Protocol for identification of *Cronartium ribicola x Cronartium comandrae* hybrids in populations of *Pinus monticola*

FRST 498 – Thesis for graduation

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Abstract

Cronartium ribicola is a rust fungi affecting soft pines throughout British Columbia. It is believed that *C. ribicola* is capable hybridizing with *C. comandrae* and is doing so in areas where the two rusts are in close proximity with each other. Establishing a means of identifying hybrid individuals is an important step in studying the hybrid individuals. Through the use of digestion enzymes and gel electrophoresis, hybrid aeciospores DNA can be differentiated from non-hybrid spore DNA. The restriction endonuclease BtsCI selectively cleaves *C. ribicola* DNA segments between the ITS1f and ITS4 binding sites while leaving *C. comandrae* segments intact. The result of the selectivity of this enzyme in the presence of *C. comandrae, C. ribicola*, and hybrid DNA is one, two, and three bands, respectively, when observed through gel electrophoresis. This relatively easy and cost effective means of identifying these hybrids will facilitate further study of these and other inter-specific hybrids, as well as the relationships of other hybrid fungi.

Key words: *hybridization, white pine blister rust, comandrae rust, BtsCI, whitebark pine,Ribes, type IIS restriction endonuclease,*

Introduction

Cronartium ribicola J.C. Fisch. and *C. comandrae* Peck are species of softwood rusts that infect soft and hard pines, respectively. *C. ribicola* is a macrocyclic heteroecious rust that affects five-needle pines, namely Western White Pine, or *Pinus monticola* Dougl. Ex D. Don, in British Columbia. Uredinia and telia are produced on *Ribes spp.,* which consists of currants and gooseberries. The distribution of *C. ribicola* follows the distribution of five-needle pines, including the high elevation regions where whitebark pine, or *Pinus albicaulis* Engelm., is a dominant species. It is widely believed to be an exotic species introduced to B.C. around 1910, and has recently been found on Rocky Mountain Bristlecone Pine trees (Blodgett, 2004). Pines are initially infected through the needles in the fall by basidiospores that have been produced on a *Ribes* spp. Trees can be infected several years before any symptoms emerge as the fungus grows through the bark and phloem of the infected tree (Allen et al. 1996). In the spring, spermatia and aecia are produced in the form of blisters on the bark of the infected pine tree. Aeciospores are not capable of re-infecting pines, as they are only capable of infecting *Ribes* spp. Shortly after infection by aeciospores, urediniospores start to be produced on the leaves of the infected plant and production continues throughout the summer months. Dikaryotic urediniospores are capable of re-infecting the plant on which they were produced. In the fall the fungus turns to producing telia that eventually give rise to monokaryotic basidiospores that go back to infecting pine trees. Spermatia function as the monokaryotic gamete stage while infection is taking place on the pines. Fusion of compatible gametes eventually gives rise to the dikaryotic aeciospores that carry the fungus back to the *Ribes spp*.

In regions where whitebark pine thrives the growing season is limited to 90-110 days (Arno & Hoff, 1989) and mortality from white pine blister have is having a marked impact on the species' ability to sustain itself in the already harsh environment at the treeline (Zeglen, 2002). The importance of whitebark pine and their seed crops on grizzly and black bear survival has been has been well studied

(Mattson et al. 1992, Kendall, 1983), and the interconnectedness of other animals in those ecosystems, such as squirrels and Clark`s nutcracker (Mattson & Reinhart, 1997, Hutchins & Lanner, 1982). The relative importance of this sensitive tree species on such a high profile mammalian species such as the grizzly bear makes *C. ribicola* a higher profile pathogen than *C. comandrae*.

C. comandrae is a rust fungus with a life cycle very similar to *C. ribicola*, but differs in its host preference. The primary aecial host of *C. comandrae* is *Pinus contorta* Dougl. Ex Loud. Var. *latifolia* Engelm., while its primary telial host varies between bastard toadflax and pale comandra. The range of this fungus is generally throughout the range of its host species, but is restricted to regions where an alternate host is present (Allen et al, 1996). Since lodgepole pine is rather ubiquitous in British Columbia, its range often overlaps with that of western white pine and occasionally whitebark pine. As a result of these overlapping ranges of tree species, there is a corresponding overlap of the ranges of the two rust fungi.

The life cycles of both *C. ribicola* and *C. comandrae* have stages where haploid spermatia are produced and there is subsequent fusion of the male and female spermatia to form a dikaryotic cell. As with other basidiomycetes, there is no nuclear fusion at this point, the nuclei from each parent gamete remain separate within the cell. The resulting aeciospores are also dikaryotic. If a spermatium from one of the rusts were to fuse with a spermatium from the other rust, the resulting cell would be dikaryotic with one nucleus from each parent fungi. The formation of hybrids through somatic cell fusion is not a novel concept in the realm of rust fungi. Hybridization among *Puccinia* spp. has been identified through pathogenic and isozymic features, as well as with the use of RAPD analysis (Park et al. 1999). While the mechanisms and consequences of hybridization among fungi seem to be poorly understood and inadequately studied, other natural examples can be found in *Melampsora* spp. and *Ophiostoma* spp. (Brasier et al. 1998, Newcombe et al, 2000). Establishing a practical means for identifying hybrid

individuals other than through pathogenic characteristics is an important step in researching inter- and intra-specific hybridization

Methods

Spore collection, desiccation and storage

C. ribicola aeciospores were collected from sites across the southern half of British Columbia. The spores were collected from individual mature aecia on P. albicaulis trees. Information recorded for each sample included the site, the tree number, the canker number, and the blister, or aecia number. Bulk samples were also collected from various sites to facilitate studies not requiring the single genotypes analyses. Aeciospores were collected by way of rupturing the individual aecia with a sterile toothpick and allowing the spores to fall into a sterile 1.5mL centrifuge microtubule.

Figure 1 indicates the locations of the sample collection sites within British Columbia. Other samples were collected from Alberta, where sites included Castle Mountain lookout in Banff National Park, Plateau Mountain, Slacker Creek near Oldman River, Porcupine Hills, and areas adjacent Carbondale River Road. Samples for analysis from Quebec were also made available to us. The samples from Quebec are those that have been confirmed to be hybrid *C. ribicola* and *C comandrae* spores (Hamelin & Joly, unpublished).

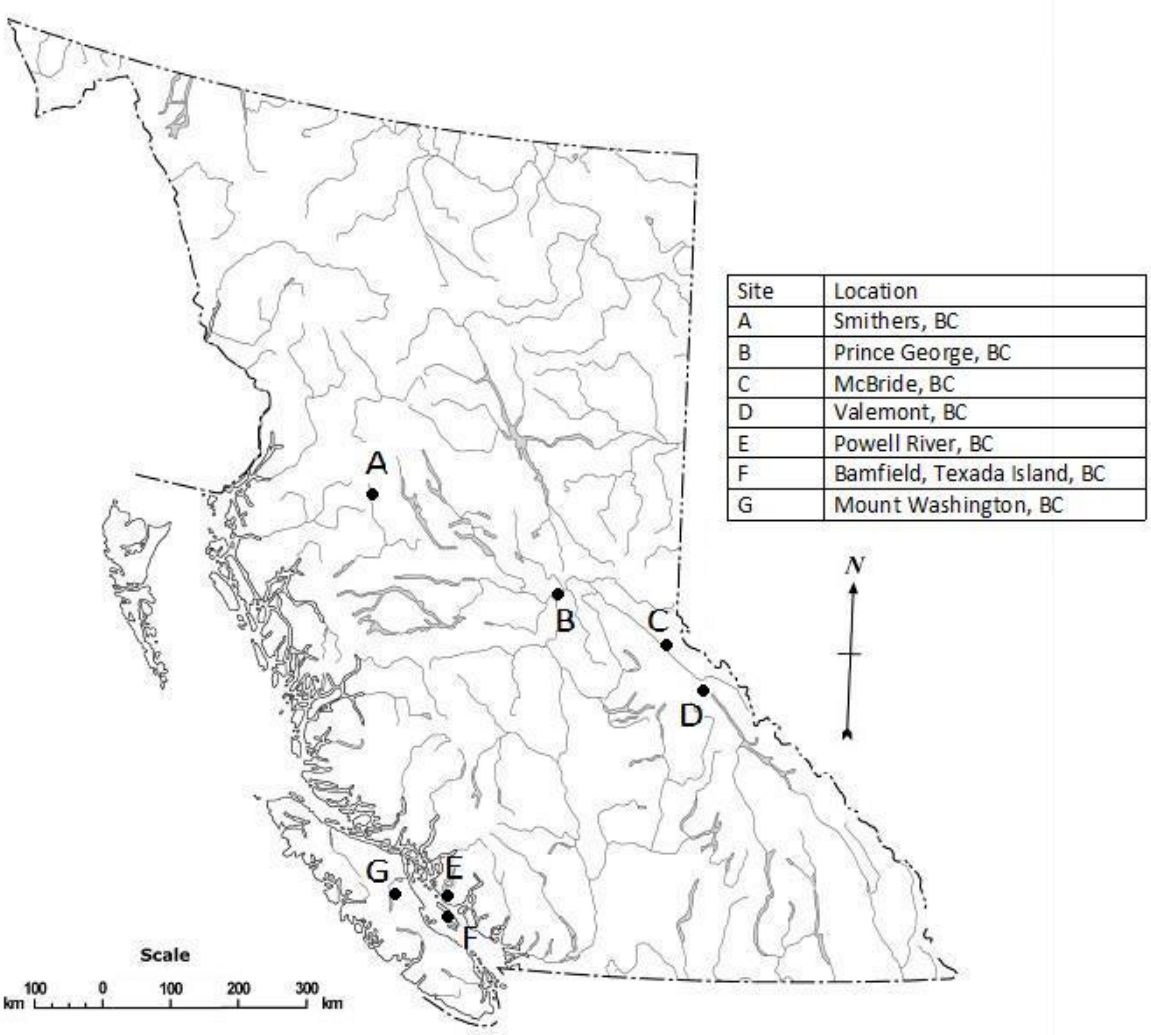

Figure 1: Location of sites for *C. ribicola* aeciospore collection.

DNA extraction

Cell Disruption

Spores were placed in a 1.5 mL centrifuge microtubule using a sterile microspatula. The quantity of spores used was approximately that which it took to cover the terminal one millimetre of the microspatula blade. A sterile bead was added to the microtubule along with a small amount of diatomatious earth. 500 μL of **AP1** buffer and 1μL RNase A (100mg/mL) was added to the microtubule. Microtubules were vortexed for 10 seconds, heat in a water bath at 80°C for 10 minutes, and the placed in a Mixer-Mill 300 for two minutes at a frequency of 30 cycles per second.

Extraction

Extraction of the fungal DNA was carried out using QIAGEN DNeasy Plant Mini Kits. Following cell disruption the spores were placed in a water bath at 100°C for five minutes. The spores were then quickly spun before the addition of 150 μL of **AP2** buffer. The sample was centrifuged at 13000rpm for one minutes, placed at -20°C for 10 minutes, then centrifuged again at 13000rpm for five minutes. The lysate was applied to the QIAshredder Mini Spin Column placed in a two mL collection tube and centrifuged for two minutes at 14,000rpm. The flow-through was transferred to a new tube without disturbing the pellet and 850μL of **AP3/E** (binding buffer) was added and mixed. 740μL of this solution was added to the DNeasy Mini Spin Column and centrifuged at 8000rpm for one minute, discarding the flow-through. The process was repeated with the remaining solution. 500μL of AW Buffer was added to the column and centrifuged for one minute at 8000 rpm to wash impurities from the membrane. This wash process was repeated a second time, discarding the flow-through both times, and the column were centrifuged again for two minutes without buffer to dry the membrane. The column was placed in a sterile 1.5mL microtubule and the DNA adhered to the membrane was eluted using 40μL of autoclaved water heated to 80°C and incubated on the membrane for one minute. The elution used a two minute centrifuge at 14,000rpm.

DNA amplification

Polymerase Chain Reaction (PCR) was carried out using ITS1f and ITS4 primers. PCR conditions included 30 cycles at 94°C, 53°C, and 72°C, for 30 seconds, 30 seconds, and 70 seconds, respectively. ITS1f and ITS4 primers yield approximately the same sized fragment when amplifying *C. ribicola* and *C. comandrae* DNA.

DNA enzyme digestion

BtsCI is a type IIS restriction endonuclease that cleaves a site within the amplified region, between the binding sites of ITS1f and ITS4, of the *C. ribicola* genome. The site specific to BtsCI is not present in the *C. comandrae* genome. The result of this characteristic of BtsCI is the selective digestion of amplified *C.*

ribicola DNA into two distinct fragments, regardless of whether *C. comandrae* DNA is present. If both *C. ribicola* and *C. comandrae* DNA is present in a sample, DNA fragments of three distinct sizes will be discernible; two fragments belonging to the *C. ribicola* DNA, and one to the *C. comandrae* DNA.

In the absence of identified hybrid DNA, amplified DNA from both species was mixed together in equal proportion prior to digestion. Samples of *C. comandrae*, *C. ribicola*, mixed DNA, and identified hybrid DNA were all subjected to the BtsCI endonuclease and then put through gel electrophoresis.

Results

Spore collection took place in regions where the range of western white pine and lodgepole pine overlapped, and there was a corresponding overlap in the range of the two rust fungi. Areas where spore collection took place were noted to contain lodgepole pine trees infected with *C. comandrae* adjacent western white pine trees infected *C. ribicola*.

155 *C. ribicola* samples from across B.C. were selected for hybrid testing, and no sample that showed an acceptable level of amplification could be identified as a hybrid. All the samples that showed suitable amplification were digested by restriction enzyme, but none showed the distinct three bands in the gel that would be expected of hybrid spores. Figure 2 shows two of the gels that were run with samples amplified and digested samples taken from individual aecia. Some samples lacked bands altogether; this is likely a result of poor amplification. Limited samples of confirmed hybrids exist, and as such only three samples could be extracted, amplified and digested. Figure 3 shows the results of the gel run with the hybrid spore samples. Two of the samples show three distinct that would be expected with a hybrid sample. The third sample showed an unexplained variation from the expected three bands.

Before samples of hybrid spores were obtained, samples of *C. ribicola* and *C. comandrae* DNA were combined and amplified together to simulate the behaviour of hybrid DNA in a gel. The result of digesting this sample was the distinct three bands that would be expected of hybrid DNA.

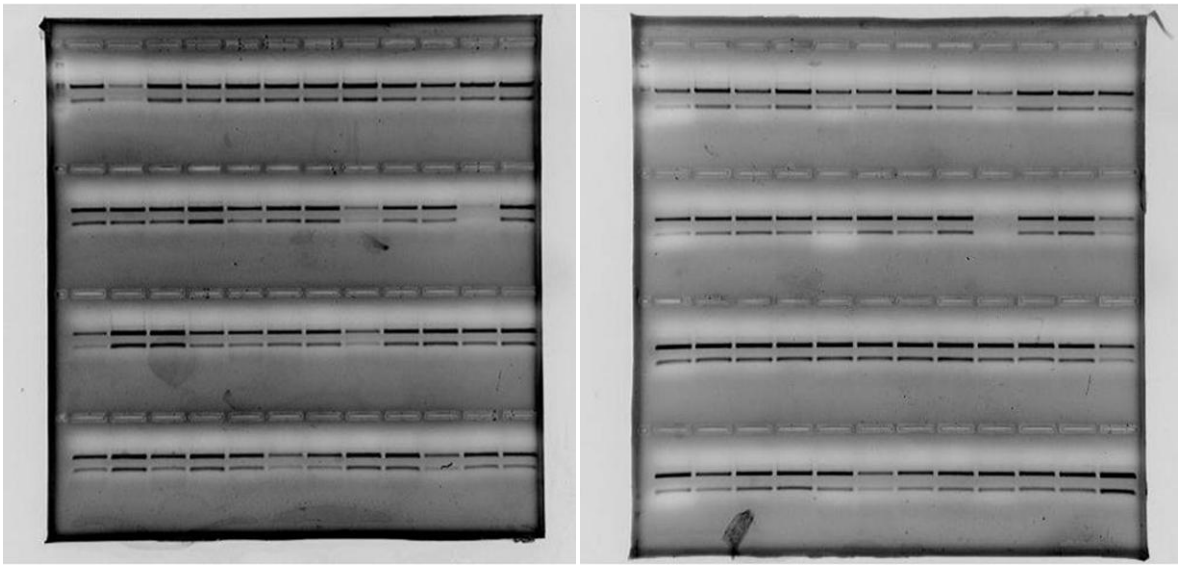

Figure 2: Stained gels showing two bands of digested DNA fragment samples C. ribicola

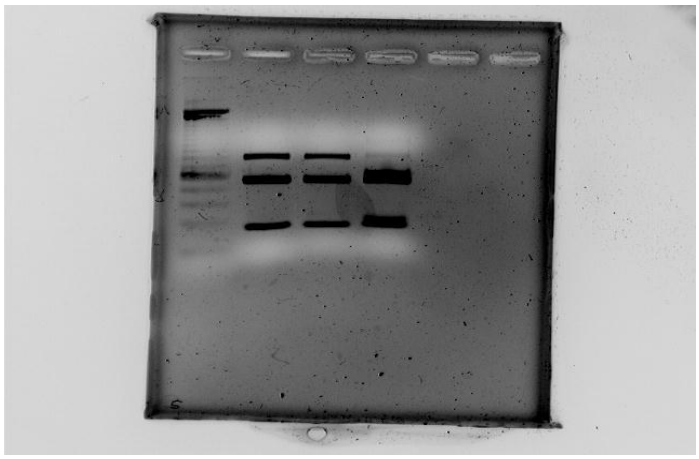

Figure 3: Stained gel showing three bands, indicating the presence of both *C. ribicola* and *C. comandrae* DNA.

Discussion and Conclusion

Two key areas of concern with this method of identifying hybrid individuals are the issues of contamination and partial digestion by the endonuclease. If spore collection is done in a sterile manner as described above, spore samples from individual aecia should be suitably free of contamination. So

long as proper techniques are used throughout the DNA extraction and amplification processes, the samples should be reasonably free of contamination. None of the samples shown in figure 2 display any signs of partial digestion. BtsCl endonuclease shows good and consistent digestion with all samples, indicating that partial digestion does not seem to be an issue.

The limited scope of this study makes it difficult to draw any inferences about the presence of hybrid individuals in wild populations. Only having three samples of confirmed hybrids as positive controls also makes it difficult to say that the protocol very effective at identifying hybrids.

With the overlapping ranges of these two rust fungi and their relative genetic closeness (Vogler & Bruns, 1998), it seems inevitable that some kind interaction might occur between them. More extensive sampling is necessary in order to determine if hybrid individuals exist in regions where the two fungi are in close proximity. Hybridization of fungi generally corresponds to some change pathogenicity, with the hybrid individual having pathogenic characteristics of either parent. If the spermatia of *C. ribicola* are fusing with those of *C. comandrae*, it does not really matter if the resulting aeciospores are not capable of infecting anything. Once hybrid spores have been identified, tests to determine pathogenicity will be necessary. Protocols for inoculation of both *Ribes spp.* and *Pinus spp.* exist and would be useful as a starting point for determining the infection capacity of the hybrid rust fungi. If sexual recombination can occur between these two species, it would have huge implication on the pathogenic variability in this group of fungi as well.

In terms of speciation taxonomy with respect to fungi, delineation has in the past been restricted to pathogenic and phenotypic characteristics, since sexual reproduction in fungi is not always present or observable. Phylogenetics has allowed scientists to determine the relative relation between different species. Recent studies have shown that *C. ribicola* and *C. comandrae* diverged fairly recently with respect to other pine stem rust fungi (Vogler & Bruns, 1998). Finding a quick and cost-effective means of identifying hybrid individuals is important as it will facilitate further research into rust hybrids. With

further testing, endonuclease digestion may prove to be an effective means for identifying *C. ribicola x C. comandrae* hybrids.

References

1. Allen, E.A., Morrison, D.J., and Wallis, G.W. 1996. Common trees diseases of British Columbia. Canadian Forest Service, Pacific Forestry Centre, Victoria, B.C.
2. Blodgett, J.T. 2004. First Report of White Pine Blister Rust on Rocky Mountain Bristlecone Pine. Plant Disease. 88(3): 311.
3. Brasier, C. M., Kirk, S. A., Pipe, N. D. & Buck, K. W. 1998. Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. Mycol. Res. 102: 45-57.
4. Hamelin, R., & Joly, D. 2010. [White pine blister rust genomic research]. Unpublished raw data.
5. Hutchins, H.E., and Lanner, R.M. 1982. The central role of Clark`s nutcracker in the dispersal and establishment of the whitebark pine. Oecologia. 55(2): 192-201.
6. Kendall, K.C. 1983. Use of pine nuts by grizzly and black bears in the Yellowstone area. Int. Conf.. Bear Res. and Manage. 5:166-173.
7. Mattson, D.J. & Reinhart, D.P. 1997. Excavation of red squirrel middens by grizzly bears in the whitebark pine zone. Journal of Applied Ecology. 34:926-940.
8. Mattson, D.J., Blanchard, B.M., & Knight, R.R. 1992. Yellowstone grizzly mortality, human habitation, and whitebark pine seed crops. J. Wildl. Manage. 56(3): 432-442.
9. Newcombe, G., Stirling, B., McDonald, S., Bradshaw, H.D. 2000. *Melampsora x columbia*, a natural hybrid of *M. medusa* and *M. occidentalis*. Mycol. Res. 104(3): 261-274.
10. Park, R.F., Burdon, J.J., Jahoor, A. 1999. Evidence for somatic hybridization in nature in *Puccinia recondita* f. sp. *tritici*, the leaf rust pathogen of wheat. Mycol. Res. 103(6): 715-723.
11. Vogler, D.R., Bruns, T.D. 1998. Phylogenetic relationships among the pine stem rust fungi (*Cronartium* and *Peridermium* spp.). Mycologia. 90(2): 244-257.
12. Zeglen, S., 2002, Whitebark pine and white pine blister rust in British Columbia, Canada. Can. J. For. Res. **32**: 1265–1274