

# Genetic diversity of poplar leaf rust populations in the north-central United States

W. Chen and T.C. Harrington

**Abstract:** Uredinia of poplar leaf rust, *Melampsora medusae* Thüm f.sp. *deltoides*, appeared in late July 1998, June 1999, and August 2000 in a cottonwood (*Populus deltoides* Bartr.) stand in Ames, Iowa. Seedlings of the alternate host (eastern larch, *Larix laricina* (Du Roi) K. Koch) set out at the site in spring 2000 formed aecia 3 months before uredinia appeared. Using three PCR-based microsatellite markers, the aecial population was genetically diverse and in Hardy–Weinberg equilibrium, typical of a population that had gone through sexual reproduction. Uredinia populations in 1998–2000 had lower levels of gene diversities (from 0.58 to 0.71) and were not in Hardy–Weinberg equilibrium. Of nine populations in Minnesota, Iowa, and Missouri during the early stage of the 1999 epidemic, the two populations with the highest gene diversity, the highest level of heterozygosity, and the greatest number of unique genotypes were within the natural geographic range of larch. However, the southernmost population in Missouri was also in Hardy–Weinberg equilibrium. Epidemics started sooner in Iowa than in Minnesota, which contradicts the hypothesis that epidemics begin in northern regions where the alternate hosts naturally overlap. Epidemics appeared to commence independently in the various locations, perhaps focused around areas where ornamental larch is in proximity to *P. deltoides*.

**Résumé :** Les urédosores de la rouille des feuilles du peuplier, causée par *Melampsora medusae* Thüm f. sp. *deltoides*, sont apparus respectivement à la fin de juillet 1998, en juin 1999 et en août 2000 dans un peuplement de peuplier deltoïde (*Populus deltoides* Bartr.) à Ames, en Iowa. Des semis de l'hôte alterne, le mélèze laricin (*Larix laricina* (Du Roi) K. Koch), installés sur le site au printemps 2000 ont formé des écies 3 mois avant l'apparition des urédosores. Selon trois marqueurs microsatellites obtenus par PCR, la population d'écies était génétiquement diversifiée et en équilibre de Hardy–Weinberg, typique d'une population issue de la reproduction sexuée. Les populations d'urédosores de 1998 à 2000 avaient un degré de diversité génétique plus faible (de 0,58 à 0,71) et n'étaient pas en équilibre Hardy–Weinberg. Des neuf populations du Minnesota, de l'Iowa et du Missouri au début de l'épidémie de 1999, les deux populations avec la plus grande diversité génétique, le degré le plus élevé d'hétérozygotie et le plus grand nombre de génotypes uniques étaient dans l'aire de répartition naturelle du mélèze. Cependant, la population la plus méridionale au Missouri était également en équilibre Hardy–Weinberg. L'épidémie a commencé plus tôt en Iowa et au Minnesota, ce qui contredit l'hypothèse selon laquelle les épidémies débutent dans les régions plus au nord où les hôtes alternes se recoupent naturellement. Les épidémies ont semblé débiter indépendamment les unes des autres dans les différents endroits, peut-être concentrées dans les endroits où le mélèze ornemental côtoie *P. deltoides*.

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## Introduction

*Melampsora medusae* Thüm f.sp. *deltoides*, the causal agent of poplar leaf rust on *Populus deltoides* Bartr. ex Marsh. (eastern cottonwood) and its hybrids (Arthur and Cummins 1962; Widin and Schipper 1980), is endemic to the eastern United States. It has been introduced into most areas where *P. deltoides* is planted, such as Australia (Walker et al. 1974), France (Pinon 1973), New Zealand (Van Kraayenoord et al. 1974), South Africa (Trench et al. 1988), and the western

United States (Newcombe and Chastagner 1993). A hybrid between *M. medusae* and *Melampsora occidentalis* H. Jacks. (= *Melampsora ×columbiana* G. Newc.) causes poplar rust on *Populus balsamifera* subsp. *trichocarpa* (Torr. & Gray) Hult. × *P. deltoides* hybrids in the Pacific Northwest (Newcombe et al. 2000). Poplar leaf rust does not appear to be a major problem in natural stands, but the disease may become devastating in plantations (Newcombe and Chastagner 1993), where the trees often have narrow genetic diversity and are planted at narrow spacing. Poplar leaf rust can develop rapidly in susceptible clones (Schipper and Dawson 1974).

*Melampsora medusae* is a heteroecious, macrocyclic rust (Ziller 1974). Uredinia (the repeating, asexual stage) are formed on *P. deltoides* and its hybrids during the summer, and the fungus overwinters as telia on the forest floor. Sexual reproduction (pycnia and aecia) occurs on the alternate host, eastern larch (*Larix laricina* (Du Roi) K. Koch) early in the season. Eastern larch is native to the Great Lakes region and further north, and there is only slight overlap between the natural distribution of *L. laricina* and *P. deltoides*,

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which is native to most of the eastern United States. In most areas of the eastern United States where *M. medusae* is known, only the poplar host is naturally present. There is no native larch in Iowa or Missouri, although susceptible *Larix* species are occasionally planted there as ornamentals.

Epidemics on *P. deltoides* in the north-central United States are assumed to originate from urediniospores from Wisconsin or Minnesota, where the fungus can overwinter as telia and complete its life cycle on larch in the spring. There are some spore trapping data (McCracken et al. 1984; Widin and Schipper 1980) that support the hypothesis that epidemics commence in the northern areas and progress southward. Alternatively, the fungus may overwinter as urediniospores on evergreen poplar leaves in southern regions and move north with prevailing summer winds (Chitzanidis and Van Arsdal 1970). Although not specifically studied in *M. medusae*, work on other rust fungi suggests that urediniospores may survive the winter in buds (Spiers and Hopcroft 1996) or on leaf debris (Gross and Venette 2001) and infect poplar in the spring. If the fungus overwinters as urediniospores in southern regions, greater genetic diversity may be expected in northern regions because of the annual sexual recombination on larch, whereas asexual overwintering (urediniospores) may lead to less diversity in the south. Asexual populations would also be expected to differ substantially from Hardy–Weinberg equilibrium, which is dependent on meiotic recombination.

Poplar leaf rust is particularly important in intensively managed, monoclonal plantations. Severe rust early in the season can lead up to 60% yield loss (Walker et al. 1974). Resistance is the most effective strategy to control this disease. However, the long-distance spore dissemination and potential adaptive ability of the pathogen may hinder deployment of resistance. *Melampsora medusae* is comprised of formae speciales, races, and pathotypes (Newcombe et al. 2001; Prakash and Thielges 1987; Shain 1988b; Singh and Heather 1982). Earlier studies indicated that the wide variation in resistance to poplar leaf rust among clones and families of *Populus* species is often associated with the origin of the host genotype. A *Populus* progeny test in Wooster, Ohio revealed that families of *P. deltoides* derived from natural stands in Missouri and Illinois were more resistant to *Melampsora* leaf rust than were those from Indiana, Ohio, and Pennsylvania (Thielges and Adams 1975). In western Kentucky, isolates of *M. medusae* f. sp. *deltoides* from the southeastern United States had broader adaptation and were more virulent on local southern host genotypes of *P. deltoides* than were isolates from the northern United States (Hamelin et al. 1992).

A better understanding of the population genetics of *M. medusae* and identification of the source of primary inoculum are needed. If the local overwintering inoculum in more southerly locations is in the form of urediniospores on leaf debris and there is no sexual stage, then we might expect the southern populations to have relatively low genetic diversity and not be in Hardy–Weinberg equilibrium. If overwintering requires sexual reproduction on larch, then we might expect that populations of the rust on poplar that are near larch will have higher levels of genetic diversity than more distant populations, where the initial populations would have gone through several generations of asexual reproduction before arrival (Bourassa et al. 1998; Samilis et al. 2001).

The hypothesis to be tested is that the epidemics south of the larch zone result from annual spread of urediniospores from northern areas where the rust can complete its life cycle. To test this hypothesis, populations of the pathogen on *P. deltoides* were collected at the early stages of local epidemics in Minnesota, Iowa, and Missouri, and microsatellite variation was used to study the population structure. To study variation in the pathogen population over time and to determine if there are localized populations south of the larch zone, we examined genetic variation in populations of *M. medusae* from a small population of *P. deltoides* on the Iowa State University campus from 1998 to 2000. Also, we investigated if there are viable basidiospores discharged from overwintered poplar leaves in Ames, Iowa.

## Materials and methods

### Sampling in Ames, Iowa, in 1998–2000

Collections were made at a single location in the midst of an abandoned coal pile in the northeastern corner of the Iowa State University campus, where 20 cottonwood trees were growing naturally within a small area of about 200 m<sup>2</sup>. No larch or Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), another potential alternate host (Ziller 1974), were within 1 km of the site.

Diseased poplar leaves were collected in 1998, 1999, and 2000. For the 1998 samples, individual pustules were excised and the spores used to inoculate detached poplar (*Populus xeuramericana* (Dode) Guinier cv. I-488) leaves, and about 10 uredinia were harvested from the detached leaves for DNA extraction. In all other samplings, however, DNA was extracted directly from individual uredinia.

On 21 July 1998, twenty-five leaves were collected from five trees, five leaves from each tree. One uredinium from each leaf was used for microsatellite analysis. On 19 July 1999, twenty uredinia were collected from two trees (five leaves per tree, two uredinia per leaf). A three-level hierarchical sampling scheme was used on 8 August 1999 and 10 October 1999. On each date, a total of 162 uredinia were sampled from six trees with three branches per tree, three leaves per branch, and three uredinia per leaf. On 19 August 2000, only four of the six trees were diseased, and 108 uredinia were sampled from the four trees.

Three-month-old *L. laricina* seedlings in trays (18 cm × 12 cm × 5 cm) were placed on the site from 15 April to 11 June 2000. The seedlings were placed directly underneath the poplar trees, where they were surrounded by overwintered poplar leaves; at 50 m southwest of the poplar trees; and at 100 m southwest of the poplar trees. After 2 weeks, the seedlings were transferred to a growth chamber set at a 16 h light : 8 h dark photoperiod and 18 °C. As soon as pycnia with droplets of pycnial nectar appeared, they were spermated by passing a small pen brush moistened with distilled water over the needles. Single aecia were used for DNA extraction. Aeciospores that formed were also used to inoculate a universally susceptible clone (*P. xeuramericana* cv. I-488) to obtain urediniospores for microscopic identification of the rust as *M. medusae* (Ziller 1974).

### Sampling in Minnesota, Iowa, and Missouri in 1999

A total of nine locations (see Fig. 2) in three states (Minnesota, Iowa, and Missouri) were sampled from natural *P. deltooides* stands at the early stages of the 1999 epidemic. Among the nine locations (populations), the two most northern (MNd and MNt) were within the natural range of eastern larch and within 1 km of mature larch trees. Samples were collected from three different sites at least 1 km apart from each other at each location. The three sites around Ames, Iowa, did not include the intensively sampled site at the Iowa State University campus. Samples were collected as close to the onset of the epidemic as possible. However, the onset and development of epidemics differed greatly among locations. Disease severity at the time of sampling was crudely estimated as low (only a small percentage of leaves infected), moderate (most leaves infected), and heavy (all mature leaves infected).

At each site, three diseased leaves were collected from each of three trees, which were at least 5 m apart. Individual uredinia (one uredinium per leaf) were cut from the air-dried leaves with a sterile scalpel and placed into 1.5 mL Eppendorf microtubes. All samples were stored at  $-80^{\circ}\text{C}$  until DNA extraction.

### Allele determination

Three polymorphic microsatellite markers *MmCAT-30*, *MmCAA-57*, and *MmCAG-11* were used (Chen 2001; Steimel et al. 2005). Flanking primers were used to amplify DNA regions containing the respective trinucleotide repeats. The sizes of the polymerase chain reaction (PCR) products were determined by polyacrylamide gel electrophoresis and GeneScan analysis at the Iowa State University DNA sequencing and synthesis facility. The three loci were unlinked, and alleles (length in base pairs) at each locus differed by three base-pair increments. The methods for DNA extraction from single uredinia, PCR amplification, and allele determination were as previously described (Chen 2001; Hamelin et al. 1996; Steimel et al. 2005).

### Analysis

For the 1999 hierarchical sampling in Minnesota, Iowa, and Missouri, samples from different locations were considered as different populations, and the three different sites per location were considered populations. The number of unique genotypes in each of these populations and subpopulations was used as an indication of asexual versus sexual reproduction.

The number of uredinia sampled in 1998–2000 at the intensively sampled site in Ames, Iowa, varied, so allele frequencies were used to generate the mean expected number of genotypes from 100 repeated samples (genotypic diversity ratio, GDR) using a computer program (Hoffmann 1986) written in S-Plus (MathSoft Inc., Seattle, Washington). In an asexual population, a low GDR is expected, whereas the GDR should be close to unity for a sexual population.

Other data were analyzed with BIOSYS-2 (Swofford and Selander 1981) and ARLEQUIN 1.1 (Schneider et al. 1997) software packages. Allelic and genotypic frequencies were estimated for each sample. Observed heterozygosities ( $H_o$ ) and Nei's expected heterozygosity (also called Nei's unbiased gene diversity,  $H_e$ ) were also calculated (Nei 1978).

Genetically identical uredinia in a single subpopulation were assumed to have originated from a common asexual lineage, thus violating Hardy–Weinberg expectations (Chen 2001). Data were thus corrected by counting each genotype in a subpopulation only once. Population structures were determined on the basis of all genotypes and, alternatively, on the basis of only unique genotypes in subpopulations.

Departure from Hardy–Weinberg expectation was tested for each locus using a  $2 \times 2$  contingency chi-square analysis. Because the microsatellite markers are codominant and the gametic phase was known, nonrandom association of haplotypes into individuals was also tested by using a modified version of the Markov-chain random walk algorithm (Guo and Thompson 1992).

To examine how asexual and sexual reproductions contribute to the rust epidemics at Ames, Iowa, we followed the guidelines given by Hebert et al. (1988), based on the departures from Hardy–Weinberg expectations. First, we calculated the mean negative  $\log_{10}$ -transformed probabilities ( $P_i$ ) of each chi-square value over three loci for deviation from Hardy–Weinberg expectation for each population. The individual-locus  $\chi^2$  test contrasts the occurrence of the observed genotypic array against a sexual population with the same gene frequencies. Sexual populations are expected to have high probabilities of the occurrence of the observed genotypic arrays, thus giving low values of  $-\log_{10}(P_i)$ , whereas asexual populations should have deviant arrays with lower probabilities and high values of  $-\log_{10}(P_i)$ .

Partition of total variance using Wright's  $F$  statistics (Wright 1969) and analysis of molecular variance (AMOVA) (Excoffier et al. 1992) on Euclidean distances were performed using ARLEQUIN 2.0 (Schneider et al. 1997). The significance of the variance components associated with different levels of genetic structure was tested using nonparametric permutation procedures. An alternative estimate of  $F_{ST}$  was also calculated by the private allele method (Barton and Slatkin 1986; Slatkin 1985). According to Wright (1969) for selectively neutral alleles, the relationship between  $F_{ST}$ , the local population size ( $N$ ), and the average rate of immigration ( $m$ ) is  $F_{ST} \sim 1/(1 + 4Nm)$ . This equation can be solved for  $Nm$ , the estimated mean number of reproducing immigrants per population per generation (Slatkin 1987).

Pairwise  $F_{ST}$  among sampling dates in Ames, Iowa, and among populations from three states in 1999 were calculated using ARLEQUIN 2.0 (Schneider et al. 1997). Nei's genetic distance based on allele frequencies was calculated between all pairs of subpopulations or populations using all genotypes using GENDIST (in PHYLIP version 3.6, Felsenstein 1989, 1993). Trees based on these genetic distance matrices were constructed using the unweighted pair group method with arithmetic averages (UPGMA), and bootstrap values for each branch were calculated based on 100 replications using SEQBOOT, also in the PHYLIP software package.

## Results

### Sampling in Ames, Iowa, in 1998–2000

Poplar rust commenced on the trees at the Iowa State University site on 16 July 1998, 24 June 1999, and 8 August 2000 (Table 1). At the only sampling in 1998, 15 single-uredinial isolates were obtained, and microsatellite analysis

**Table 1.** The date of first appearance of *Melampsora medusae* on poplar trees at a site in Ames, Iowa, sampling dates, spore stages, number of unique genotypes, genotypic diversity ratio (GDR) with standard deviation (SD), the average of logarithms of probability of deviation from Hardy–Weinberg equilibrium, and Nei's gene diversity ( $H_e$ ).

Onset of epidemic	Sample date	Spore stage	No. of pustules sampled	No. of unique genotypes	GDR±SD	$-\log(P_i)$	$H_e$
16 July 1998	21 July 1998	Uredinia	15	10	0.665±0.017	3.72	0.71
24 June 1999	19 July 1999	Uredinia	20	8	0.442±0.032	> 5	0.58
	8 August 1999	Uredinia	162	66	0.413±0.025	> 5	0.65
	10 October 1999	Uredinia	162	87	0.604±0.021	3.52	0.70
	22 April 2000	Aecia	45	44	0.993±0.039	1.09	0.77
8 August 2000	19 August 2000	Uredinia	108	52	0.504±0.022	> 5	0.71

**Table 2.** Analysis of molecular variance to compare uredinia populations of *Melampsora medusae* collected on 8 August and 10 October 1999 at a site in Ames, Iowa, using all genotypes and unique genotypes only.

Source of variation	df	Sum of squared deviations	Variance components	Proportion of variance components (%)	$p^a$
All genotypes					
Among sampling dates	1	3.787	0.004	0.37	0.192
Among trees	10	25.324	0.028	2.68	<0.001
Within trees	636	645.574	1.016	96.95	<0.001
Unique genotypes only					
Among sampling dates	1	1.650	0.004	0.32	0.143
Among trees	10	10.117	0.002	0.20	0.649
Within trees	372	401.765	1.080	99.48	0.504

<sup>a</sup>The  $p$  value is for the null hypothesis that there is no significant variation within that level based on 1023 permutations.

revealed an average gene diversity for the three loci of 0.71 (Table 1) and 10, 8, and 4 alleles at *MmCAT-30*, *MmCAA-57*, and *MmCAG-11*, respectively. Ten unique genotypes were detected (GDR = 0.665), and  $-\log_{10}(P_i)$  was 3.72 (Table 1), indicating a significant deviation from Hardy–Weinberg expectations.

In 1999, the 20 uredinia collected on 19 July had 5, 4, and 3 alleles at *MmCAT-30*, *MmCAA-57*, and *MmCAG-11*, respectively. Eight unique genotypes were detected (GDR = 0.442), and  $-\log_{10}(P_i)$  was 5.0 (Table 1), indicating a significant deviation from Hardy–Weinberg expectations. The 162 uredinia collected on 8 August 1999 had 9, 9, and 7 alleles found at *MmCAT-30*, *MmCAA-57*, and *MmCAG-11*, respectively. The genotypic diversity ratio and  $-\log_{10}(P_i)$ , from the 8 August sample were similar to those of the 19 July sample (Table 1). The 162 uredinia collected on 10 October 1999 had 12, 9, and 8 alleles at loci *MmCAT-30*, *MmCAA-57*, and *MmCAG-11*, respectively. The 10 October sample showed more unique genotypes (GDR = 0.604), but the value of  $-\log_{10}(P_i)$  indicated that the 10 October population deviated from Hardy–Weinberg expectations. The gene diversity for the three dates were 0.58, 0.65, and 0.70, respectively, indicating increasing diversity as the epidemic progressed (Table 1).

Both the 8 August and 10 October 1999 samples included 162 uredinia. The August samples had 66 unique genotypes, and the October samples had 87 unique genotypes (Table 1). Only 18 genotypes were shared between the two sampling dates. The detection of 68 new genotypes in the second sampling may suggest substantial immigration. However, the AMOVA test, both with all individuals included and with only unique genotypes, indicated that the two sampling dates were not a significant source of variation and that most of

the variation was found within trees (Table 2). There was a significant difference among the trees when all genotypes were included but not when only unique genotypes were analyzed (Table 2). When the sampling dates were analyzed separately (Table 3), there were significant differences among trees and among individuals within branches in the 8 August sampling but not in 10 October sampling (Table 3). No significant difference among branches was detected in either the August or October populations (Table 3).

Three-month larch seedlings placed under poplar trees on 15 April 2000, after two warm and humid weeks, became infected, with pycnia formed on the top needles of all seedlings in the tray. Seedlings placed 50 m away on 15 April developed light infections (pycnia formed on only a few seedlings), and seedlings placed 100 m away did not develop pycnia. Fewer pycnia developed on seedlings placed under the poplar trees the following 2 weeks, and no seedlings placed at 50 m or 100 m away developed pycnia. No pycnia formed on larch seedlings placed on the site from 14 May to 11 June. Aecia formed 3 or 4 days after spermatization of pycnia. Urediniospores were produced on poplar leaves after being inoculated with aeciospores from the infected larch seedlings. Microscopic observation confirmed that the fungus was *M. medusae* based on the size (23–31  $\mu\text{m} \times 16$ –21  $\mu\text{m}$ ), the shape, and the equatorial bald spot of the urediniospores (Newcombe and Chastagner 1993; Ziller 1974).

The aecia population had 8, 10, and 8 alleles at locus *MmCAT-30*, *MmCAA-57*, and *MmCAG-11*, respectively. Average gene diversity of the 2000 aecia population was 0.77, slightly higher than that of the October 1999 uredinia population (Table 1). Forty-four unique genotypes were detected from the 45 aecia sampled (GDR = 0.993). Although there

**Table 3.** Analysis of molecular variance of uredinia populations of *Melampsora medusae* collected at a site in Ames, Iowa, on two dates in 1999.

Sampling dates	Source of variation	df	Sum of squared deviation	Variance components	Proportion of variance components (%)	$p^a$
8 August	Among trees	5	17.549	0.045	4.34	<0.001
	Among branches	12	12.944	0.005	0.50	0.273
	Within branches	306	301.722	0.986	95.16	<0.001
10 October	Among trees	5	7.281	0.009	0.88	0.057
	Among branches	12	11.463	0.005	0.50	0.684
	Within branches	306	321.444	1.050	98.68	0.333

<sup>a</sup>The  $p$  value is for the null hypothesis that there is no significant variation within that level based on 1023 permutations.

**Table 4.** Contingency chi-square values for departure from Hardy–Weinberg equilibrium for each locus with all genotypes included or only unique genotypes for populations of *Melampsora medusae* from a site in Ames, Iowa.

Sampling dates	Spore stage sampled	All genotypes included			Unique genotypes only		
		<i>MmCAT-30</i>	<i>MmCAA-57</i>	<i>MmCAG-11</i>	<i>MmCAT-30</i>	<i>MmCAA-57</i>	<i>MmCAG-11</i>
21 July 1998	Uredinia	18.65*	16.21*	15.33*	13.10	11.26	9.83
19 July 1999	Uredinia	16.96**	15.62**	12.11**	7.88	6.58	5.12
8 August 1999	Uredinia	152.31**	113.52**	47.54*	42.23	35.66	25.78
10 October 1999	Uredinia	108.10**	56.82*	52.11*	42.16	40.10	34.21
22 April 2000	Aecia	10.22	9.13	6.70	10.01	8.92	6.56
19 August 2000	Uredinia	50.99**	97.23**	35.54*	25.92	40.29	21.25

\*Significant departure from Hardy–Weinberg equilibrium ( $p \leq 0.05$ ).

\*\*Significant departure from Hardy–Weinberg equilibrium ( $p \leq 0.01$ ).

**Table 5.** Analysis of molecular variance (AMOVA) of *Melampsora medusae* uredinia populations collected in 1998, 1999, and 2000 at a site in Ames, Iowa.

Source of variation	df	Sum of squared deviations	Variance components	Proportion of variance components (%)	$p^a$
All genotypes					
Among sampling dates	4	26.224	0.018	1.70	<0.01
Among trees	13	32.245	0.027	2.49	<0.001
Within trees	1005	1036.311	1.031	95.81	<0.001
Unique genotypes only					
Among sampling dates	4	8.754	0.006	0.53	<0.01
Among trees	13	15.325	0.003	0.25	0.276
Within trees	615	671.966	1.093	99.22	0.078

<sup>a</sup>The  $p$  value is for the null hypothesis that there is no significant variation within that level based on 1023 permutations.

were only three microsatellite loci tested, we expected each of the 45 aecia to be genetically distinct because of the high number of alleles found. However, the two aecia with identical microsatellite alleles may have resulted from cross-spermatization of adjacent pycnia, which would have resulted in identical dikaryons. The value of  $-\log_{10}(P_i) = 1.09$  for the aecia population indicated that it was in Hardy–Weinberg equilibrium.

Uredinia were conspicuous at the site later in 2000 than in 1998 or 1999. The first appearance of uredinia in 2000 was on 8 August, 3 months after infection of the larch seedlings. From a total of 108 uredinia sampled on 19 August 2000, 9, 11, and 7 alleles were found at loci *MmCAT-30*, *MmCAA-57*, and *MmCAG-11*, respectively. Average gene diversity was 0.71 (Table 1). Fifty-two unique genotypes were detected among 108 uredinia sampled in 2000 ( $GDR = 0.504$ ), and the value of  $-\log_{10}(P_i)$  was greater than 5, more typical of

the uredinia samples of 1998 and 1999 than the aecia samples of April 2000 (Table 1).

Contingency chi-square analysis revealed significant deviations from Hardy–Weinberg equilibrium for all uredinia populations in all 3 years, whereas the 2000 aecia population was in Hardy–Weinberg equilibrium (Tables 1 and 4). After removal of identical genotypes (only unique genotypes included in the analysis), all the populations were in Hardy–Weinberg equilibrium for all three loci (Table 4).

Nested AMOVA of uredinia populations (calculated both with all genotypes and with only unique genotypes at the population level) confirmed that most (>95%) of the total genetic diversity was attributable to the diversity within populations (Table 5). There was a significant difference among the samples from different trees when all genotypes were included but not when only unique genotypes were included in the analysis (Table 5). There was a slight but significant dif-

**Table 6.** Population pairwise  $F_{ST}$  values among samples of *Melampsora medusae* collected at a site in Ames, Iowa, on different dates.

	21 July 1998	19 July 1999	8 August 1999	10 October 1999	22 April 2000
19 July 1999	0.013				
8 August 19 99	-0.007	0.040*			
10 October 1999	-0.009	0.022*	0.002		
22 April 2000	-0.005	0.022*	0.004	0.002	
19 August 2000	-0.006	0.023*	0.008	0.005	0.009*

\*Significant difference among populations ( $p \leq 0.05$ ).

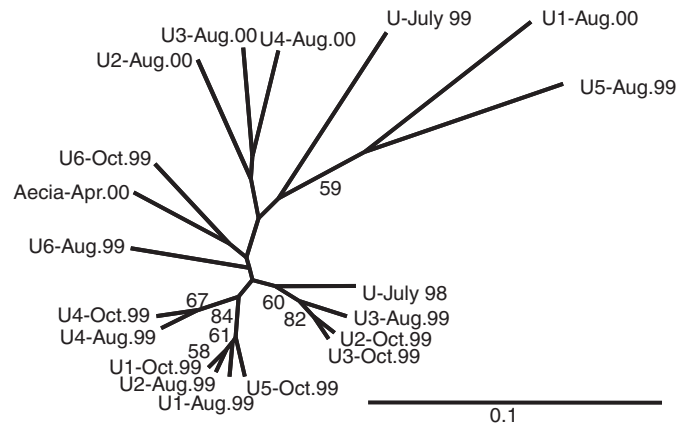
ferentiation among populations from different sampling dates (Table 5). The  $F_{ST}$  analysis also suggested little variation among the sampling dates, but significant pairwise  $F_{ST}$  values were found between the July 1999 population and the populations sampled in August 1999, October 1999, April 2000, and August 2000 (Table 6). The August 2000 uredinia sample significantly differed from the aecia sample in April 2000 (Table 6).

The UPGMA cluster analysis based on pairwise genetic distance (allele frequencies) also suggested that samples from different trees and sampling dates did not differ greatly from each other, i.e., the branch lengths were relatively short (Fig. 1). In general, samples from adjacent trees or from the same tree at different dates did not group together with strong bootstrap support. Only a few branches had bootstrap values greater than 80. For instance, samples from the adjacent trees U2 and U3 on October 1999 were similar, but there was no bootstrap support for the any branches connecting the April 2000 aecia population with any of the poplar samples collected in 1999 or 2000.

### Sampling in Minnesota, Iowa, and Missouri in 1999

The poplar rust epidemic began relatively early in Ames, Iowa, in 1999. Uredinia on poplar leaves were first noticed on 24 June, the epidemic developed quickly in July, and the epidemic was well advanced by 1 August. A comparable level of infestation was apparent in the Westalton, Missouri, population (MOa) at the 5 August sampling, but the Minnesota epidemics apparently developed later (Fig. 2). The two populations (MNd and MNt) near natural larch stands in Minnesota started late in the season; the most northerly epidemic (MNd) had just barely begun by 26 August (Fig. 2). Contrary to expectations, there was very little poplar rust between central Iowa and the MNd and MNt sites in mid-August 1999, but a low level of infestation was found at one site (MNa) in southern Minnesota on 10 August.

The microsatellite markers proved highly polymorphic across the three states, with 15, 15, and 8 alleles at *MmCAT-30*, *MmCAA-57*, and *MmCAG-11*, respectively. There was a high level of genotypic diversity, with 174 genotypes observed among the 243 uredinia sampled in 1999. Twenty-five genotypes appeared more than once; only three genotypes were shared among populations (locations), five were shared among subpopulations (sites), and the rest were shared only within subpopulations. The three genotypes that were found in more than one population were probably not truly clonal but merely identical at the three loci due to chance. The two northernmost populations had more alleles and more unique genotypes than did southern populations (Table 7). In the northernmost population (MNd), each of

**Fig. 1.** Unweighted pair group method with arithmetic averages (UPGMA) analysis of genetic distance based on allele frequencies calculated from an aecial (Aecia) population on larch seedlings or from uredinia (U) populations of *Melampsora medusae* on six individual poplar trees (second digit) collected in 1998, 1999, and 2000 at Ames, Iowa. Bootstrap values greater than 50% are indicated on the branches.

the 27 sampled uredinia was genetically unique, suggesting aeciospores as the inoculum. The other population near natural larch (MNt) had 26 unique genotypes among the 27 uredinia sampled. In contrast, two subpopulations (sties) in Missouri (MOe2 and MOt2) had only one genotype present (Fig. 3).

Observed mean heterozygosities and Nei's  $H_e$  (Nei 1978) were generally high but variable among the nine populations, with values of  $H_e$  ranging from 0.550 to 0.797 (calculated with all individuals; Table 7). The greatest heterozygosity and gene diversity were found at the northernmost population (MNd), within the natural range of larch. The three Missouri populations had relatively low gene diversity values. Gene diversities were significantly higher for two Missouri populations after elimination of identical genotypes (MOt:  $p = 0.018$ ; MOe:  $p = 0.010$ ). Even after removal of identical genotypes, however, these Missouri populations had the lowest levels of heterozygosity and gene diversities (Table 7).

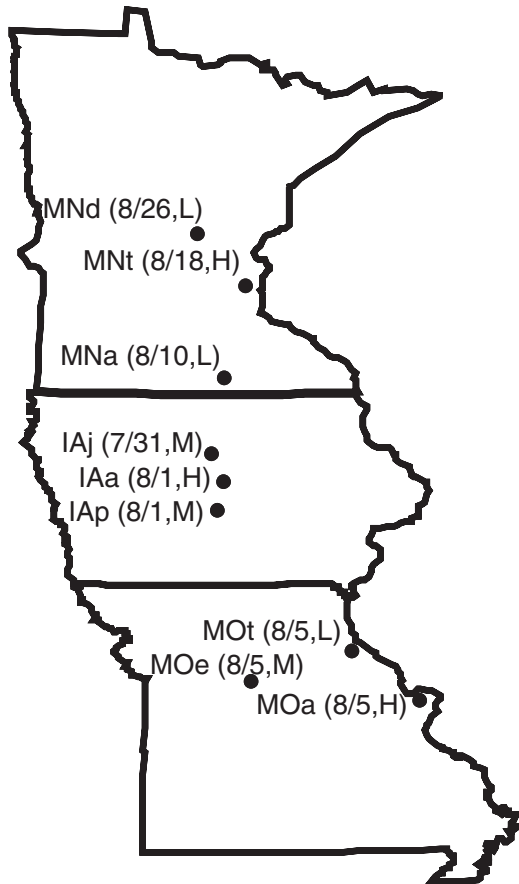
Deviations from Hardy–Weinberg equilibrium were used to further test for levels of asexual versus sexual reproduction within the populations. Based on chi-square analysis, only the two northernmost (MNd and MNt) and the southernmost (MOa) populations were consistent with Hardy–Weinberg expectations at all three loci (Table 8). One population in Iowa (IAj) was under Hardy–Weinberg equilibrium when tested with all three loci combined, but it deviated

**Table 7.** Number of unique genotypes, observed mean heterozygosities ( $H_o$ ), and expected heterozygosities (gene diversities,  $H_e$ ) with standard deviations in nine *Melampsora medusae* populations from Minnesota, Iowa, and Missouri.

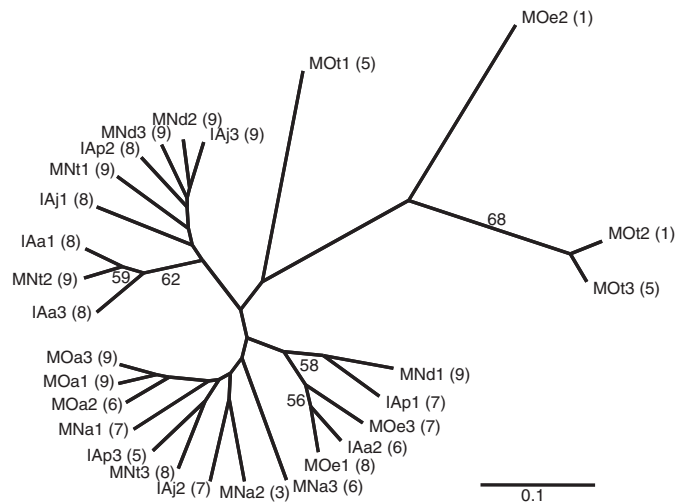
Populations	No. of unique genotypes ( $n = 27$ )	All individuals		Unique genotypes only	
		$H_o$	$H_e$	$H_o$	$H_e$
MNd	27	0.815±0.119	0.797±0.047	0.815±0.119	0.797±0.047
MNt	26	0.778±0.077	0.776±0.064	0.769±0.080	0.779±0.064
MNa	14	0.778±0.119	0.748±0.069	0.810±0.104	0.788±0.057
IAj	24	0.778±0.043	0.792±0.038	0.792±0.048	0.788±0.039
IAa	21	0.743±0.075	0.743±0.048	0.778±0.063	0.748±0.051
IAP	19	0.728±0.086	0.756±0.047	0.772±0.088	0.773±0.048
MOt	14	0.605±0.163	0.594±0.098	0.619±0.086	0.667±0.071
MOe	9	0.506±0.192	0.550±0.136	0.630±0.134	0.686±0.108
MOa	23	0.765±0.139	0.681±0.107	0.797±0.113	0.721±0.096

**Note:** See Fig. 2 for population abbreviations. Values for heterozygosities are means ± SEs.

**Fig. 2.** Distribution of the nine 1999 sampling sites for *Melampsora medusae* populations. Each population is identified by the state abbreviation and the first letter of the city or area (Minnesota: Dalbo State Wildlife Management Area (MNd), Twin Cities near Minnesota River (MNt), Albert Lea (MNa); Iowa: Jewell (IAj), Ames (IAa), Polk City (IAP); Missouri: Ted Shanks Conservation Area (MOt), Eagle Bluffs Conservation Area (MOe), Westalton near Mississippi River (MOa)). Sample date (month/day) and disease severity (H, heavy; M, moderate; L, Slow) are given in parentheses.



**Fig. 3.** Unweighted pair group method with arithmetic averages (UPGMA) analysis of genetic distance based on allele frequencies among 27 uredinia subpopulations of *Melampsora medusae* on poplar. See Fig. 2 for location abbreviations. Each subpopulation's name is designated as a number (1–3). The values in parentheses indicate the number of unique genotypes out of nine samples for each subpopulation. Bootstrap values greater than 50% are indicated on the branches.



from Hardy–Weinberg equilibrium for the *MmCAG-11* locus. After elimination of identical genotypes from subpopulations, all nine populations were in Hardy–Weinberg equilibrium (Table 8).

The among-population variance parameter  $F_{ST}$  can serve as a measure of genetic distance among populations. The low  $F_{ST}$  values (0.013, 0.043, 0.040, and 0.032 for *MmCAT-30*, *MmCAA-57*, *MmCAG-11*, and all three loci combined,

**Table 8.** Contingency chi-square values for departure from Hardy–Weinberg equilibrium with all individuals included or only unique genotypes for nine populations from Minnesota, Iowa, and Missouri.

Populations	All individuals				Unique genotypes only			
	<i>MmCAT-30</i>	<i>MmCAA-57</i>	<i>MmCAG-11</i>	All three loci	<i>MmCAT-30</i>	<i>MmCAA-57</i>	<i>MmCAG-11</i>	All three loci
MNd	13.52	19.28	3.67	36.48	13.52	19.28	3.67	36.48
MNt	23.60	22.47	5.94	52.00	23.35	22.45	5.94	51.73
MNa	30.91**	65.19**	22.60*	118.70**	12.06	26.41	16.46	54.93
IAj	17.75	30.69*	17.20	65.63	16.46	24.11	14.03	54.60
IAa	32.36*	24.20	15.95	72.51*	23.18	18.23	9.53	50.93
IAP	45.71**	16.43	15.76*	77.89**	26.11	12.89	6.79	45.79
Mot	38.61**	31.92**	14.92	85.45**	14.67	12.12	3.28	30.07
Moe	45.31**	43.90**	15.73*	104.94**	15.84	14.29	5.09	35.22
MOa	18.59	13.93	6.62	39.14	14.33	12.35	5.03	31.71

**Note:** See Fig. 2 for population abbreviations.

\*Significant departure from Hardy–Weinberg equilibrium ( $p \leq 0.05$ ).

\*\*Significant departure from Hardy–Weinberg equilibrium ( $p \leq 0.01$ ).

**Table 9.** Analysis of molecular variance (unique genotypes only at subpopulation level) for poplar leaf rust from Minnesota, Iowa, and Missouri.

Source of variation	df	Sum of squared deviations	Mean squared deviation	<i>F</i>	Variance components	Proportion of variance components (%)	$p^a$
Among populations	8	19.067	2.383	0.023	0.026	2.29	<0.001
Among subpopulations	18	22.666	1.259	0.009	0.010	0.86	0.104
Within subpopulations	351	394.867	1.125	0.031	1.125	96.85	<0.001

<sup>a</sup>The  $p$  value is for the null hypothesis that there is no significant variation within that level based on 1023 permutations.

**Table 10.** Population pairwise  $F_{ST}$  among nine *Melampsora medusae* populations from Minnesota, Iowa, and Missouri.

	MNd	MNt	MNa	IAj	IAa	IAP	Mot	MOe
MNt	<0.001							
MNa	0.007	0.011						
IAj	–0.007	0.004	0.021					
IAa	0.019*	0.008	0.046*	0.029*				
IAP	–0.006	0.001	<0.001	<0.001	0.012			
Mot	0.048*	0.049*	0.024	0.066*	0.021	0.024		
MOe	0.050*	0.063*	0.004	0.059*	0.088*	0.030	0.033	
MOa	0.033*	0.045*	–0.013	0.047*	0.080*	0.021*	0.041*	<0.001

**Note:** See Fig. 2 for population abbreviations.

\*Significant difference among populations ( $p \leq 0.05$ ).

respectively) as calculated according to Weir and Cockerham indicate that there was little genetic differentiation among the nine populations. The  $F_{ST}$  value estimated by the private allele method for all three loci combined was somewhat higher ( $F_{ST} = 0.048$ ) than that calculated by the Weir and Cockerham method ( $F_{ST} = 0.032$ ). By converting  $F_{ST}$  to estimates of mean numbers of reproducing immigrants ( $Nm$ ) per population, both  $F_{ST}$  calculations suggest considerable immigration (Weir and Cockerham method: an overall mean of eight reproducing migrants per generation; private allele method: five migrants per generation).

Partition of population variation in a nested AMOVA (Table 9) showed that total genetic diversity was mostly (97%) attributable to within subpopulations. However, the analysis also revealed a low (2.3%), but significant, level of genetic diversity among populations. No significant difference was found among the three subpopulations within a population.

Pairwise  $F_{ST}$  and gene diversities among populations also suggested rather limited differentiation among the populations (Table 10). The two most northern populations in Minnesota (MNd and MNt) significantly differed ( $p = 0.05$ ) from the three Missouri populations. Two Iowa populations (IAj and IAa) also differed from the Missouri populations and from each other, and the IAa population differed from the northernmost Minnesota population (MNd). Although there was no significant difference between Missouri populations MOt and MOe, the Westalton population (MOa) significantly differed from the MOt population.

UPGMA cluster analysis of genetic distances among subpopulations did not reveal clear geographic components (Fig. 3), except that four subpopulations with low genetic diversity (MOt1, MOe2, MOt2, and MOt3) from Missouri were not closely related to other subpopulations (had long branch lengths). Also, the three MOa subpopulations grouped

closely together, with short branch lengths. However, no strong bootstrap support was found for any of the branches. The strongest support (bootstrap value of 68) was for the branch connecting two subpopulations in Missouri (MOt2 and MOt3).

## Discussion

Poplar leaf rust populations in the north-central United States are genetically diverse and interrelated. The estimated number of new immigrants per generation was high, suggesting substantial long-distance intermingling of urediniospore populations. All sampled uredinia populations were in Hardy–Weinberg equilibrium when only unique genotypes were analyzed, indicating that they had earlier gone through sexual reproduction. Although it has been hypothesized that epidemics begin in the north where there are natural stands of the alternate host, epidemics from Minnesota to Missouri appear to be initiated independently.

Even in the narrow zone of overlap of the natural ranges of *L. laricina* and *P. deltoides*, these tree species are not commonly in close proximity. We had difficulty locating poplar trees within 1 km of larch in Minnesota. South of this range of overlap, there are scattered plantings of ornamental *Larix* species and Douglas-fir, another possible alternate host (Ziller 1974). Aeciospores of *M. medusae* have been found on ornamental *Larix* species in Kentucky, which is far south of the natural range of larch (Shain 1988a). However, we were unable to find aecia on planted *Larix* species in the Ames area in 1999 or 2000, apparently because we were unable to find *Larix* species that were in close proximity to *P. deltoides*. Compared with aeciospores and urediniospores, viable basidiospores likely do not travel far. Larch seedlings at 50 m, but not at 100 m, from overwintering poplar leaves with telia in 2000 became infected by basidiospores. Proximity of ornamental larch and poplars may be a limiting factor in the initiation of poplar rust epidemics.

The data suggest that urediniospore populations build slowly on poplars during the summer from rare production of aeciospores in the spring. When larch seedlings were placed near overwintering inoculum in Ames, there was a 3 month lag between basidiospore infection on the larch seedlings and the first appearance of uredinia on poplar, which would be sufficient time for 10 or more generations of urediniospores on poplar under ideal weather conditions (Spiers 1978; Widin and Schipper 1980). Sexual reproduction on ornamental or natural larch trees may be taking place tens or hundreds of kilometres away from most poplar stands, and there may be many urediniospore generations before the initial inoculum enters the stand.

The uredinia populations in the north-central United States are generally only conspicuous in middle to late summer. In addition to the proximity of overwintering inoculum, local weather conditions and prevailing winds are likely major determinants of the date of onset of the poplar leaf rust epidemics. The summer of 2000 was relatively dry in central Iowa, and the epidemic at the Ames study site started 6 weeks later than in the unusually early 1999 epidemic (24 June). Some of the variation in date of onset of the epidemics at the nine locations from Missouri to Minnesota was likely due to variation in local weather conditions. Spore trapping studies

(McCracken et al. 1984; Widin and Schipper 1980) have also shown substantial variation in the onset of poplar leaf rust epidemics.

The two northernmost populations in Minnesota, within the natural range of the alternate host, had high gene diversity values and were in Hardy–Weinberg equilibrium, suggesting recent sexual reproduction. Poplars at the MNd site had very low levels of infection when sampled on 26 August, perhaps because of the late infection of larch needles at this relatively cold site. Surprisingly, the southernmost population, sampled from heavily infected trees on 5 August near Westalton, Missouri, was also in Hardy–Weinberg equilibrium, typical of a population that had gone through a recent sexual stage on larch. Pairwise  $F_{ST}$  values suggested that the Westalton population differed from other populations, and the three subpopulations were genetically related, suggesting a single source of initial inoculum, perhaps from one or a few ornamental larch trees in close proximity.

The poplars at the other Missouri sites (MOt and MOe) were not as heavily infected as those of the Westalton sites, there were fewer unique genotypes and relatively low gene diversity, and these populations were not in Hardy–Weinberg equilibrium. Further distance from diseased larch might explain the late arrival and low genetic diversity of the populations at the MOt and MOe sites. The southernmost Minnesota epidemic (at MNa, far south of the natural range of larch) was similarly late in developing, the population had relatively few unique genotypes, and the population was not in Hardy–Weinberg equilibrium. The Iowa populations in 1999 had intermediate gene diversity values, and the onset of the epidemics in Iowa was somewhat earlier than in Minnesota, but the Iowa populations also deviated from Hardy–Weinberg equilibrium unless only unique genotypes were analyzed. Thus, these six populations were largely asexual but were derived from populations that had gone through sexual reproduction.

Although local epidemics may have different sources of initial inoculum, the genetic structure of the populations suggests substantial intermingling of urediniospore populations throughout the region. Gene diversities were high for most of the populations we sampled, genetic differentiation among populations was very low, and immigration rates (estimated from  $F_{ST}$  values) were high. Urediniospores of rust fungi are known to travel far. A single, long-distance jump of *Melampsora larici-populina* and *M. medusae* urediniospores from Australia to New Zealand was indicated by circumstantial evidence (Van Kraayenoord et al. 1974; Wilkinson and Spiers 1976).

Although the 1998–2000 populations at the sampling site in Ames were interrelated, there was genetic differentiation among the sampling dates, suggesting different external sources of inoculum. The 2000 uredinia subpopulations were not closely related to the 2000 aecia population on the test seedlings or to the uredinia populations in October 1999. Genetic differentiation among the sampled trees in the 8 August 1999 population suggests that the initial inoculum consisted of a limited number of genotypes and that some of the initial population build up during the first 6 weeks of the epidemic was due to spread of urediniospores within individual trees. However, in the 10 October 1999 sampling, there was no genetic differentiation among the trees, perhaps

because of immigration of new genotypes into the stand. The number of new alleles and unique genotypes increased during the 1999 epidemic, as did the gene diversity of the Ames population. Thus, although many populations may begin with a limited number of genotypes building on a few trees, long distance dispersal of urediniospores likely homogenizes poplar leaf rust populations as the season progresses.

Our observations, and the fact that prevailing summer winds in this region are from the south, challenge the hypothesis that southern populations originate from urediniospore showers from poplar stands in the natural range of larch. Poplar leaf rust first appeared in central Iowa before it appeared in northern Iowa and southern Minnesota in both 1998 and 1999. In the sampling of populations in 1999, the epidemic at the southernmost site in Missouri was further advanced than the later sampled epidemics in Minnesota. However, most of our study was in a single year, and different weather conditions may bring inoculum from other sources. An earlier spore trapping study (Widin and Schipper 1980) found that aeciospores of *Melampsora* sp. were detected only at a Wisconsin site (Rhineland) within the natural range of larch, and urediniospores were detected earlier at Rhineland than at the more southern sites of Rosemont, Minnesota, and Ames, Iowa. Another spore trapping study (McCracken et al. 1984) across a broader area also detected urediniospores of *Melampsora* sp. following aeciospores at Wisconsin and Minnesota sites within the natural range of larch, and urediniospores were detected later at most southern sites. However, they also found there was a yearly progress of urediniospores from south (Yoakum, Texas) to the north (West Helena, Arkansas), and the urediniospores in the south were detected at the same time as aeciospores were found in the north. The southern populations may have been initiated by urediniospores on a few evergreen poplar leaves (Chitzanidis and Van Arsdel 1970) or by urediniospores surviving in bud scales, as has been postulated for other *Melampsora* species (Spiers and Hopcroft 1996). It is possible that urediniospores may survive the winter on decayed leaves or in bud scales in more northern sites (Gross and Venette 2001), but we did not find evidence for this in Iowa.

Our data suggest that stands of poplars near native larch in Minnesota were infected late in the season and are not initial sources of inoculum for Iowa and Missouri epidemics. It appears that the fungus is going through sexual reproduction in areas south of the natural range of larch, apparently on ornamental larch, Douglas-fir, or some other member of the Pinaceae (Ziller 1974). For commercial plantings of *P. deltoides* in areas south of natural larch, ornamental plantings of larch may lead to the instability of single gene resistance because of (i) the potential early onset of epidemics (ii) a more genetically diverse population with a greater potential for evolution of new, virulent races; and (iii) local year-to-year selection of virulent races. In the absence of nearby ornamental and natural larch, however, it may be safe to deploy single gene resistance in these regions, even on a large scale. If there is no local overwintering survival as urediniospores, then any selection for virulence to planted resistant clones of *P. deltoides* may be lost the next season if there are no larch in proximity.

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