or any prior information of

genome. This method can be difficult and may not be accurate or the SNP are found in intergenic regions in many cases. The second method is gene based screening; this involves PCR amplification of a specific region in the genomic DNA and then direct sequencing of this region. This method is more costly, because it requires a number of primers to be tested on a copious amount of sequencing. The second method is often preferred to the first as it allows researchers to focus on specific regions of interest (Haga 2002).

The most common method of SNP discovery is to sequence PCR products targeting a specific region in different dikaryotic individuals. By sequences among individuals, polymorphic positions can be identified by the presence of double peaks in the chromatogram, indicating a heterozygous individual. Once SNPs have been identified, different techniques can be used to genotype large numbers of individuals. The most widely used method is a two-step protocol that allows allele identification (Vignal 2002).

## 1.9 Objectives

Previous characterizations of *C. ribicola* populations have revealed some barriers to gene flow between eastern and western populations, but little genetic differentiation among populations within eastern and western regions. In addition, western populations were shown to have a much lower genetic diversity than eastern populations. However western C. ribicola populations are subjected to a much more variable environment than eastern populations. Indeed, there are more host species, landscape variations, and climates in western North American than in eastern North America. The ability of white pine blister rust to infect multiple species of white pine at different elevations and landscape could indicate that certain strains of rusts are adapted to infect trees in different environmental conditions. The sampling

of the populations and the low number of markers employed in earlier studies could result in fine scale patterns being missed.

The Objectives of the present study were to characterize *C. ribicola* populations in eastern and western North America using SNPs to test the following hypotheses:

1) Populations of *C. ribicola* from western North America are more structured than eastern populations because of the more varied landscape, hosts, and climates.

2) Populations from eastern and western North America are genetically differentiated and eastern populations have a higher genetic diversity than western populations.

## 2 Materials and Methods

### 2.1 Sampling

There were two sources of samples for this study. Samples were obtained from a previous continent-wide sampling (Hamelin et al 2000). In addition, samples were collected from 25 locations in British Columbia and Alberta in 2009 and 2010 to explore the landscape patterns in western North America. The sampling was structured to sample from various landscapes; in particular, sampling was structured to cover high and low elevation sites from coastal and interior regions (Table 1).

Sampling was conducted prior to the aecial blisters opening, to ensure that there was no cross-contamination. This allowed direct sampling of unique dikaryotic individuals (Hamelin et al 1995). A toothpick was used to rupture the aecial blister and the dry spores were collected in a 1.5 ml eppendorf tube. Blisters were ruptured from the bottom of the canker first to minimize contamination. At most sites at least 3 aecia per canker and 10 trees per site were samples. However in some sites, in particular the natural stands, less than 10 trees were infected. Each eppendorf tube with aeciospores was labeled with the site name, tree, canker and aecia. The samples were placed in plastic boxes designed to hold 96 tubes and these boxes were placed in a larger plastic container with pouches of calcium sulphate (dryrite) to keep the samples dry for transport. In the lab, the aecia were dried in desiccation chambers with a layer of water saturated with calcium chloride at the bottom of the chambers and then placed in a -20°C freezer for storage.

## 2.2 DNA Extraction

DNA was extracted from the frozen spores using the Qiagen DNeasy Plant Mini extraction kits® (Qiagen Inc,. Toronto, Ontario Canada) with some modification to the protocol provided. The frozen aeciospores spores were placed in Eppendorf tubes, along with a sterile bead, 500 μL of AP1 buffer, 1ul of Reagent DX and 1μL RNase. The spores were mechanically disrupted using a mixer-mill for 2 minutes, and then placed in an 80°C water bath for 10 minutes. The spores were shaken again in the mixer-mill for another 2 minutes and then placed in a beaker of boiling water for 5 minutes to complete the disruption step of the extraction. Once removed from the boiling water, 150 μL of AP2 was added to the spore mixture, centrifuged and moved to the -20°C freezer for 10 minutes, and centrifuged for 5 minutes. The rest of the extraction follows the protocol provided starting at the step 11 which involves transferring the supernatant to the QIAshredder Mini Spin Column. A subset of the extracted DNA was checked on 0.8% agrose gel and the concentration was quantified using a nanodrop spectrophotometer to ensure the presences of DNA.

## 2.3 SNP Discovery

Single nucleotide polymorphisms (SNP) markers were designed from anexpressed sequence tag (EST) library and from a draft of a genome sequence and used to genotype the aeciospores. SNP markers identified single base pair differences between haplotypes, which can be used to identify unique individuals and population structure.

The EST library was constructed from aeciospores bulked from individual cankers (and therefore containing a mosaic of individuals) collected from multiple cankers on eastern white pine in May 2008. The aeciospores have been germinated on detached *Ribes* leaves for

16 hours. The germinated spores were peeled off the leaves, centrifuged and flash frozen with liquid nitrogen. The spores were disrupted using a mix-a-mill and the RNA was extracted with a Qiagen RNeasy Plant Mini Kit® (Qiagen Inc,. Toronto, Ontario Canada). Duo-range insert sizes normalized cDNA libraries were made by Bio S&T (Montréal, QC). Clones were arrayed in 384 wells plates and sequenced at the CHUQ sequencing plateforme (Québec, QC).

The library was assembled into contigs and searched for polymorphisms. Because of the paucity of polymorphisms in *C. ribicola* (Hamelin et al 2000, Kinloch et al 1998), the EST library did not contain enough polymorphisms to design sufficient assays for SNP genotyping. To supplement this data, we performed a single lane Illumina sequencing on a sample of *C. ribicola* obtained by collecting all aecia from a single canker, which have been shown to be genetic mosaics (Hamelin et al. 1996) and searched for SNP polymorphisms in the assembled scaffolds.

From the EST sequences and the genome assembly, potential SNPs were identified and primers were designed to target the SNP regions. The software Primers 3 (Rozen and Skaletsky 2007) was used to design the primers. A sequence contig containing an SNP(s) was uploaded into Primer 3, the SNP was targeted and primers were designed around the desired region. The primers were optimized using a panel of sixteen aecial samples, twelve samples from western Canada and four from eastern Canada. PCR amplification was performed in 20µl volumes that contained 1X buffer, 1.5mM $MgCl_2$. 0.5mM of each dNTP, 0.2µl of oligonucliotides, 2µl of DNA and 0.5 units of Taq DNA polymerase (Invitrogen). The amplification process was performed in thermal cycler (Applied Biosystems Gene Amp PCR System 9700). The program was set for denaturing at 94°C for 3 minutes and 30 cycles

of 94° for 30 seconds, annealing temperature (different for all primers) for 30 seconds followed by 72° for 1 minutes and 10 second. The reaction ended with an extension period of 72° for 10 minutes. The PCR products were run on 2% agrose gels to verify amplification and only samples with a single band were sent for sequencing. Sequencing was conducted at the Université of Laval in Québec using the ABI 3730 xI data analyzer.

## 2.4 SNP Validation

The sequence files were uploaded into Geneious (Drummond et al 2012), a software used to align sequences to confirm the presence of SNPs. Each sequence was aligned and the chromatograms were visually checked for the presence of double peaks indicating the presence of SNPs. Once all the sequences had been reviewed and corrected manually (the ends trimmed and other ambiguities fixed) all the individuals for that primer that had clean sequences were aligned to check if the SNP was found throughout the panel or if it was a rare SNP or possibly a sequencing error. For an ideal SNP, there would be one third with the double peaks, one third with one allele and then last third with the other allele. There were some sequences where insertion/deletion events had occurred; these sequences were excluded from this study.

Once the SNPs were confirmed on the panel, they were used to genotype all the individual aecia collected as well the collections from eastern North America. The genotyping was conducted at McGill University and Genome Québec Innovation Centre platform (Montreal, QC, Canada; http://genomequebec.mcgill.ca/centre.php) using an assay based on the iPLEX primer extension protocol on a MassARRAY® Compact system (Sequenom®).

## 2.5 Data Analysis

Various genetic analyses, including frequency and likelihood based methods, were performed to infer the population structure and to determine the effects of landscape on genetic diversity of *C. ribicola*. Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were also assessed to ensure the data set meets assumptions of some of the analyses. For all frequency based analyses, only populations with seven or more individuals were used. However, all individuals were included in the likelihood based analyses.

GenAlEx 6.4 (Peakall 2006) was used to calculate allele frequencies, the number of polymorphic loci (Table 1), to verify Hardy-Weinberg equilibrium and to perform a Principal Coordinates Analysis (PCoA). The PCoA converts a multivariate dataset of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components that can be plotted to obtain a visual representation. This allows inspecting the data and discovering trends without *a priori* assumptions.

The program Structure 2.3.2 uses a Bayesian Monte Carlo Markov Chain to infer population structure based on genotypic data (Prichard et al 2000). The program assumes all loci are in Hardy-Weinberg equilibrium and linkage equilibrium, and there is an unknown number of population clusters K that are characterized by a genotype. The dataset was run with the number of clusters set from K=1 to 15 and each K was replicated 10 times, with a burn-in length of 100,000 generations and 800,000 generations to verify the likelihood values of each K . Structure was run with the admixture (mixed ancestry) or independent allele frequencies model. Both models were run and replicated, with the results from the admixture model presented. Structure Harvester (http://taylor0.biology.ucla.edu/struct_harvest/) was

used to estimate the K value with highest likelihood (Evanno et al. 2005). Loci with missing data were excluded and Structure was re-run with the same parameters.

Geneland (Guillot et al. (2005a,b, 2008), Guillot([2008), Guillot and Santos (2010), Guedj and Guillot (2011)) was used to detect population structuring based on differences in allele frequency with the option of incorporating geographic data. To determine the number of clusters, K was set between 1 and 100 and the algorithm was run with and without geographic data. Once K was determined both a correlated and non-correlated allele frequency model was used with 1,000,000 iteration and 500,000 burnin length.

Genepop on the web (Raymond and Rousset 1995) was used to test for isolation by distance. Genotypic and geographic data was used to compute the relationship between geographic and genetics distances. Because of the large genetic differentiation between the eastern and western populations analyses of isolation-by-distance were run separately to determine if there was a barrier to gene flow within each cluster.

GeneClass 2 (Piry et al 2004) was used to assign individuals to a population based on allele frequency and detect migrant individuals. GeneClass was used to assign any admixed individuals found in Structure and individuals from populations found between the two major clusters (i.e., South Dakota, Wyoming, Wisconsin, and Minnesota) to a population. The model was run on the entire dataset using different algorithms; a frequency based model (Paetkau et al 1995) and a Bayesian method (Rannala et al 1997). Small populations with less than eight individuals were combined with other geographically neighbouring populations to ensure population size did not affect the results.

Arlequin (Excoffier et al 2010) was used to test for linkage disequilibrium and analysis of molecular variance (AMOVA). Linkage disequilibrium tests for non-random associations of alleles at different loci and AMOVA evaluates that population's genetic structure. AMOVA was used to investigate amount of variation between the eastern and western North America and on a finer scale the landscape features of elevation, coast vs interior and host species in the western data set.

Barrier v 2.2 (Manni et al 2004) was also used to detect any potential barriers to gene flow and search for patterns that could be correlated with landscape features. Coordinated for each sampling population along with Nei's genetic distances and 100 bootstrap matrices generated from Microsatellite Analyzer (MSA) was used to detect any barriers.

## 3 Results

A total of 1341 dikaryotic aecia of *C. ribicola* from 76 populations across the distribution range of the pathogen in North America were collected for genotyping. Two hundred and sixty nine primer pairs covering 134,500 base pairs were designed and screened for SNPs using PCR and sequencing. The average SNP frequency was one SNP every 2000 base pairs; however SNP frequency was highly variable in the genome regions sampled. The SNPs were annotated to 20 genome contigs and four EST (Table 2). 21 SNPs were found in introns or intergenic regions. Five were classified as retrotransposable element and five SNPs were found in exons. Of the five SNPs found in exons, two were synonymous and three were non-synonymous changes (Table 3). All loci were in Hardy-Weinberg equilibrium in all eastern populations, whereas some loci deviated from Hardy-Weinberg equilibrium in western populations. Of 31 SNPs genotyped, 21 loci in New Mexico were monomorphic. A single private allele (8649rare) was found in the western populations and two private alleles (8759Beast and 8759Deast) were detected in the eastern populations. The genotypic data was used to assess population parameters, to measure and compare genetic diversity and to search for associations between patterns of population structure and geography or landscape features.

### 3.1 Higher Genetic Diversity in Eastern than Western Populations

There was a clear geographic pattern in heterozygosity (Table 4). The expected and observed heterozygosity in eastern populations was 0.342 and 0.386 respectively, but it was only 0.225 and 0.230 in western populations. All eastern populations had higher heterozygosity than western populations, with the exception of the Minnesota population,

which had both observed and expected heterozygosity of approximately 0.230, comparable to western populations (Table 4). The population with the lowest heterozygosity was New Mexico, with observed and expected heterozygosities of approximately 0.100. The population with the highest heterozygosity was Ste-Camille-de-Bellechasse, in Québec, with observed heterozygosity of approximately 0.460. But this population was not an outlier as there were 14 eastern populations with heterozygosities higher than 0.400 (Table 2). Although sample size varied among populations, there was no correlation between sample size and heterozygosity ($R^2 < 0.01$; results not shown). Some of the smallest populations (e.g. Perch Lake, Ruisseau Tortue) had the largest heterozygosity, while some of the populations with the largest sample sizes (New Mexico, Puddingburn, Smithers) have the smallest heterozygosity (Table 4).

### 3.2 Population Clustering

To determine population structuring without *a priori*, the software Structure was used. The optimal value of K (cluster) was estimated to be two, based on the $\Delta K/ K$ values calculated by (Evanno et al 2005) implemented in Structure Harvester. The clusters were resolved clearly along geographic lines, with eastern populations comprising individuals that belonged to one cluster and western populations to a second cluster (Figure 2). Most individuals in the western populations were clearly assigned to a single cluster and showed low levels of admixture compared to eastern populations that comprised relatively high levels of admixture. To determine if additional population structuring is present within these two clusters but is masked by the strong east-west differences, each cluster was analyzed separately with Structure. Two clusters were found within each of the eastern and western

clusters. Within the western cluster, New Mexico formed one cluster and the rest of the western populations formed the other. In the east, Minnesota comprised one cluster and the second cluster comprised the rest of the eastern populations.

Population clustering pattern was also evaluated using Geneland using models with and without geographic data with various parameters. Five clusters were found in the analysis including geographic data that included two western clusters, New Mexico as a distinct cluster, one large eastern cluster that was comprised of all populations except Minnesota, and one cluster comprising of Minnesota. There were no obvious patterns of population in the two western clusters.

The principal coordinates analysis using pairwise genetic distances mirrored the geographic distribution (Figure 3). All western populations were grouped in the left quadrants while all eastern populations were in the right quadrants. However, Minnesota was an outlier population in the right quadrants and New Mexico in the left quadrants. Both of these populations had the lowest heterozygosity within their cluster (Table 4) and the greatest number of monomorphic loci, 21 in New Mexico and 6 in Minnesota.

The test of isolation by distance was examined separately in eastern and western populations because of the significant differentiation and lack of equilibrium between eastern and western population clusters. The result confirmed that geography does influence population differentiation. The geographic versus genetic distance plots showed weak but significant correlation in both the western ($R^2$- value of 0.0677) and eastern populations ($R^2$-value of 0.0638), R-values indicating a general association between genetic and geographic distances (Fig. 4). There was a pattern in the data points within eastern populations, with some of the largest geographic distances showing some of the largest genetic distances. Most

of this pattern can be accounted by the outlier population, Minnesota. The program Barrier detected two barriers in the landscape, one between the eastern and western population and a second between Minnesota and eastern populations (Fig 2).

The AMOVA analysis indicated a large proportion of genetic diversity among populations (Table 5). However, when populations are grouped into an eastern and western cluster, most of the variation (22.95%) is attributed to the clusters, with a much smaller proportion (4.47%) attributed to the differentiation among populations within clusters.

### 3.3 Population Structure in Western Canada

Because of the large variation in landscape, climate, and hosts in western North America, an analysis to compare and contrast the population structure within the western cluster was preformed. When Structure was run only using western populations, no clear population structuring that could be explained by landscape features, hosts, or climate was found. The optimal number of cluster was k= 1, and each cluster comprised a mixture of populations from the B.C. coast and interior, from high and low elevation and from different hosts (results not shown). AMOVA analysis was performed on these different comparisons. There was no significant genetic differentiation between populations sampled at high and low elevation or between populations sampled from the Coast or the Interior (Table 5). There was also no significant difference between populations sampled on different host species (Table 5).

Although there was no clear association between genetic patterns at the population level and landscape features or hosts, it is possible that there is incipient adaptation, drift, or differentiation. One SNP (8649rare) was rare and found only in a few western populations. It

was found in single individuals as heterozygotes in Prince George and the Kootenay and in 6 individuals (five heterozygotes and one homozygote) in Smithers, the northernmost population sampled and also the northernmost range of distribution of white pines. This SNP was absent from all other populations and is the only SNP that is unique to the West (results not shown).

### 3.4 Individual Assignment

Results from Structure identified several individuals with admixture (Fig. 2). Each admixed individual was assigned to one cluster though a portion of that individual belonged to the other cluster. Geneclass was used to assign individuals to populations. All of the admixed individuals in the eastern population were assigned to populations from the east, however, a majority of the admixed individuals in the west were also assigned to eastern populations (results not shown).

## 4 Discussion

*Cronartium ribicola* was introduced to North America over a century ago and has since dispersed across thousands of kilometers over varied landscapes, infecting various hosts. The pathogen has recently expanded its range significantly, with new reports of the pathogen in the South West. This study was designed to further understand the population structure of *C. ribicola* in North America, in particular contrasting the contribution of landscape features to genetic differentiation. This is the most extensive population study of *C. ribicola* both in terms of sample size (1341 samples from 76 populations) and markers used (31 SNP markers).

This study confirms previous observations of differentiation between eastern and western North American *C. ribicola* populations, and the higher diversity in eastern than western populations. Several lines of evidence suggest that this is due to different founder events in eastern and western North America, followed by reduced gene flow among these populations. Our study also highlights some novel observations, such as additional barriers to gene flow and reduced diversity in outlier populations.

### 4.1 Genetic Diversity

Genetic diversity was approximately twice as high in the eastern populations compared to the western populations. The most likely explanation for this difference is that there has been a more severe founder effect in western than in eastern populations. *Cronartium ribicola* has been introduced repeatedly over several years in eastern North America from nurseries in Europe, notably from Germany and France (Hummer 2000). By

contrast, there is only a single documented introduction of the pathogen into western North America (Hummer 2000).

Two outlier populations have lower genetic diversity than populations from the same regions. Minnesota had expected and observed heterozygosity similar to western populations, in spite of the fact that it clearly clusters with eastern populations in both assignments and genetic distance-based analyses. Minnesota samples were collected from eastern and western sites within the State and very similar genetic profiles were observed in these two sites; therefore, it is unlikely that the observed reduced genetic diversity was an artifact of our sampling. In addition, the Minnesota population is at Hardy-Weinberg equilibrium, with expected and observed heterozygosities approximately the same. The most likely explanation for this reduced heterozygosity is genetic drift from natural spread of a few individuals from the eastern cluster, and a founder effect possibly following pathogen introduction on infected nursery material. To maintain such a different composition to neighboring populations, an additional barrier to gene flow has to be invoked. Even a limited amount of gene flow would result in homogenization relative to geographically closely related populations. It is possible that the Great Lakes represent a landscape barrier to rust migration.

The New Mexico population had the lowest observed and expected heterozygosity of all *C. ribicola* populations studied. White pine blister rust was first reported in New Mexico in 1990, but old infections indicate the rust may have been present since 1970 (Hawksworth 1990, Van Arsdel 1998). This is the southernmost population of *C. ribicola.* It has been hypothesized that the pathogen was either introduced there from nursery seedlings, or that spores were blown from across the Sierra Nevada (Geils 2000, Van Arsdel et al 1998). Either scenario could have resulted in extreme population bottleneck. In support of this possibility,

a high level of inbreeding ($F_{is}$=0.113) was observed in that population. The current analysis does not allow differentiating between these scenarios. The New Mexico population was assigned within the western cluster. The migration of rust spores across the Sierra Nevada certainly appears possible as rust spores are known to travel hundreds of km. A study also found that favorable meteorological conditions for the rust to cross the Sierra Nevada occurred multiple times per year (Geils 2000). Additional sampling, in particular on the west side of the Sierra Nevada, would allow testing of these different hypotheses.

There was no clear isolation by distance (IBD) pattern within the eastern and western population clusters as indicated by the low correlation between genetic and geographic distances. The IBD analysis was conducted separately for the two clusters because of the bimodal nature of the distribution and the disequilibrium between eastern and western populations. Nevertheless, the comparison included in the analysis comprised populations that are separated by over 1000 km, yet display very low genetic distances. Long distance dissemination of spores has been well documented in many species of fungi, and in particular for rusts (Nagarajan and Singh 1990). Spore dispersal patterns are complex and there are many factors such as climate, environment and stand features that affect the distances of spore dispersal. *Endocronartium harknessii* (J.P. Moore) an autoecious rust found on pine dispersal patterns was investigated and found the relationship of the concentrations of spores and distances formed an inverse J-shape (Blenis et al 1993). A majority of spores are locally disseminated but spores that are caught in wind currents are able to travel great distances.

Accordingly, examples of single long-distance jumps of hundreds of km are rare but well documented. *Melampsora larici-populina* Kleb. and *Melampsora medusa* both were introduced in New Zealand from Australia. Climate and wind pattern analysis show that

these pathogens were carried 3000km by wind (Close et al 1978; Spiers and Hopcroft 1994). Wind transport of *Puccinia graminis tritici* from Mexico to the Canadian Prairies a distance of 1000km along the "Puccinia path" occurs annually. Changes in agricultural practices, eradication of the alternate host and deployment of rust resistant material in the Great Plains has aided in the long distance dispersal of this pathogen (Chen 2005; Nagarajan and Singh 1990; Roelf 1989). *Cronartium ribicola* clearly has a capacity to disperse over long distances. The progression of the pathogen from a single introduction in Vancouver to cover thousands of km of landscape in Western North America represents a good case for this capacity. Although human dissemination is also involved in *C. ribicola* dispersal, it has spread to areas that were not subjected to reforestation and where infections could also have occurred through natural spore dispersal.

An important factor in rust epidemiology is the annual alternation on two hosts. In several studies, this was found to influence the population structure of the pathogen. *Melampsora larici-populina* poplar rust epidemic in the Durance River Valley in France began in the upstream area of the valley, where the alternate host, larch occurs naturally, and gradually moved downstream (Xhaard et al. 2012). Genetic diversity was found to decrease along a North-South gradient that corresponded to the rust migration and the distance away from the alternate host. Similarly, genetic diversity in *M. medusa* f. sp. *deltoidae* was highest in areas of sympatry between the telial and aecial hosts in North America, possibly the result of annual migration from the source of primary inoculum, or inbreeding in the populations that are most distant to the inoculum source (Bourassa et al 2007). *Melampsora larici-populina* spread from its native European population to Iceland and eastern Canada, and both introduced populations showed decreased genetic diversity (Barrès et al 2008). Although the

current study was not designed to test the effect of alternate hosts abundance on the rust population structure, we have not observed a decrease in diversity in high elevation populations, in areas where there is a paucity of alternate hosts and unfavorable conditions for basidiospore germination and infection might prevail.

## 4.2 Population Structure

Distance based (e.g. PCA) and Bayesian (e.g. Structure and Geneland) analyses consistently revealed the presence of two clusters in North America. Hamelin et al (2000) reported the presence of distinct clusters in eastern and western North America. The present study sampled more populations, used a larger number of markers and confirms the presence of distinct eastern and western clusters. However, the present study also revealed some substructure within these clusters, essentially caused by the presence of two outlier populations within each of the eastern and western clusters.

It was hypothesized that further structuring was possible in western North America that might not have been discovered in previous studies. There are more landscape features, climatic variation and telial and aecial host species in western North America than in eastern North America. Extensive population sampling within British Columbia was undertaken, covering various landscapes across latitudinal and altitudinal clines and from Coastal and Interior sites. Whenever possible, high and low elevation sites were matched within a geographic region.

There was no clear pattern of clustering that corresponded to the various landscape features. Within the two main clusters, different Bayesian analyses offered slightly different results, an indication that the population structure signal was not robust. The analysis

conducted with Structure did not detect any sub-clustering within the two main clusters aside from New Mexico and Minnesota as the two outlying populations in the west and east respectfully. Analysis with Geneland using multiple parameters discovered two clusters within western Canada. The two clusters were not delineated by obvious landscape features such as elevation, host species, location or stand type. It is possible that other features, including presence of alternate host species, or microclimatic factors, are responsible for these additional clusters. Alternatively, it is possible that these additional clusters represent artifacts of the assignment algorithms.

The various hypotheses tested regarding sub-structuring, including elevation, host, or locations, were all rejected by AMOVA, indicating these factors do not influence genetic structure. The lack of significant contrasts in the AMOVA for location (Interior vs Coast) and stand type (natural stand or plantation) in the western population of white pine blister rust are consistent with other studies that found no difference between stand type in eastern Canada (Hamelin et al 1995). This data follows similar trends found in eastern Canada, where RAPD markers have been used to describe the genetic structure and found very little genetic differentiation between populations with most of the diversity present within populations (Et-touil et al 1999).

Population structuring has been reported in Fusifom rust (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. Fusiforme). This macrocyclic heteroecious fungus is an important disease of loblolly pine ( *Pinus taeda* L) and slash pine (*Pinus elliotti* Engelm) in the southeastern United States and alternates on oak (*Quercus spp*). Unlike *C. ribicola*, fusiform rust is a native pathogen. However these rusts share many similarities in biology and infection process. A distinct difference between these two pathogens is that *C. ribicola*

cankers are caused by a single basidiospore (Hamelin 1998) whereas *C. quercuum* galls are often caused by infection of multiple basidiospore (Kubisiak et al 2004). Population clusters using RAPD markers showed that the population of fusiform rust in the USA was uniform and there was little genetic differentiation based on geography (Hamelin et al 1994). A later study found four genetically distinct populations an eastern, mid-eastern, western and a mid-western cluster of this rust using microsatellite markers (Kubisiak 2004). Similar to white pine blister rust, most of the genetic variation of this rust was found within a population (Hamelin et al 1994; Kubisiak 2004), however fusiform rust showed regional population structure with little gene flow between regions. A possible explanation for the difference between these two rust population structures is that fusiform rust has co-evolved with its host allowing for local adaptation of the rust, whereas *C. ribicola* is still in the invasive/expansion mode.

Another possibility is that resistant pines are broadly deployed in the southern U.S., possibly resulting in an impact on the rust population. A study of *Melampsora larici-populina* a rust pathogen of *Populus spp.*, was conducted in France to determine the impact of the release of a cultivated rust resistant species of poplar rust (Xhaard et al 2011). Three distinct clusters of rust were found in France; the first is found in northern France where the rust is virulent and reproduces sexually. The second cluster is comprised of an avirulent, sexual rust in southern France and the third also in the southern part of France is an avirulent, asexual rust (Xhaard et al 2011). This pattern may be due to selective pressure on the rust by the host. Such a pattern has not been observed so far in British Columbia. Cr2 resistant trees from Champion Mine, where this resistant gene is naturally occurring were planted in BC. The rust in Oregon has been able to overcome the resistance, whereas in BC the trees are still

showing resistance after over 15 years. A new cultivated host for resistance was introduced into a system where both the poplar and *Melampsora larici-populina* are native to France adding a new factor of selection for the rust. White pine blister rust is an introduced pathogen in North America, the host and pathogen have not co-evolved; however resistance in the host is naturally occurring in the population.

Population structuring is not only found in rust fungi. In *Grosmannia clavigera*, a symbiont of the mountain pine beetle, the populations were much more genetically structured, over a narrower geographic range, than *C. ribicola*. *G. clavigera* is completely dependent on the insect for dispersal. The beetle has been expanding its range in the recent epidemic, carrying its fungal associate along. Four clusters where found within the mountain pine beetle epidemic that correspond to geographic regions. Due to range expansion, in two of the four cluster many of the fungal isolates were admixtures from all populations this may be due to range expansion (Tsui et al 2012). Although some admixture were found in some individual of white pine blister rust this was due to those individual in the east being more homozygous at certain loci that made them more similar to the western cluster.

## 4.3 Barriers to Migration

A barrier to migration was hypothesized to be responsible for maintaining the east-west differentiation in *C. ribicola* (Hamelin 2000). The paucity or absence of hosts in the Great Plains and the Canadian Prairies was the most plausible explanation for this barrier. The more extensive sampling conducted in the current study allows us to assess more closely this barrier. In all analyses, both South Dakota and Wyoming individuals were assigned to the western cluster and Minnesota individuals were assigned to the eastern cluster. Somewhat

surprisingly, no admixture between these geographically close populations was found. This may be the result of the recent eastern migration of *C. ribicola* from populations within the western cluster into South Dakota and Wyoming. White pine blister rust was first discovered in the Black hills of South Dakota on Limber pine in 1992 (Lundquist et al 1992) and in Wyoming in 1978 (Schwandt et al 2010). Previous studies have suggested that the Great Plains act as a physical barrier of gene flow between the eastern and western populations (Hamelin 2000). The Great Plains extends from the prairies in Canada down through South Dakota. The lack of *Ribes* and the pine hosts restricts movement of the rust. All white pine blister rust infections in South Dakota occur within the Black Hills which are located west of the Great Plains (J.Ball personal communication). The absence of host provides a barrier to gene flow between the east and the west, which was confirmed by our analysis with Barrier.

The epidemic of the *C. ribicola* began in Vancouver in 1910, from where it spread south to Washington, Oregon and was found in California in 1930 (Hunt 2009; Schwandt et al 2010). The rust spread east to Alberta, Wyoming, to North and South Dakota then to Colorado. An isolated population of rust was first reported in New Mexico in 1990, and most recently in Arizona in 2003 (Schwandt et al 2010). In general, populations with the most recent introduction show the highest levels of inbreeding, such as New Mexico. The population in Smithers showed the highest level of inbreeding in this study, this population is at the northern most extent of the rust.

## 4.4 Admixture

Although most individuals were clearly assigned to one cluster, there were some individuals that displayed admixture. There are two possible explanations for this: 1) there is some cryptic gene flow between the eastern and western cluster; or 2) because of the common origin from Europe last century, the eastern and western clusters all share most of the same alleles, and by chance some individuals possess patterns characteristic of the other cluster. The first explanation is unlikely, given the evidence presented previously, but the second explanation is more likely. Six out of the 31 SNP markers used in this study had the greatest discriminatory weight when assigning individuals to populations. In the eastern cluster, all admixed individuals were homozygous at more than one of these six discriminant loci, which is characteristic of individuals in the western cluster. The admixed individuals in the east were assigned to populations using Geneclass and all individuals were assigned to an eastern population. The admixed individuals in the western cluster had great heterozygosity at these six loci, however in Geneclass some individuals were assigned to eastern populations. The individuals are not really admixed and this represents a weakness of the assignment software, or an incomplete set of markers. Additional discriminant markers would probably resolve these individuals.

## 4.5 Unique Polymorphisms

There were very few SNPs that were private in this study, i.e. occurring uniquely in one population or one cluster. There were two private SNPs present in the east and 3 other SNPs that were present in the east but very infrequent in the west. These SNPs could be old alleles that were introduced from Europe and have spread throughout the eastern populations

but were eliminated in the west by drift or selection. The only unique SNP in the western cluster is particularly interesting. It was only found in western North America in 3 populations in the Interior of BC. It was most frequent in Smithers, the northernmost population sampled at the extreme northern range of distribution of Whitebark pines. Given the extensive survey we have conducted, it is likely that this is a SNP that appeared in these western populations following the introduction. Without a more extensive survey of polymorphisms across populations, it is difficult to determine if this SNP is involved in adaptation in this marginal population, or simply the result of genetic drift. A more intensive sampling of populations across latitudinal and altitudinal gradients with a larger number of SNPs will be necessary to properly address this question.

This study confirmed previous findings of two clusters of *C. ribicola* in North America, a western cluster and an eastern cluster. The two clusters are separated by the Great Plains where the absence of the host acts as a barrier to gene flow. Aside from the two main clusters, Minnesota and New Mexico were found as outliers and formed their own distinct cluster. No regional structuring was detected in western BC with Structure, but Geneland detected two clusters in BC. The two clusters were not delineated by geography, host species, or elevation. Further research using more markers and populations from western US to determine regional structuring would be needed. Extensive sampling of the area surrounding the barrier between the east and west would be interesting to see if the barrier is "leaking" allowing for some migration between the east and west.

## 5 Conclusion

Understanding the population structure of *Cronartium ribicola* is an important basis for management strategies. Population genetics can provide knowledge about pathogen spread and about migration patterns in addition to informing breeders about pathogen genetic diversity, one of the most important parameters that determines disease resistance stability. White pine blister rust was introduced into North America over a century ago and has been able to spread and cause high levels of mortality of its host. Great effort has been put into breeding programs for rust resistant trees. Knowledge of the genetics of the rust populations is essential for successful deployment. The eastern and western clusters of the rust are genetically differentiated, with greater genetic diversity found in the east. A barrier (the Great Plains) separate these two populations, however admixed individual found within each cluster may be an indication that there may be a leak in the barrier. Movement of rust between the eastern and western cluster would allow for new alleles that were not previously detected become present in that population. Intensive sampling and genotyping of individuals around the barrier in areas such as North and South Dakota and Wyoming would be necessary to confirm movement across the barrier.

Although no structuring was found within the western cluster, a rare SNP in the northern most population of Smithers indicated there may be some cryptic regional differentiation. This would be useful for breeders if these rare alleles lead to a new race of pathogen that has great virulence or is able to overcome resistance in the host. To further investigate rare alleles and fine scale population differentiation more markers covering a greater number of genes would be needed.

This study of the population structure of white pine blister rust is a building block for future research. Advances in genomics will make information more accessible. Comparative studies of genes of interest in the two clusters would be possible as well as studies on host/pathogen interaction with respect to virulence genes would be possible. This future work will help guide management strategies of white pine blister rust.

Table 1. Location, host, stand type, and geographic coordinates of populations of *Cronartium ribicola.*

| Population Name | Location | Host | Stand Type | Geographic coordinates | |
|---|---|---|---|---|---|
| | | | | Longitude | Latitude |
| Gander River (GR) | Newfoundland | *P. strobus* | Natural | -54.8133 | 49.0260 |
| Little Grand Lake (LG) | Newfoundland | *P. strobus* | Plantation | -57.7428 | 48.5589 |
| Perch Lake (PL) | Nova Scotia | *P. strobus* | Plantation | -65.1012 | 44.4949 |
| Trafalgar (TF) | Nova Scotia | *P. strobus* | Plantation | -62.6597 | 45.2883 |
| Moncton (NB) | New Brunswick | *P. strobus* | Plantation | -64.8030 | 46.1155 |
| QC (QC) | Quebec | *P. strobus* | Natural | -74.3399 | 47.6603 |
| Chesterville (CH) | Quebec | *P. strobus* | Natural | -71.8616 | 45.9629 |
| Corte-Réal (CR) | Quebec | *P. strobus* | Natural | -64.5999 | 48.9076 |
| Cowansville (CO) | Quebec | *P. strobus* | Natural | -72.7503 | 45.2081 |
| LaTuque (LT) | Quebec | *P. strobus* | Natural | -72.7856 | 47.4338 |
| Plessisville (PV) | Quebec | *P. strobus* | Plantation | -71.7721 | 46.2195 |
| Plessisville-97 (PV) | Quebec | *P. strobus* | Plantation | -71.7731 | 46.2199 |
| Quatre-Chemins (QU) | Quebec | *P. strobus* | Natural | -70.5093 | 46.2297 |
| Rivière Lièvre (RL) | Quebec | *P. strobus* | Natural | -75.1222 | 46.8691 |
| Ruisseau Tortue (RT) | Quebec | *P. strobus* | Natural | -76.1846 | 46.4166 |
| St.Alexisde Montcalm(AM) | Quebec | *P. strobus* | Plantation | -73.6209 | 45.9335 |
| St.Cyprien (SY) | Quebec | *P. strobus* | Plantation | -69.0190 | 47.8963 |
| St.Cyprien97 (SY) | Quebec | *P. strobus* | Plantation | -69.0290 | 47.8973 |
| St.Camille de Bellechasse (CB) | Quebec | *P. strobus* | Natural | -70.2185 | 46.4935 |
| Minden (MI) | Ontario | *P. strobus* | Natural | -78.7238 | 44.9248 |
| Temagomi (TE) | Ontario | *P. strobus* | Natural | -79.7816 | 47.0667 |
| Sault Sainte Marie (SS) | Ontario | *P. strobus* | Natural | -84.3500 | 46.5330 |
| Minnesota (MN) | Minnesota | | Plantation | -93.1365 | 47.1627 |
| New Mexico (NM) | New Mexico | *P. strobiformis* | Natural | -105.6907 | 32.8851 |
| Mosca Pass (MP) | Colorado | *P. aristata* | Plantation | -105.5497 | 37.6290 |
| Banff (BA) | Alberta | *P. albicaulis* | Natural | -115.9431 | 51.3001 |
| Carbondale River Road (CA) | Alberta | *P. albicaulis* | Natural | -114.5355 | 49.3796 |
| Plateau Mountain (PM) | Alberta | *P. albicaulis* | Natural | -115.9449 | 50.1913 |
| Porcupine Hills (PH) | Alberta | *P. flexilis* | Natural | -113.8802 | 49.7870 |
| Slacker Creek (SL) | Southern Interior | *P. albicaulis* | Natural | -114.5910 | 50.0813 |
| Puddingburn (PB) | Southern Interior | *P. albicaulis* | Natural | -116.4073 | 49.5601 |
| Nelson (NE) | Southern Interior | *P. albicaulis* | Natural | -117.3022 | 49.5338 |
| Quartz Gravel Pit (QG) | Southern Interior | *P.monticola* | Natural | -117.3676 | 51.4896 |
| Bombi Summit (BS) | Southern Interior | *P.monticola* | Natural | -117.5211 | 49.2382 |

| Population Name | Location | Host | Stand Type | Geographic Coordinates | |
|---|---|---|---|---|---|
| | | | | Longitude | Longitude |
| Little Slocan (LS) | Southern Interior | *P.monticola* | Natural | -117.7163 | 49.6161 |
| Kootnays (KO) | Southern Interior | *P.monticola* | Natural | -116.0194 | 50.9078 |
| Springer Creek (SP) | Southern Interior | *P.monticola* | Natural | -117.4495 | 49.7790 |
| McBride (MB) | Northern Interior | *P. albicaulis* | Natural | -120.1302 | 53.3369 |
| Valemount (VA) | Northern Interior | *P.monticola* | Natural | -119.0833 | 52.7014 |
| Prince George1 (PG1) | Northern Interior | *P.monticola* | Plantation | -122.1741 | 53.9920 |
| Prince George2 (PG2) | Northern Interior | *P.monticola* | Plantation | -121.8700 | 53.4128 |
| Prince George3 (PG3) | Northern Interior | *P.monticola* | Plantation | -122.1063 | 53.9067 |
| Smithers (SI) | Northern Interior | *P. albicaulis* | Natural | -126.7465 | 54.8498 |
| Pemberton (PE) | Coastal | *P.monticola* | Plantation | -122.8000 | 50.3207 |
| Texada (TX) | Coastal | *P.monticola* | Plantation | -124.4375 | 49.6544 |
| Powell River (PR) | Coastal | *P.monticola* | Plantation | -124.4883 | 49.9030 |
| Mt.Washington (MW) | Coastal | *P.monticola* | Natural | -125.2337 | 49.7401 |
| Oregon (OR) | Coastal | *P. lambertiana* | Natural | -122.6819 | 45.5200 |

Table 2. Primer sequence and gene targeted for DNA amplification and sequencing in *Cronartium ribicola*.

| Locus | Primer Sequence | No. of SNPs | Homology |
|---|---|---|---|
| 10600F | CTGCTCAGCCAGATCTCAAC | 1 | phosphatidylinositol 3-kinase tor2 |
| 10600R | GTCATGAAAGGGAAGCCAGA | | |
| 11481F | CTCAGCCCTCAAGAAGAACG | 1 | mrna splicing factor (prp1 zer1) |
| 11481R | TTCGTACCCACCGAATAAGC | | |
| 15194F | GTGATAATTTCGTCATCGTCATC | 1 | retrotransposon unclassified |
| 15194R | ACATACCCATGGCTGAGAGG | | |
| 15337AF | CGGACCAACTTTCCTAGTCG | 2 | phenol 2-monooxygenase |
| 15337AR | TTTGTGGACTTGCAGGTTTG | | |
| 16015F | TCACTGGGTTACGAGCCAAT | 1 | probable signal peptidase |
| 16015R | TTCAATCCACTTCTCACCAATG | | (endopeptidase sp18) |
| 24835F | GGTTGCCAGAAATTCTCAAAGG | 1 | sec1 family superfamily protein |
| 24835R | GGTCCAACATTCCACCCTAA | | |
| 30763F | CGCTTTGCTTCCACTTTTTAGC | 1 | clathrin-coated vesicle protein |
| 30763R | TGTGGGACCGGTATTCTCTC | | |
| 43bshortF | CCCCAAAATCACCCAATATG | 2 | hypothetical protein MELLADRAFT_94370 |
| 43bshortR | AGCGACCGGTCCTTTAACTT | | [Melampsora larici-populina 98AG31] |
| 4859F | GCTGAACTGGATCAAGCAG | 1 | hypothetical protein MELLADRAFT_108574 |
| 4859R | AAAAGGCCTACTGACATGCAA | | [Melampsora larici-populina 98AG31] |
| 5403F | TTGCGCAATTGACAAAAGAG | 1 | kinetochore spindle checkpoint protein ndc80 |
| 5403R | AAAACCCAGACCTATCACCAAA | | |
| 5741AF | GCTGCTCTGGGAATGGTTAC | 2 | gag-pol polyprotein |
| 5741AR | CCGATGCTCCTAGTCAGACC | | |
| 6209F | GCTCCCTTTGGTGGTCTTTAC | 1 | retrotransposable element tf2 155 kda |
| 6209R | TTCGATGGGTCCATGGTAAT | | protein type 1-like |
| 6296F | GCCTGAACCCAAATTGTTTCC | 1 | hypothetical protein E5Q_05065 |
| 6296R | ACCTCTCGGAACCCATTCAA | | [Mixia osmundae IAM 14324] |
| 6765F | CTTGATGCATTGGAAGATCG | 1 | hypothetical protein MELLADRAFT_103633 |
| 6765R | TGTCACCTCAGTGAGCAAGG | | [Melampsora larici-populina 98AG31] |
| 7313F | CCGCACTGGTGTTTGTACTG | 1 | hypothetical protein MELLADRAFT_108332 |
| 7313R | TGCTTGCTTTCATTGTGAGG | | [Melampsora larici-populina 98AG31] |
| 7390F | ACCTGAAGGCCTTGACACAC | 1 | hypothetical protein MELLADRAFT_47787 |
| 7390R | CAAACACAACACGTCCAAGC | | [Melampsora larici-populina 98AG31] |
| 8291A F | GGCGGACATGCTATGTTCTTG | 1 | ornithine carbamoyltransferase |
| 8291A R | CGTTGAAAGTTCCCGCTTAC | | |
| 8291B F | GCGCAGCCTCAAAAGATTTC | 1 | hypothetical protein MELLADRAFT_33703 |
| 8291B R | GGAAGTGGGCTGAAATCAAA | | [Melampsora larici-populina 98AG31] |
| 8649F | GCTGCAGCAGTCGACGTATC | 1 | eukaryotic translation initiation factor 3 subunit |
| 8649R | GATGGAGGGTCAGCGATAAA | | |
| 8759BF | GCTGCTCTGGGAATGGTTAC | 2 | family 18 glycoside hydrolase |

| Locus | Primer Sequence | No. of SNPs | Homology |
|---|---|---|---|
| 8759BR | CCGATGCTCCTAGTCAGACC | | [Melampsora larici-populina 98AG31] |
| 9887F | GCGGACATGGTGTAAGTG | 1 | tpr domain protein |
| 9887R | TCCAGCAACATCTTCCTTCC | | |
| SNPB5AF | GTGGGTGGTGCCTATGAAGC | 1 | aspartyl aminopeptidase |
| SNPB5AR | CAGCAGGAGAGGATGGTGAT | | |
| SNPC23BF | GGTCCTTCTGGGAACCTAACC | 1 | glycoside hydrolase family 31 protein |
| SNPC23BR | ATCCCAAATGCTTGGAACTG | | |
| SNPH13AF | CTGCTCTCAACCTACAAGCTAATG | 1 | macrofage activating glycoprotein |
| SNPH13AR | TAGCAGGGATGACAGCAGTG | | |
| SNPM5AR | CCTACAACAATATGGCCTCCTC | 1 | dihydrolipoamide succinyltransferase |
| SNPM5AF | CGCCTCACACCCAACTTATT | | |

Table 3. Characteristics of the SNP genotyping assays.

| Locus | SNP position/ identity | synonymous/ non-synonymous | iPlex Primers Sequence | Probe Sequence |
|---|---|---|---|---|
| 10600b | 2041, A/G | Retrotran-sposon | ACGTTGGATGCAGGTAAAAGGTGTGTTGGG<br>ACGTTGGATGAAAATGGCTAGACTCTGCCC | GTGAAAAGAGTGGATAGTCTTTTCATAT |
| 11481A | 1484, A/G | Intron | ACGTTGGATGTATCTAGCCGTGAATGAGGG<br>ACGTTGGATGGGACAGAGAGAGTTGAGTTC | GAGGGGAGACGGTTAGGTAATTAATAT |
| 15194A | 30, C/T | Retrotran-sposon | ACGTTGGATGGTGATAATTTCGTCATCGTC<br>ACGTTGGATGGCCTTCTATCAATGCTCGTG | GGGAATTTCGTCATCGTCATCATCC |
| 15337A | 1177, G/T | Intergenic | ACGTTGGATGGGCTCTCAACTCAAGCTATC<br>ACGTTGGATGACAGGGTGTAGTCCAGTTTC | AACTCAAGCTATCACAAAGTT |
| 15337B | 1337, A/G | Intergenic | ACGTTGGATGGTAATCTGAACAGCTATCCC<br>ACGTTGGATGTGCACAGATTGGATGGGATG | GGGATAAATCCCTATTTGCACTACAAC |
| 16015A | 1467, C/T | Non-synonymous | ACGTTGGATGCTGACCCTATTCTTACACCC<br>ACGTTGGATGAAGACCACTAACAAGACCTG | CCCCTGGTTCAACAAGTTCATCT |
| 24835A | 3176, A/G | Intergenic | ACGTTGGATGCTGTGTGAGGAAAAGAGTGG<br>ACGTTGGATGAGGTAGGTTGAAACAAGGGC | TTGTTCAAAAGCACGGGT |
| 30763B | 3298, C/T | Intergenic | ACGTTGGATGGTACTTATTGTGATGGACCT<br>ACGTTGGATGCTTGGTGAGTAGAGATGTAAC | GGAAGCAAGTTGATCAAACCATTTTAATA |
| 43shortA | 173, A/G | Intergenic | ACGTTGGATGAGAGGTTGTCATTTGTCAGG<br>ACGTTGGATGGCTGGCTAGTATCGAACTTC | GGGGACTCCATCTGGAAAC |
| 43shortB | 224, G/T | Intergenic | ACGTTGGATGTCCTGACAAATGACAACCTC<br>ACGTTGGATGCCTGTGGGTAGATCAAGTAG | GGAAGCCTAGTGCTACAGGA |
| 4859A | 970, C/T | Non-synonymous | ACGTTGGATGCAATTGAACTCTCCTCTCGG<br>ACGTTGGATGGTGGCTTTTGAGTTGATGGG | GTATGGCTAGAATTACTGCAACATCG |
| 4859C | 1146, A/G | Intergenic | ACGTTGGATGGGCATCTATCAGTCTGTCAC<br>ACGTTGGATGCCAAAAAAATCACGGGCTGG | GATTAGATGTGCTGTTACAGTACA |

| Locus | SNP position/ identity | synonymous/ non-synonymous | iPlex Primers Sequence | Probe Sequence |
|---|---|---|---|---|
| 5403A | 1077, A/T | Intergenic | ACGTTGGATGCTGTGGCCCATATGAGAGAA<br>ACGTTGGATGATCCACCACTTGAGAATCAC | AAAGAAGGCACACTACC |
| 5741A east | 852, C/T | Retrotran-sposon | ACGTTGGATGCTGAAATGGACGGCACCAAG<br>ACGTTGGATGGACAAAAATCTTGCCCAAGG | CAAGTCCATCTGGCTGAT |
| 5741B east | 1124, C/T | Retrotran-sposon | ACGTTGGATGCTGAGGCCGAAGATTTGTTC<br>ACGTTGGATGGGTCACATGATAAGGGACTG | GGGTTCTTGTAGTCGGCTAAAGTA |
| 6209A | 926, A/G | Retrotran-sposon | ACGTTGGATGGTGAGCCATTCATCACCTTC<br>ACGTTGGATGCTTCGTCAATTATAGGGCTG | GTCCGGGCACTCAAAGCAGATTGAATG |
| 6296B | 971, A/G | Retrotran-sposon | ACGTTGGATGGCGCTGTGTTAGGTAAAGTC<br>ACGTTGGATGGCAAGCTGGTTGGCTAAATG | GAAAGTCAGCTTGTGTAACAAA |
| 6765A | 1125, C/G | Intergenic | ACGTTGGATGTGCACCCTCTTGTAGTTCTC<br>ACGTTGGATGCTCTTGCCCTTGTGAGAATC | ACATTCTAGCTTGCCTCTCG |
| 7313A | 157, C/T | Intergenic | ACGTTGGATGACTCCCCTCTCTTCTTCATC<br>ACGTTGGATGCTAGTGACTGAGAGATCTAC | AAACCTCAACAGGCTGAATA |
| 7390B | 902, G/T | Intergenic | ACGTTGGATGGGATCCATTAGTTGAGCCTG<br>ACGTTGGATGCAAACAGGCCTTAACAGCTC | GGGCATCATATGGAGTGGATTG |
| 7390C | 1032, A/C | Intergenic | ACGTTGGATGTTGCAGCAGAAGTCCTTGAC<br>ACGTTGGATGGGATTGGTATACTTTGAGGC | TCCTTGACAGCAGTATAATATAA |
| 8291A | 362, C/T | Synonymous | ACGTTGGATGATCCTTGCCGATCTTGTCAC<br>ACGTTGGATGTTTGAATCACCGATCCAGGC | CTGTCACACTTCTTGAAAC |
| 8291a | 1713, C/T | Intergenic | ACGTTGGATGTTTGAATCACCGATCCAGGC<br>ACGTTGGATGATCCTTGCCGATCTTGTCAC | GAGAATGCGGGAAGAA |
| 8649 rare | 1393, A/C | Non-synonymous | ACGTTGGATGATCACGCGCAAGATTCAACC<br>ACGTTGGATGGAAAGAGACGTGTCATCACC | TCCTTCAACGCCTTGG |
| 8759B east | 1010, A/G | Intergenic | ACGTTGGATGGTCTCTCTCGACTTCCTTAG<br>ACGTTGGATGCCTTGCAATTCTTTCTGGGC | AAGATATTGAAGGCCGGC |

| Locus | SNP position/ identity | synonymous/ non-synonymous | iPlex Primers Sequence | Probe Sequence |
|---|---|---|---|---|
| 8759D east | 1112, C/T | Intergenic | ACGTTGGATGTCATATCCGAATAGGT GAGC<br>ACGTTGGATGACCGGATCAGTAAGGA GAAC | CTCATTTATCGATACCTTGCAGGAA |
| 9887H | 452, A/C | Intergenic | ACGTTGGATGATTGAACCAGTCAGTC CACC<br>ACGTTGGATGGGCAGTGACAGCAATT CTTC | ACCAGAAGGAAGTGTTTGAA |
| SNP B5A | 510, A/G | Intron | ACGTTGGATGCCCAGACCGATCAACT AAAG<br>ACGTTGGATGAGGTTGTACCTAGCGC AAAC | CCGTCGTCAATGTTTTCAATTGCAA AGT |
| SNP C23B | 831, C/T | Synonymous | ACGTTGGATGTGGCCTCAACGTTAAG ACAG<br>ACGTTGGATGACTCGAATTCCCGGGT ATTG | TCTCAAAGTTTATCATACCCA |
| SNP H13A | 803, A/C | Intron | ACGTTGGATGAGCTTCGCTTTGTCAA CGTG<br>ACGTTGGATGCCGATTCGGTCATATA CGTC | TTGTCAACGTGCGTATATTTTTTTAA CAAA |
| SNP M5A | 272, C/T | Intron | ACGTTGGATGTGTCTCGAATCTGCCA AAGC<br>ACGTTGGATGGGAAAGCCAGACTAA GTCAC | GGCGCGTTTTTACAAT |

Table 4. Population genetic parameters in 46 populations of *C. ribicola* genotyped for 31 SNP loci.

| Population Name | Location | N | $H_o$ | $H_e$ | $F_{is}$ | Polymorphic Loci (%) |
|---|---|---|---|---|---|---|
| Gander River (GR) | Newfoundland | 7 | 0.329 | 0.343 | 0.039 | 93.5 |
| Little Grand Lake (LG) | Newfoundland | 9 | 0.358 | 0.361 | 0.008 | 90.3 |
| Perch Lake (PL) | Nova Scotia | 7 | 0.424 | 0.362 | -0.174 | 93.5 |
| Trafalgar (TF) | Nova Scotia | 14 | 0.356 | 0.359 | 0.008 | 96.8 |
| Moncton (NB) | New Brunswick | 12 | 0.387 | 0.350 | -0.105 | 93.5 |
| QC (QC) | Quebec | 9 | 0.398 | 0.355 | -0.119 | 87.1 |
| Chesterville (CH) | Quebec | 8 | 0.375 | 0.375 | -0.001 | 90.3 |
| Corte-Réal (CR) | Quebec | 8 | 0.431 | 0.375 | -0.151 | 93.5 |
| Cowansville (CO) | Quebec | 8 | 0.454 | 0.401 | -0.134 | 96.8 |
| LaTuque (LT) | Quebec | 10 | 0.456 | 0.365 | -0.251 | 90.3 |
| Plessisville (PV) | Quebec | 28 | 0.373 | 0.378 | -0.070 | 93.5 |
| Quatre-Chemins (QU) | Quebec | 7 | 0.316 | 0.372 | 0.15 | 93.5 |
| Rivière Lièvre (RL) | Quebec | 7 | 0.320 | 0.358 | 0.104 | 96.8 |
| Ruisseau Tortue (RT) | Quebec | 7 | 0.434 | 0.391 | -0.111 | 93.5 |
| St.Alexisde Montcalm(AM) | Quebec | 13 | 0.410 | 0.413 | 0.007 | 96.8 |
| St.Cyprien (SY) | Quebec | 29 | 0.387 | 0.393 | -0.021 | 96.8 |
| St.Camille de Bellechasse (CB | Quebec | 9 | 0.465 | 0.376 | -0.234 | 93.5 |
| Minden (MI) | Ontario | 9 | 0.409 | 0.351 | -0.167 | 93.5 |
| Temagomi (TE) | Ontario | 15 | 0.384 | 0.37 | -0.037 | 96.8 |
| Sault Sainte Marie (SS) | Ontario | 12 | 0.417 | 0.371 | -0.124 | 96.8 |
| **Average Eastern**[1] | | 302 | 0.386 | 0.342 | -0.054 | 94.3 |
| Minnesota (MN) | Minnesota | 9 | 0.232 | 0.231 | -0.006 | 80.6 |
| New Mexico (NM) | New Mexico | 28 | 0.095 | 0.107 | 0.113 | 32.3 |
| Mosca Pass (MP) | Colorado | 9 | 0.214 | 0.208 | -0.031 | 64.5 |
| Banff (BA) | Alberta | 17 | 0.246 | 0.221 | -0.112 | 64.5 |
| Carbondale River Road (CA) | Alberta | 16 | 0.233 | 0.211 | -0.100 | 64.5 |
| Plateau Mountain (PM) | Alberta | 52 | 0.235 | 0.241 | 0.024 | 77.4 |
| Porcupine Hills (PH) | Alberta | 20 | 0.204 | 0.210 | 0.031 | 64.5 |
| Slacker Creek (SL) | Southern Interior | 11 | 0.179 | 0.160 | -0.115 | 51.6 |
| Puddingburn (PB) | Southern Interior | 26 | 0.195 | 0.202 | 0.038 | 64.5 |
| Nelson (NE) | Southern Interior | 34 | 0.225 | 0.203 | -0.108 | 71.0 |
| Quartz Gravel Pit (QG) | Southern Interior | 28 | 0.239 | 0.234 | -0.021 | 71.0 |
| Bombi Summit (BS) | Southern Interior | 20 | 0.202 | 0.191 | -0.056 | 61.3 |
| Little Slocan (LS) | Southern Interior | 26 | 0.232 | 0.220 | -0.051 | 74.2 |
| Kootnays (KO) | Southern Interior | 38 | 0.232 | 0.239 | 0.030 | 67.7 |
| Springer Creek (SP) | Southern Interior | 28 | 0.263 | 0.250 | -0.055 | 71.0 |

| Population Name | Location | N | Ho | He | FIS | Polymorphic Loci (%) |
|---|---|---|---|---|---|---|
| McBride (MB) | Northern Interior | 169 | 0.244 | 0.244 | 0.003 | 96.8 |
| Valemount (VA) | Northern Interior | 104 | 0.224 | 0.231 | 0.030 | 64.5 |
| Prince George1 (PG1) | Northern Interior | 12 | 0.224 | 0.207 | -0.081 | 51.6 |
| Prince George2 (PG2) | Northern Interior | 38 | 0.245 | 0.231 | -0.062 | 71.0 |
| Prince George3 (PG3) | Northern Interior | 34 | 0.245 | 0.259 | 0.052 | 80.6 |
| Smithers (SI) | Northern Interior | 37 | 0.195 | 0.241 | 0.193 | 77.4 |
| Pemberton (PE) | Coastal | 29 | 0.253 | 0.251 | -0.007 | 74.2 |
| Texada (TX) | Coastal | 109 | 0.232 | 0.244 | 0.050 | 77.4 |
| Powell River (PR) | Coastal | 52 | 0.255 | 0.230 | -0.111 | 77.4 |
| Mt.Washington (MW) | Coastal | 53 | 0.239 | 0.236 | -0.012 | 83.9 |
| Oregon (OR) | Coastal | 11 | 0.262 | 0.239 | -0.095 | 87.1 |
| **Average West[2]** | | 973 | 0.230 | 0.225 | -0.024 | 71.2 |

[1] The average east calculations does not include Minnesota

[2] The average west calculation does not include New Mexico

Table 5. AMOVA results for 55 *C. ribicola* populations sampled in 2009 and 2010. Degrees of freedom (df), sum of squares (SS), Variance components (Var.), and percentage variation (%).

| | df | SS | Var. | % | p-values |
|---|---|---|---|---|---|
| **Eastern and western clusters comparisons[a]** | | | | | |
| Between Groups | 1 | 805.090 | 0.994 | 22.95 | <0.001 |
| Among populations within groups | 49 | 606.519 | 0.194 | 4.47 | <0.001 |
| Within populations | 2451 | 7702.022 | 3.142 | 72.57 | <0.001 |
| **Total** | 2501 | 9113.631 | 4.330 | | |
| **Elevation comparisons (high and low)[b] within the western cluster** | | | | | |
| Between Groups | 1 | 13.856 | -0.003 | -0.14 | >0.001 |
| Among populations within groups | 21 | 199.157 | 0.098 | 4.83 | <0.001 |
| Within populations | 1885 | 3660.217 | 1.941 | 95.31 | <0.001 |
| **Total** | 1907 | 3873.230 | 2.037 | | |
| **Coast and interior comparisons [c] within the western cluster** | | | | | |
| **Between Groups** | 1 | 38.039 | 0.012 | 1.52 | <0.001 |
| Among populations within groups | 24 | 190.357 | 0.0861 | 4.19 | <0.001 |
| Within populations | 1932 | 3743.079 | 1.937 | 94.29 | <0.001 |
| **Total** | 1957 | 3971.506 | 2.0548 | | |
| **Pines species comparisons[D]** | | | | | |
| Between Groups | 1 | 8.230 | -0.003 | -0.17 | >0.001 |
| Among populations within groups | 19 | 159.767 | 0.095 | 4.74 | <0.001 |
| Within populations | 1475 | 2823.555 | 1.914 | 95.43 | <0.001 |
| **Total** | 1495 | 2991.552 | 2.00 | | |
| **Natural stand vs plantation comparison[E]** | | | | | |
| Between Groups | 1 | 33.570 | 0.026 | 1.24 | <0.001 |
| Among populations within groups | 21 | 179.443 | 0.086 | 4.14 | <0.001 |
| Within populations | 1885 | 3660.217 | 1.942 | 94.59 | <0.001 |
| **Total** | 1907 | 3873.230 | 2.053 | | |

[a] eastern group contains individuals from GR, LG, PL, TF, NB, QC, CH, CR, CO, LT, PV, QU, RL , RT, AM, SY, CB, MI, TE, SS, MN. Western group contained individuals from NM, MP , BA, CA, PM, PH, SL, PB, NE, QG, BS, LS, KO, SP, MB, VA, PG1, PG2, PG3, SI, PE, TX, PR, MW, OR.

[b] High Elevation group includes samples from BA, CR, PM, PH, PB,NE, KO, QG, SI, LS, BO and MB. Low elevation contains individuals from PE, PR, MW, TX, VA, PG1, PG2, PG3 and SP.

[c] Coastal group includes individuals from PE, PR, TX, AND MW and the interior group includes individuals from SI, PG1, PG2, PG3, VA, MB, BO, LS, SP, KO, QG, PB, NE, BA, CR, PM, and PH.

[D] Individuals collected on *P.monticola* QG, BO, LS, KO, VA, PG1, PG2, PG3, PE, TX, PR, and MW. Individuals collected on *P. albicaulis* BA, CA, PM, SL, PB, NE, MB and SI. PH was collected from *P. flexilis.*

[E] Group contains individuals collected from a natural stand BA, CR, PM, PH, PB, NE, QG, BO. LS, KO, SC, MB, SI, MW, and VA. Individual collected from a plantation PG1, PG2, PG3, PE, TX, and PR.

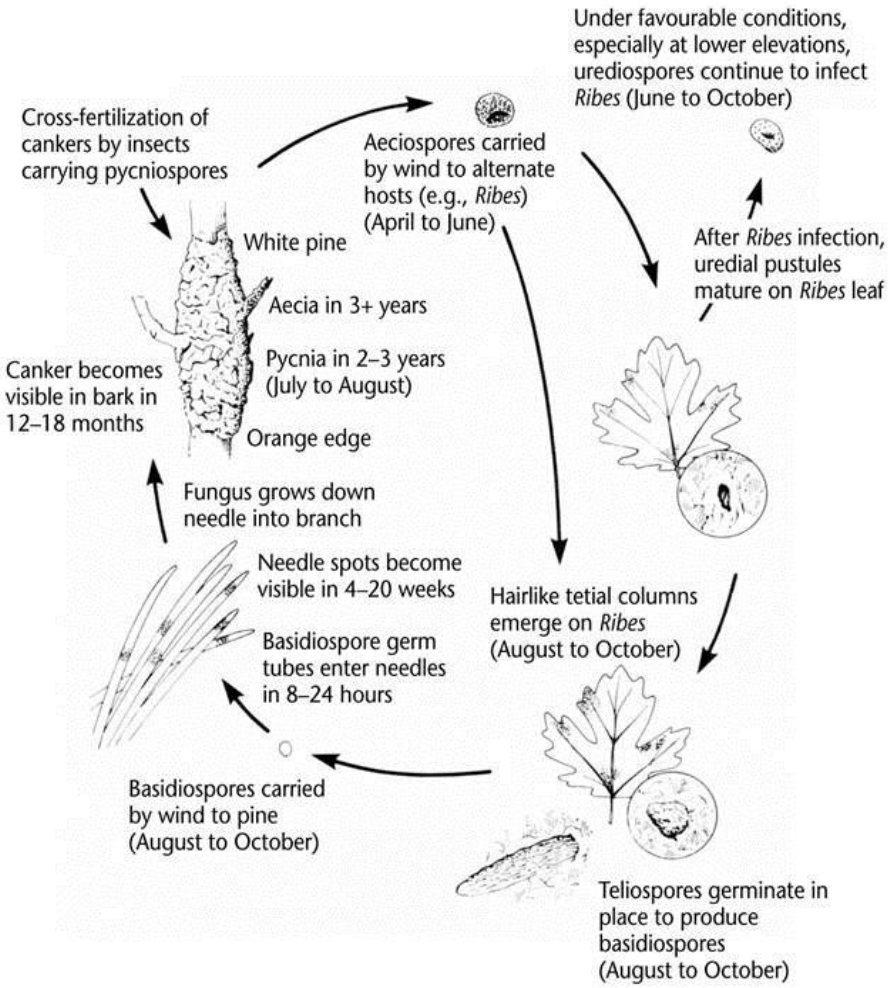


Figure 1. Life cycle of *C. ribicola*. Showing all five spore stages and alternate host. Modified from Zeglan et al 2009.

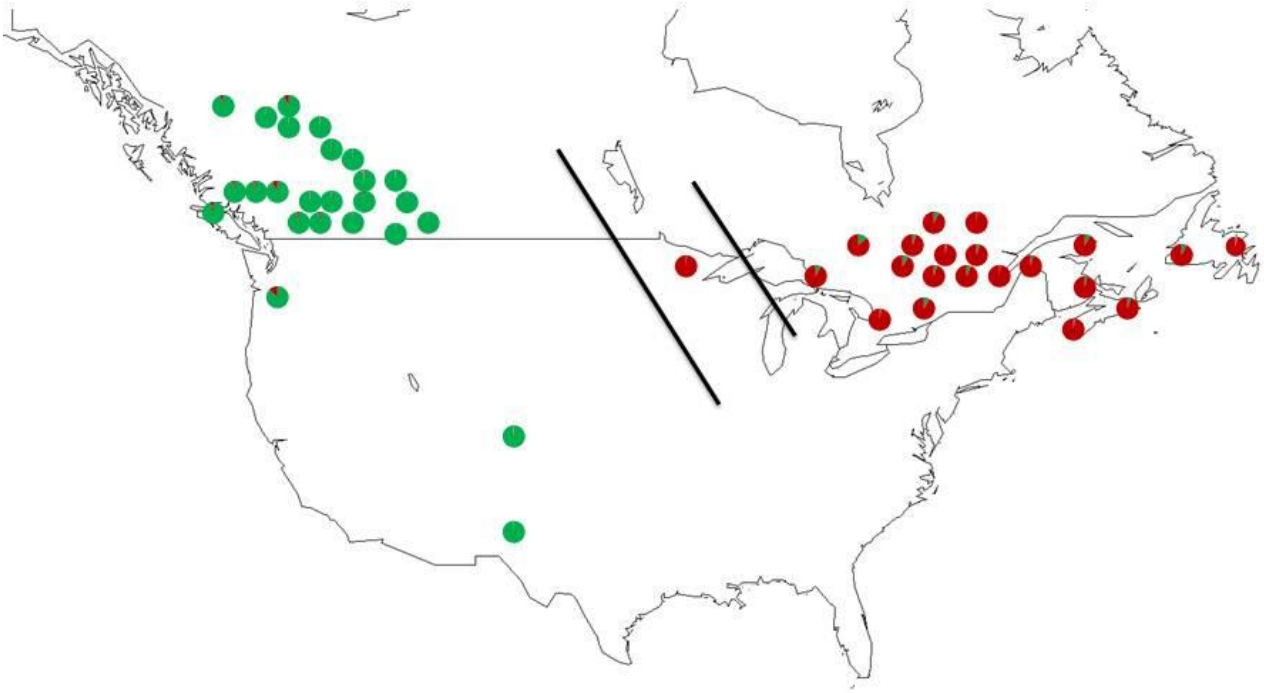

Figure 2. Location of *C. ribicola* populations evaluated in this study. The pie charts show the clustering results from Structure red is contributed from the eastern cluster and green is contributed from the western cluster. The two black lines show the two barriers to gene flow the longer line is between the eastern and western populations and the shorter line is between Minnesota and the rest of the eastern population.

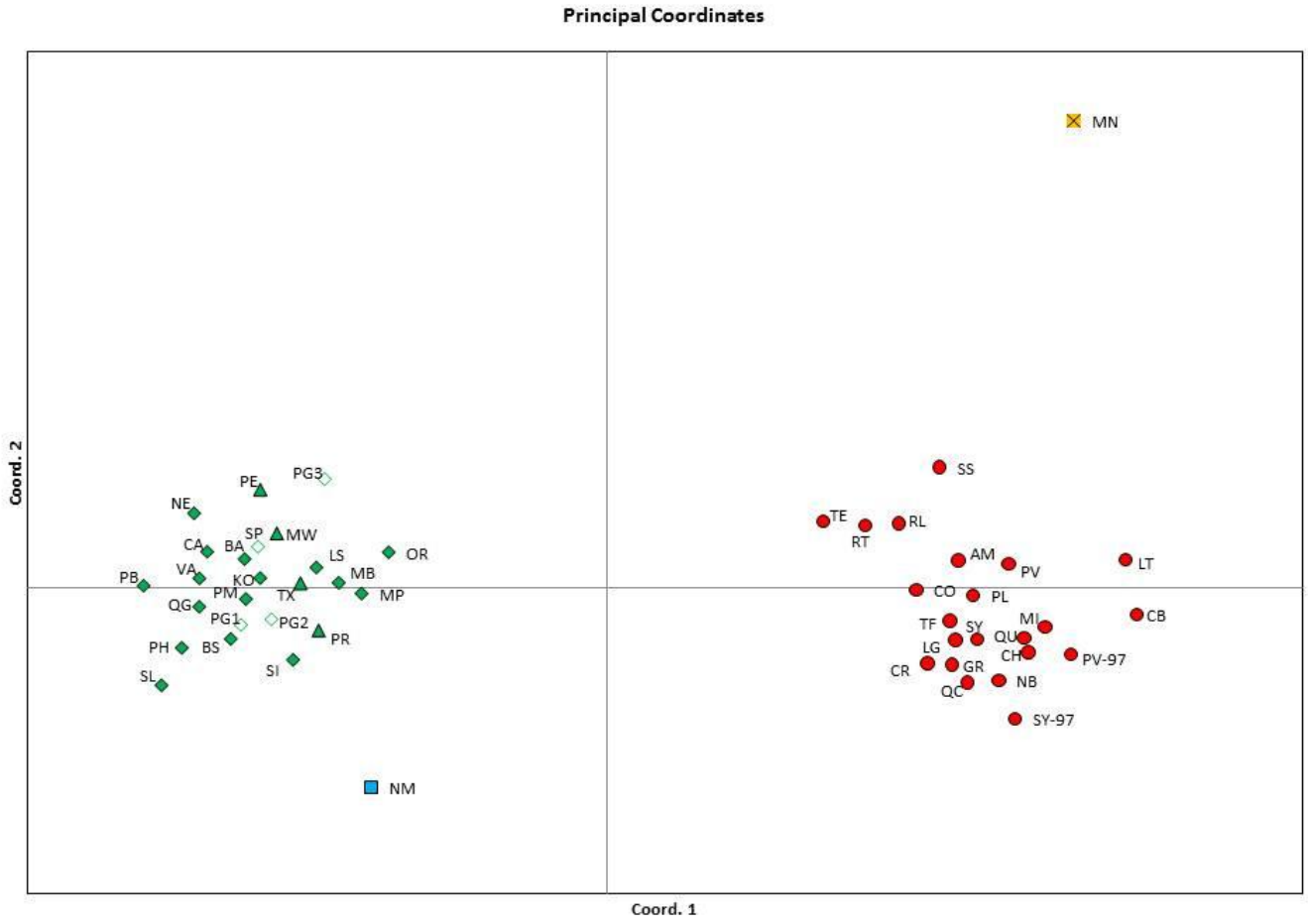


Figure 3. Principal Coordinates Analysis (PCoA) among 55 populations based on Nei's genetic distance using GenAlEx. Eastern Cluster (red circles), western cluster (green), coastal populations (green triangles), low elevation (open diamonds) and high elevation (solid green diamonds). The two outlying populations New Mexico (blue square) and Minnesota (yellow square with "x").

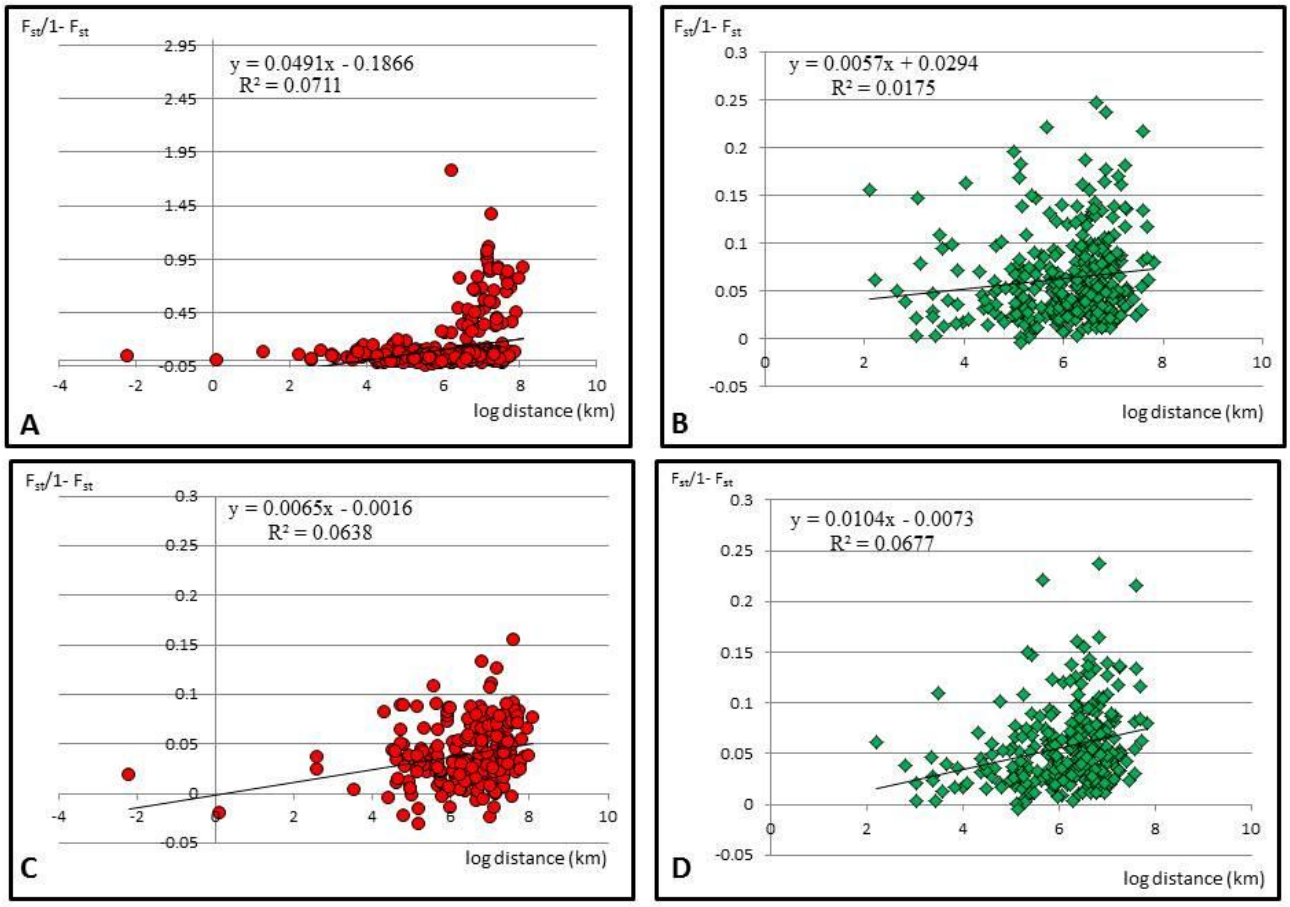


Figure 4. Plot of isolation-by-distance for **(**A) eastern population, (B**)** Western population, (C**)** eastern population excluding Minnesota and (D**)** western population excluding New Mexico.

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