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Genetic Resistance to White Pine Blister Rust in Limber Pine (*Pinus flexilis*): Major Gene Resistance in a Northern Population

Richard A. Sniezko^{1*}, Robert Danchok¹, Douglas P. Savin¹, Jun-Jun Liu², Angelia Kegley¹

¹USDA Forest Service, Dorena Genetic Resource Center, 34963 Shoreview Road, Cottage Grove, Oregon, 97424, U.S.A (rsniezko@fs.fed.us; rdanchok@fs.fed.us; dpsavin@fs.fed.us; akegley@fs.fed.us)

²Pacific Forestry Centre, Canadian Forest Service, Natural Resources Canada, 506 West Burnside Road, Victoria, BC, V8Z 1M5, Canada, jun-jun.liu@canada.ca

* Corresponding, email: rsniezko@fs.fed.us, Phone: (541) 767-5716, Fax: (541) 767-5709

Draft

Abstract

Limber pine, *Pinus flexilis*, a wide-ranging tree species in western North America, is highly susceptible to white pine blister rust (WPBR), caused by the non-native fungal pathogen *Cronartium ribicola*. The Canadian populations in particular have been heavily impacted, and in 2014 limber pine was designated *Endangered* in Canada by COSEWIC. Little is known about genetic resistance to WPBR in limber pine, but major gene resistance (MGR) has been characterized in some populations in the U.S.A. This study examines resistance in seedling families from 13 parent trees from British Columbia, Alberta and Oregon, representing the northern and northwestern-most populations. Most families were susceptible with 100% of the seedlings cankered, but one family from Alberta segregated 1:1 for cankered and canker-free. This is the first report of (a) MGR in Canada of any of the four species of five-needle pines native to Canada, and (b) any resistance in limber pine in Canadian populations, and is the northern-most known incidence of putative R-gene resistance in a natural stand of any five-needle pine species. Many of the Canadian selections were from stands with high incidence of WPBR infection, and their high susceptibility in this trial suggests that further infection and mortality is likely in the Canadian populations.

Keywords: major gene resistance, *Cronartium ribicola*, *Pinus flexilis*, limber pine, white pine blister rust

31 Introduction

32 Limber pine (*Pinus flexilis* James) is a wide-ranging forest tree species, occurring from southern
33 California in the United States to British Columbia and Alberta in Canada (Fig. 1). It occurs at
34 elevations ranging from 850 to 3810 m, in a wide variety of habitats from subalpine to upper
35 alpine tree line (Steele 1990; COSEWIC 2014; Tomback and Achuff 2010). Limber pine is
36 highly susceptible to white pine blister rust (WPBR), caused by the non-native fungal pathogen
37 *Cronartium ribicola* J.C. Fisch (Smith et al. 2013). Other threats to this species include habitat
38 loss due to fire suppression and climate change as well as mortality from mountain pine beetle
39 (*Dendroctonus ponderosae* Hopkins) (Tomback and Achuff 2010). In the Canadian portion of its
40 range, limber pine was designated *Endangered* in November 2014 by the Committee on the
41 Status of Endangered Wildlife in Canada (COSEWIC), and since 2009 it has been designated
42 *Endangered* under Alberta's Wildlife Act (Government of Alberta 2014). A species recovery
43 plan is in place in Alberta (Alberta Whitebark and Limber Pine Recovery Team 2014).

44 Limber pine has been affected in much of its range by WPBR infection and mortality (Schwandt
45 et al. 2010). In Canada WPBR incidence is notably high, with many pine populations with
46 greater than 60 percent infection and over 40 percent mortality (Smith et al. 2011; 2013). The
47 ultimate deterioration and possible extirpation of these populations by WPBR will depend on
48 factors such as conducive environmental conditions for disease spread and intensification, levels
49 of natural regeneration of limber pine, and frequency of durable genetic resistance to WPBR in
50 extant populations.

51 The maintenance of limber pine as a viable species component in forest stands in much of its
52 range will be heavily dependent on the frequency and types of genetic resistance to WPBR and

the geographic distribution of resistance. Maintaining genetic diversity within the species will also be important. On sites of high WPBR hazard, the best chance for successful restoration efforts will be dependent on the availability of seedlings with genetic resistance to WPBR. However, relatively little is known for limber pine about the types of resistance to WPBR and their frequency, and previous testing has encompassed only a small portion of the species' geographic range (Schoettle et al. 2014). In earlier tests at Dorena Genetic Resource Center (DGRC), limber pine from some populations in southern Wyoming to southern Colorado had major gene (MGR) or R-gene resistance (Schoettle et al. 2014; Kinloch and Dupper 2002). Major gene resistance restricts infection to the needles, and progeny of open-pollinated parent trees heterozygous for MGR segregate in a 1:1 ratio for cankered and canker-free seedlings. In this study we report on the first examination of half-sib families from populations of limber pine from both Canada (BC and AB) and Oregon for resistance to WPBR. These populations are hundreds of kilometers from areas previously documented to have the *Cr4* gene associated with the MGR in limber pine in southern populations of the species. To our knowledge this is the first WPBR testing of half-sib families of individual parent trees of limber pine from these three geographic areas.

Materials and Methods

Open-pollinated progeny of five parent trees from Alberta, five from British Columbia, and three from Oregon were included in a WPBR resistance test at Dorena Genetic Resource Center, Cottage Grove, Oregon, U.S.A. The Canadian seedlots represent the northern extent of the species range, while the Oregon seedlots represent the most northwestern population of the species (Fig. 1). Seed from all but one of the Canadian parent trees were collected in 2010 and 2011; the other Canadian parent had seed collected in 1999. All of the Oregon parent trees had

seed collected in 2013. The British Columbia and Alberta seedlots originated from six populations, with one or two parent trees per population. The two parents from Prairie Bluff were prostrate shrubs, less than 0.5 m tall. Previous surveys have noted the level of WPBR to be very high, 82, 94, and 96 percent in or near one British Columbia and two Alberta populations (Crowsnest Pass, Prairie Bluff, and Kananaskis, respectively) and low (0 to 13 percent) in the Abraham Lake population (Jodie Krakowski, personal communication, 2016). WPBR was present, but at undocumented levels, in the other British Columbia populations (Don Pigott, personal communication, 2016). An inactive canker was noted on the Kananaskis parent tree; WPBR infection was noted on both Crowsnest Pass parent trees, and previous records noted an inactive canker on Andy Good Creek #151 (Don Pigott and Barb Gass, personal communication, 2016). The three Oregon seed collections were from parent trees on the Eagle Cap Ranger District of the Wallowa-Whitman National Forest. No WPBR infection was noted in the Oregon population during cone collection (Andy Bower, personal communication, 2016).

Seed were placed into stratification at 2° C on 1 April (Oregon seedlots) and 7 May 2014 (Canadian seedlots). Stratified seed from the Oregon lots were placed in the germinator on 17 May 2014, and seed began to germinate on 19 May. Stratified seed from the Canadian lots were placed in the germinator 9 June 2014 and began germinating 12 June. Germinated seed were transplanted into Ray Leach cone-tainers (164 cm³) in an unheated greenhouse. Prior to inoculation, seedlings were placed into a randomized complete block design, with three blocks and up to eight seedlings per family per block in row plots; most families had 21 seedlings, one family had only 17 seedlings, and one family had 22 seedlings (Table 1). All of the young seedlings had cotyledons and primary needles present, and 44 of the 270 seedlings (16 percent) also had secondary needles.

99 Inoculation followed standard Dorena Genetic Resource Center protocols (Snieszko et al. 2011).
100 Seedlings were moved into the inoculation chamber on 8 September 2014 to acclimate.
101 Concurrently, native *Ribes hudsonianum* var. *petiolare* (an alternate host for WPBR) leaves
102 naturally infected with *C. ribicola* were collected from areas near whitebark pine and limber pine
103 on the Wallowa-Whitman National Forest. In late afternoon on 11 September, the infected *Ribes*
104 leaves were placed on wire screens above the pine seedlings at DGRC, telial side down, to
105 release basidiospores. Spore fall was monitored at periodic intervals by examining spore density
106 on microscope slides placed among the seedlings. The chamber was maintained at approximately
107 16.7°C and 100% relative humidity during seedling acclimation and spore fall. *Ribes* leaves were
108 removed once the target spore density of 10,000 spores/cm² was observed on the monitoring
109 slides, approximately 10.5 hours after the *Ribes* leaves were placed over the pines. Once the
110 target inoculum density was reached for the last frame, the temperature was raised to 20°C and
111 relative humidity maintained at 100% to promote germination of the basidiospores; the seedlings
112 were left in the inoculation chamber approximately 48 hours to promote spore germination and
113 needle infection. The seedlings were removed from the chamber on 14 September and returned
114 to the unheated greenhouse for the duration of the trial. Actual spore density for the trial,
115 assessed from slides after the infected *Ribes* leaves were removed, averaged 10,740 spores/cm²
116 (with rep means ranging from 9600 to 12,400 spores/cm²), and basidiospore germination,
117 assessed on agar plates, was noted at 100 percent.

118 Assessments of seedling infection started after needle spots were visible on most seedlings.
119 Seedlings were assessed four times for disease symptoms and mortality: 28 January, 2 March, 23
120 April and 19 November 2015. Each seedling was evaluated for spot type (a) normal yellow,
121 susceptible (non-MGR) spots, (b) spots with necrotic bands or brown necrotic spots usually

associated with MGR (Schoettle et al. 2014), or (c) indeterminate spot type, as well as three categories of spot counts (0 spots; 1 to 10 spots; more than 10 spots), canker class (none; possible canker; probable canker; definite canker), number of cankers, and five categories of seedling vigor (vigorous; moderately vigorous; sickly; dead from WPBR; dead from non-WPBR causes). The presence/absence of pycnia was noted at 3rd assessment (April). In May 2015 after the third assessment, the severely cankered seedlings were destructively sampled for separate work related to a comparative genomic study of MGR between northern and southern populations. Only those seedlings that were canker-free or had less severe cankers at the 3rd assessment (and not destructively sampled) were assessed a fourth time (November). Canker-free seedlings were left for further observation.

Statistical tests were performed using R version 3.2.2 software (R Core Team 2015). Binomial tests were performed to see if any families differed from a 1:1 segregation in cankered and canker-free traits that would suggest control by a single major dominant gene such as *Cr4*.

Results

Inoculation was successful with 267 of 270 of the seedlings showing needle spots (the first symptom of infection) on primary needles at the first assessment. By second assessment (2 March) all seedlings had needle spots (Fig. 2). Most seedlings (267 of 270) had more than 10 needle spots at one or more inspection, with the majority (257 of 270 seedlings) having more than 10 spots at first assessment (Fig. 2). Secondary needles also showed a high level of needle spotting, with 40 of 44 seedlings having spots on secondary needles.

At the first assessment, most needle spots were classified as normal, yellow, and diffuse or spreading (susceptible phenotype, Fig. 2c), but spots for 13 of the 267 seedlings were classified

as 'some necrotic' (n=1) or 'indeterminate' (n=12) (Fig. 2b). All three seedlings with no spots and 12 of the 13 seedlings with indeterminate spot type at the first assessment were progeny of one Alberta parent tree, Prairie Bluff #2. By second assessment all seedlings of twelve of the 13 families had distinctly susceptible spots (Table 1). In contrast, 15 seedlings of Prairie Bluff #2 were still characterized as indeterminate (Table 1); two of the Prairie Bluff #2 seedlings with indeterminate spots in March were later characterized as having susceptible spots at third assessment (23 April).

Stem infection was high for this trial; 100 percent of seedlings in 10 of the 13 families developed cankers (Table 1, Fig. 2d), and two other families had greater than 85% cankering. Cankering increased between January (1st assessment) and April (3rd assessment), and most of the observed cankers were present by March (2nd assessment). Overall, 53.0%, 90.4%, and 94.7% of the seedlings had cankers at the 1st, 2nd, and 3rd assessments, respectively. Number of cankers per seedling varied from 0 to 6, 0 to 9, and 0 to 9 at the 1st, 2nd, and 3rd assessment (mean=0.77, 2.95, and 3.33 cankers per seedling, respectively), although counting cankers became more difficult as time progressed, and cankers began to merge. At the 3rd assessment, pycnia were noted on 72.2 percent of cankered seedlings.

All of the families had a ratio of cankered to canker-free seedlings at 3rd assessment significantly different from 1:1 ($p<0.0001$) except for Prairie Bluff #2 ($p=0.26$). This suggests that Prairie Bluff #2 is segregating for cankered and canker-free seedlings and is putatively heterozygous for MGR. Prairie Bluff #2 also showed a perfect correspondence between cankered/canker-free seedlings and susceptible/necrotic spot type; in this trial, analysis of spot phenotype yields identical results to analysis of cankered phenotype. Seven canker-free seedlings died from

unknown causes during the trial; three of these were from Prairie Bluff #3, three from Prairie Bluff #2, and one from Crowsnest Pass #49.

Seedlings in Prairie Bluff #2 (the putative MGR family) also tended to have fewer early needle spots relative to the other families in this trial at the first assessment; nine of the 13 trees with 10 or fewer spots at first assessment were from this family. The odds of having ten or fewer spots at 1st assessment were significantly greater for this family than for the group of the other families ($p < 0.0001$, Fisher's exact test). The odds of having 10 or fewer spots at 1st inspection were estimated to be between 11 and 224 times greater for the MGR family (95% confidence interval). By second assessment, only four seedlings (including three from Prairie Bluff #2) had 10 or fewer needle spots.

Within the putative MGR family (Prairie Bluff #2), the odds of developing cankers by the 3rd inspection were significantly lower for the seedlings that had 10 or fewer spots at the 1st inspection ($p = 0.015$ from two-sided test). None of the nine seedlings in this family with 10 or fewer spots at first inspection displayed cankers later in the trial.

Mortality was first observed at 3rd assessment, with 26 cankered seedlings dead and 4 seedlings dead from unknown causes (three of these from Prairie Bluff #3 and one from Prairie Bluff #2). Delayed mortality could be an indicator of partial resistance; however, trends in mortality over time were not investigated in this trial because the majority of cankered seedlings were destructively sampled in May 2015.

Discussion

Limber pine is very susceptible to WPBR both in its natural forest environment as well as in inoculation trials utilizing young seedlings. However, only a few seedling inoculation trials have

188 been undertaken to examine the types and frequency of resistance and its geographic distribution
189 (Snieszko et al. 2011; Schoettle et al. 2014; Kinloch and Dupper 2002). In a trial including
190 populations from the southern Rocky Mountains, the frequency of the *Cr4* allele averaged five
191 percent but varied from 0 to 13.9 percent over sites, and 14.3% of the 105 seed parents showed
192 segregation ratios suggesting MGR (Schoettle et al. 2014).

193 This trial examined young seedling families from the northern part of the geographic range of
194 limber pine for the presence of MGR. During the trial, 100 percent of the seedlings displayed
195 needle infections, and only three of the 13 families had one or more canker-free seedlings,
196 potential evidence of genetic resistance to WPBR. One of the families segregated 1:1 for
197 cankered and canker-free seedlings, suggesting that one of the parent trees from Alberta has
198 MGR, putatively from the same *Cr4* gene identified in southern populations in earlier trials at
199 DGRC (Schoettle et al. 2014). Spots on the canker-free seedlings in this family (Prairie Bluff
200 #2) were characterized as “questionable” because they were not spreading like a susceptible spot
201 yet lacked the necrotic ring typical of spots on canker-free MGR seedlings in other white pine
202 species such as western white pine and sugar pine (Fig. 2a, 2b). However, the spots were similar
203 to those noted in our previous trials of limber pine from southern populations (Schoettle et al.
204 2014). The spots on the MGR seedlings in Prairie Bluff #2 in this trial appeared as defined,
205 discrete, and brown compared with the normal spreading yellow susceptible spots on cotyledons
206 and primary and secondary needles (Fig. 2). The necrotic spot phenotype could generally be used
207 to indicate MGR in limber pine, but canker-free status would provide additional confirmation.
208 Many of the canker-free seedlings in this family also showed latency in the development of
209 needle spots, having ten or fewer spots at first assessment. Although in this study the latency was
210 primarily observed in seedlings with the MGR phenotype, future studies will be needed to

confirm its relationship with MGR. Two other seedling families showed just one to three canker-free seedlings (out of 21 seedlings tested), suggesting that those parent trees are susceptible but may have been pollinated by a MGR parent tree in the vicinity or have some other type of resistance. One of these two parent trees (Prairie Bluff #3) is located ~100 m from the MGR parent (Prairie Bluff #2) identified here, and presumably pollen exchange could easily occur between these two trees. However, it is notable that those four seedlings never showed the necrotic spot type. The populations for these three families are in southern Alberta and British Columbia, and previous surveys have shown these areas to have very high levels of WPBR infection in the vicinity of the parent trees. Progeny of canker-free parent trees in highly infected stands can be strong candidates to show genetic resistance in inoculation trials since a high level of natural 'pre-screening' has been done in the forest.

To our knowledge, this is the first report of MGR to WPBR in Canada in natural populations of any of the four white pine species present there (*P. flexilis*, *P. monticola*, *P. albicaulis*, and *P. strobus*), the first report of any type of genetic resistance to WPBR in the northernmost populations of limber pine, and the northern-most known incidence of putative R-gene resistance in a population of any five-needle pine species. Prior to this report, MGR in limber pine had only been confirmed to be present in some populations present in Colorado and Wyoming. The results here extend the known geographic range of MGR, putatively affiliated with the same *Cr4* gene as that of the Colorado and Wyoming populations, to one of the northern-most populations of the species, >700 km north of the previously documented occurrence of *Cr4*. In the other three species of white pines where MGR has been documented, there has been no confirmation of MGR in the northern-most populations (Kinloch 1992; Kinloch et al. 2003; Snieszko, unpublished). The previous northernmost documentation of MGR in any of the North American

species was in *P. monticola* in southern Washington (Kinloch et al. 2003), more than 250 km south of the Prairie Bluff #2 parent tree. Future genetic mapping efforts will help determine whether MGR in the Alberta population is due to the same gene (*Cr4*) noted previously in limber pine or to a separate R-gene.

Surveys of several of the Canadian populations whose progeny were tested here have indicated that 85 to 96 percent of the trees are infected or dead from WPBR. WPBR cankers had been noted on four of the 10 Canadian parent trees whose progeny were evaluated here, and 100 percent of the seedling progeny of those parents were cankered in this trial. It should also be noted that progeny of only one or two parent trees per population was evaluated in this trial; it is possible that other parent trees within the population may have MGR or other types of genetic resistance. Finding one parent with MGR is encouraging, but the susceptibility of the progeny of the other parents from these native stands with high infection levels suggests that (a) even higher infection and mortality will occur in those stands, or (b) resistances other than MGR (e.g. partial resistance) may be present and difficult to detect in the very young, small seedlings utilized here.

The eastern Oregon population of limber pine represents an outlying, disjunct population at the northwestern-most portion of the species range. The three families tested showed no indication of MGR.

Complete resistance from a major (single, dominant) gene has been found in four of the nine species of white pines present in the U.S. (Kinloch and Dupper 2002; Kinloch et al. 2003; Schoettle et al. 2014). More extensive trials are now underway at DGRC to examine the frequency and geographic distribution of MGR and partial resistance to WPBR in limber pine.

The information from these trials will provide key knowledge to help resource managers better manage their populations.

To be of maximum utility, genetic resistance in limber pine has to be durable. Virulence to MGR has been previously documented in *P. monticola* and *P. lambertiana* (Kinloch and Dupper 2002; Kinloch et al. 2004) but not in either *P. flexilis* or *P. strobiformis* thus far. Limber pine parent trees identified as having MGR can serve as monitors for the potential evolution of virulence or a change in efficacy of this type of resistance with a changing climate. In addition, parent trees identified as susceptible can serve as monitors of the spread or intensification of WPBR. The data from such monitoring will provide managers with information to guide recovery efforts of this species in Alberta and restoration efforts throughout the range of limber pine.

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334 Table 1. White pine blister rust infection (needle, stem) and mortality for 13 limber pine families.

Population and Tree number	Number seedlings	Number with susceptible needle spots	Number dead (non-rust) ^a	Number cankered	Mean number of cankers ^b
Kicking Horse #1	21	21	0	21	4.0
Andy Good Creek #123	22	22	0	22	2.5
Andy Good Creek #151	17	17	0	17	2.1
Crowsnest Pass #49	21	21	1 ^c	20	2.8
Crowsnest Pass #51	21	21	0	21	3.5
Prairie Bluff #2	21	7	2 ^c , 1 ^d	7	0.78 (2.0 ^e)
Prairie Bluff #3	21	21	3 ^d	18	2.1
Kananaskis #9	21	21	0	21	2.8
Abraham Lake #1	21	21	0	21	3.5
Abraham Lake #8	21	21	0	21	4.3
Hurricane #1	21	21	0	21	5.0
Hurricane #3	21	21	0	21	3.5
Hurricane #6	21	21	0	21	5.6

335 ^aSee text for discussion of rust-related mortality.
336 ^bMean number of cankers is based on the maximum number of cankers observed for any assessment on
337 individual seedlings, excluding dead (non-rust) trees.
338 ^cMissing at 4th assessment (presumably no canker).
339 ^dDead at 3rd assessment (no canker).
340 ^eMean number of cankers excluding dead (non-rust) and canker-free trees.
341

342 Figure 1. Locations of limber pine seed trees of 13 seedling families tested for blister rust resistance at
343 Dorena Genetic Resource Center. Previous locations (Wyoming and Colorado) where major gene
344 resistance (MGR) in limber pine had been documented are indicated with 'X' in the small insert map
345 (Limber pine range data from Whitebark Pine Ecosystem Foundation. 2014. Available online from
346 <http://whitebarkfound.org>).

347 Figure 2. Differential MGR spot types on canker-free seedlings of (a) western white pine and (b) limber
348 pine family Prairie Bluff #2. Also shown is a limber pine seedling with yellow, susceptible type needle
349 spots typical on cankered seedlings (c) and a heavily cankered seedling (d).

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350
Figure 1.

351

352

Figure 2.