European Journal of Plant Pathology **109:** 183–190, 2003.

Host-specific genetic composition of *Melampsora larici-epitea* populations on two *Salix viminalis* varieties in a mixture trial

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Accepted 11 October 2002

Key words: AFLP, AMOVA, genetic diversity, leaf rust

Abstract

The genetic composition of *Melampsora larici-epitea* populations on two *Salix viminalis* varieties in monoculture and in mixed stands of *Salix* was studied using amplified fragment length polymorphism (AFLP). A total of 88 isolates collected from a large-scale mixture trial in Northern Ireland were analyzed. Genetic analyses were based on polymorphism for 63 AFLP markers. Differences in genetic composition of *M. larici-epitea* populations between the two *S. viminalis* varieties were indicated by all population characteristics used. In neighbor-joining analysis and principal component analysis, isolates from the same variety tended to group together. Analysis of molecular variance indicated a substantial differentiation between varieties ($\Phi_{ST} = 0.20$) and differences in genotypic composition was indicated by the non-random distribution of clonal isolates between the two varieties. The detection of host specialization with selectively neutral DNA markers was ascribed to predominant asexual reproduction. No differences in gene or genotypic diversity between *M. larici-epitea* populations in mixed and monoclonal stands were found for any of the two *S. viminalis* varieties.

Introduction

Short-rotation coppice willow (*Salix* spp.) used for bioenergy is a vegetatively propagated crop with a life span of at least 20 years. One of the most damaging parasites of willow is the biotrophic fungus causing leaf rust, *Melampsora larici-epitea* Kleb. (*Melampsora epitea* var. *epitea* Thüm.) (Hunter et al., 1996; McCracken and Dawson, 1998; Verwijst, 1990). The leaf rust spreads through repeated cycles of asexual production of wind-dispersed urediniospores during the cropping season, while sexual reproduction occurs on the alternate host (*Larix*) in the spring. Willow is usually cultivated in large monoclonal plantations, which increases the risk of rapid adaptation of the pathogen populations to specific willow clones with increased disease epidemics as a consequence. There are examples of willow clones that have lost their resistance after 8–10 years of cultivation, due to virulence changes in the rust populations (McCracken and Dawson, 1998; Pei et al., 2000). In a perennial crop like willow, durable resistance is especially important, since varieties that become susceptible to disease cannot readily be replaced.

A control strategy that has potential to give a more sustainable resistance is the use of host species/variety mixtures. Delayed development of epidemics caused by *M. larici-epitea* and reduced infection levels have been indicated in experimental *Salix* mixtures (McCracken and Dawson, 1998; McCracken et al., 2000). When variety mixtures of other crops (e.g., barley and rice) have been cultivated over large

areas, the reduction of disease and increase in yield have in some cases been comparable to that typically obtained with the use of fungicides (Wolfe, 1992; Zhu et al., 2000). Three important mechanisms for disease reduction in mixtures have been pointed out (Wolfe, 1985; Finck et al., 2000): (i) increased distance between susceptible host plants, (ii) resistant plants acting as barriers to pathogen spread (the effect is reciprocal, i.e. plants of one host genotype will act as barrier for the pathogen specialized to a different genotype and vice versa), and (iii) resistance induced by the maintenance of avirulent pathogens. To be functional for disease reduction, the mixtures should be composed of varieties or species of the host that differ in their susceptibility to the pathogen (Wolfe, 1985), and the greatest effect has been suggested for race-specific resistance (Newton and Thomas, 1992; Xu and Ridout, 2000).

To ensure a functional diversity with regard to the leaf rust, the experimental willow mixtures have usually been composed of different *Salix* species and species hybrids, that are known to host genetically separate populations of *Melampsora* (McCracken et al., 2000; Pei et al., 1997; 1999). From an applied point of view, it would be valuable to have mixtures which are composed, to a large extent, of varieties of *S. viminalis*, the most important species in short-rotation coppice. It is well documented that *S. viminalis* varieties vary in resistance to *M. larici-epitea* (McCracken and Dawson, 1992; Gullberg and Ryttman, 1993; Lascoux et al., 1996; Rönnberg-Wästljung and Gullberg, 1999), but it is not known to what extent specific interactions are involved. If all varieties of *S. viminalis* are infected by the same genotypes of the pathogen, a pure *S. viminalis* mixture would be less likely to reduce disease. However, some degree of specificity has been indicated. For example, a study by McCracken et al. (2000) showed differences in pathotype composition between two *S. viminalis* varieties in a mixture experiment.

To develop appropriate mixtures, there is a need for more information on the interactions between *Melampsora* and *Salix*. In this current study, amplified fragment length polymorphism (AFLP) was used to analyze the genetic composition of *M. larici-epitea* populations on two *S. viminalis* varieties in monoculture and in mixed stands of *Salix*. Two basic questions were addressed: (i) are there any differences in genetic composition of *M. larici-epitea* populations on different *S. viminalis* varieties and (ii) are there any differences in genotypic diversity between the pathogen populations in monoculture and in mixed stands?

Materials and methods

Mixture trial

The experiment was performed in a large-scale mixture trial (in total ca. 7 ha) in Castlearchdale (54°28′N 7°43′W) in Northern Ireland (McCracken and Dawson, 1998), where 20 varieties (i.e., different clones) of *Salix* were planted both as monoclonal plots and as intimate, random mixtures (polyclonal plots). The complete trial includes mixtures with various numbers of *Salix* varieties, but for this study only those where all 20 varieties were included were used. Eleven of the varieties were from *S. viminalis* and the other nine were from *S. burjatica*, *S. dasyclados* × *aquatica*, *S. dasyclados* × *caprea*, *S. mollissima-undulata*, *S. schwerinii* × *aquatica*, *S. schwerinii* × *viminalis* × *dasyclados*, *S. viminalis* × *aquatica*, and *S. viminalis* × *caprea*. The trial consists of two replications (block I and block II) located approximately 500 m apart. Block I was planted in 1994 and block II in 1995. Block I was cut back at the end of both the first and the second year (1994, 1995) and block II at the end of the first year (1995). The size of the polyclonal plots was 1500 m^2 (30 m × 50 m in block I, and about 21 m × 70 m in block II) and the monoclonal plots were 100 m^2 (10 m × 10 m). The planting density of the investigated plots was 15,000 plants/ha.

Rust was sampled on September 9–10, 1997 from two *S. viminalis* varieties, '78118' and '78183', that normally show moderate rust levels. Both varieties originate from earlier Swedish willow cultivation for wickerwork. Samples were collected both in polyclonal and monoclonal plots in each of the two blocks (in total six plots). Samples were taken from eight plants in each plot, at least 2 m apart (distances between plants were usually larger in the polyclonal plots). Three leaves from each plant were picked and a single uredinium from each leaf was cultivated, resulting in a total of 24 samples from each plot. The leaves were kept separately in a cooled box for 2–3 days, and then stored frozen until cultivation of the rust.

Spores from one uredinium were placed on a greenhouse-cultivated leaf of the same willow clone. The leaf was kept in a Petri dish on a water-soaked filter paper at 18 °C with a 12-h light period, until new uredinia developed, about one week later. Inoculation of spores from a single uredinium to a new leaf, was repeated twice to ensure that each rust isolate consisted of a single genotype. Urediniospores were multiplied on several leaves. They were collected by

gently tapping the leaf and letting the spores fall on a paper. After drying in a dessicator, spores were stored at −20 °C.

DNA extraction and AFLP analysis

DNA was extracted from urediniospores using a CTAB procedure (Chen et al., 1993) with modifications (Samils et al., 2001a). AFLP reactions were performed principally as described in the protocol from the Perkin-Elmer/Applied Biosystems AFLP™ plant mapping kit for small genomes. It is based on the method of Vos et al. (1995) but uses non-radioactive fluorescent dyes to label the primers. Modifications to the protocol and sequences of oligonucleotides, i.e., adapters and primers, have been described (Samils et al., 2001a). The same two primer combinations as in the previous study were used (E-TG/M-CAA and E-TA/M-CAG). GeneScan Analysis software (PE Applied Biosystems) was used to visualize and score the digital profiles. DNA fragments were sized, by means of the internal size standard included in each lane, using the local Southern size calling option in the software. After initial analysis, sample files were imported into Genotyper version 2.0 (PE Applied Biosystems), and all samples were normalized. Initially, potential markers were generated by the software, followed by a manual selection of loci with clearly separated size ranges and overall high signals (fragments with scaled peak heights mainly above 100), that could be scored unambiguously for all samples. Scoring was done by the software, with presence of a fragment (marker allele) in a sample denoted as 1 and absence (null allele) as 0, resulting in a binary data matrix of the different AFLP multilocus phenotypes.

Data analysis

As urediniospores are dikaryons and AFLP markers are dominant (i.e., they do not distinguish between dominant homozygotes and heterozygotes), the data were treated as dominant markers in diploids.

Genotypic diversity was calculated by a normalized Shannon's diversity index (H_S) as described by Goodwin et al. (1992): $H_S = -\sum P_i \ln P_i / \ln N$, where P_i is the frequency of the ith multilocus genotype and N is the sample size. This diversity index corrects for differences in sample size (Sheldon, 1969). Values for H_S range from 0 (single genotype) to 1 (each isolate in the sample is unique).

Pairwise distances between all isolates were calculated from the binary data matrix using Nei and Li's (1979) similarity coefficient: $S_{xy} = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of fragments in common between isolates x and y, and n_x and n_y are the total numbers of fragments in isolates x and y, respectively. Similarity values were converted to distances as $(1 - S_{xy})$. Calculations were done with the RAPDistance package (Armstrong et al., 1996). Phenograms based on the resulting distances were constructed using the neighbor-joining method (Saitou and Nei, 1987) as implemented in the program Neighbor from the Phylip package (Felsenstein, 1993) and drawn with TreeView (Page, 1996). The binary data set was also subjected to bootstrap analysis with 500 replications using the program TREECON (Van de Peer, 1994). Principal component analysis, PCA (JMP software, SAS Institute Inc., Cary, North Carolina), was conducted directly on the binary data matrix, to study relationships among the isolates.

Analysis of molecular variance, AMOVA (Excoffier et al., 1992), was used to estimate variance components for AFLP phenotypes and for partitioning the variation within populations and among populations. The analysis is based on an Euclidean distance matrix between all pairs of AFLP multilocus phenotypes. The resulting parameters, the so-called Φ-statistics, are analogues to F-statistics. Significance levels for variance component estimates and Φ_{ST} are computed by nonparametric permutation procedures. AMOVA analyses were conducted with the Arlequin software (Schneider et al., 1997). Analysis was made both on entire samples and on clone-corrected samples, i.e., where only one representative of each multilocus phenotype was included.

Results

A total of 88 rust isolates were obtained for analysis (Table 1), after some losses in the procedure of isolation and bulking-up of spore samples. The two AFLP primer combinations produced 63 distinct DNA fragments, of which 16 were monomorphic for all isolates in the data set. Identical multilocus phenotypes were considered as belonging to the same clone, since the probability that two isolates should have identical phenotypes for all 47 polymorphic loci by chance is negligible when the actual band frequencies and the sample size are taken into account (data not shown). The clonal fraction of the entire sample was 54%, and

Table 1. Distribution of *M. larici-epitea* multilocus phenotypes among experimental plots

S. vim. clone	Plot	Block	Sample size	No. of isolates of phenotype (clone)											No. of single-copy phenotypes	No. of different phenotypes	Shannon's index
				C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11			
78118	Mono	I	9	4	—	—	—	2	—	—	2	—	—	—	1	4	0.58
		II	15	1	—	—	—	2	—	1	—	3	1	2	5	10	0.85
	Poly	I	8	1	—	—	—	—	3	3	1	—	—	—	—	4	0.60
		II	9	2	2	2	—	—	—	—	—	—	1	—	2	6	0.79
78183	Mono	I	9	2	6	1	—	—	—	—	—	—	—	—	—	3	0.39
		II	13	2	2	1	3	—	—	—	—	—	—	—	5	9	0.82
	Poly	I	10	4	3	3	—	—	—	—	—	—	—	—	—	3	0.47
		II	15	3	4	2	—	—	—	—	—	—	—	—	6	9	0.75
Total			88	19	17	9	3	4	3	4	3	3	2	2	19	40	0.61

the normalized Shannon's index was 0.61 (Table 1). The genotypic diversity, as measured by a normalized Shannon's index, was of equal level in monoclonal and polyclonal stands (Table 1). For all types of plots, however, the Shannon's index was higher in block II than in block I.

The three most frequent clones (C1, C2, and C3) represented 22%, 19%, and 10% of the entire sample, respectively. Clone C1 occurred in fairly equal proportions on both willow varieties, both in monoclonal and polyclonal plots. Clones C2 and C3 occurred in higher numbers on variety '78183' than on '78118'. A chi-square test showed that clone C2 differed significantly ($P < 0.01$) from equal occurrence on the two willow varieties, while the difference for clone C3 was not significant. Other clones were sampled in small total numbers (four isolates or less), and were found exclusively on one or the other willow variety and only in one or two of the plots. In only three cases out of 37 possible, isolates collected from the same plant were clone mates (one pair of clone C1 in a monoclonal plot and two pairs of clone C2, one in a monoclonal and one in a polyclonal plot).

The relationship among isolates is visualized in a neighbor-joining phenogram (Figure 1). The isolates formed a structured tree, where isolates from the same willow variety tended to group together. Among the exceptions were isolates of clones C1, C2, and C3 from variety '78118', who grouped with isolates from '78183'. Bootstrap analysis produced high (or moderately high) values only for clusters at the lower branching levels and most of those clusters did comprise multiple isolates of the same AFLP phenotype. In the PCA, the two first components accounted for 37.9% of the total variation (Figure 2). Similar relationship among isolates as in the phenogram was indicated. Most isolates from variety '78118' were separated from variety '78183' on one or both component axes, while a few phenotypes were placed among phenotypes from the other willow variety.

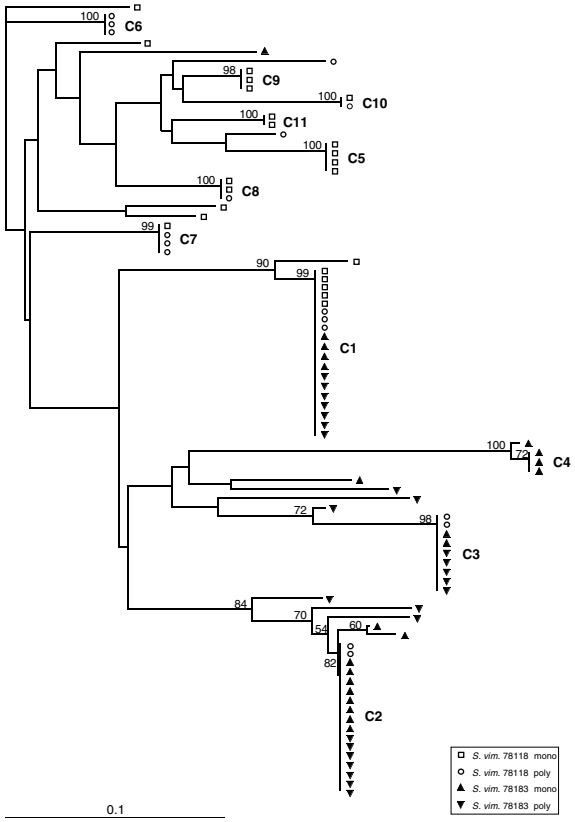

Figure 1. Neighbor-joining phenogram of the *M. larici-epitea* isolates. *S. viminalis* clone and stand type (mono- or polyclonal) are indicated with symbols. Numbers above branches indicate the percentage of bootstrap values that support the branch. Bootstrap values are only provided for those branches with >50% support.

The AMOVA revealed a significant differentiation between the two willow varieties ($\Phi_{ST} = 0.198$; $P < 0.001$) (Table 2), while no differentiation between monoclonal and polyclonal plots was detected. A similar result was obtained when the AMOVA was performed on clone-corrected samples, i.e., only one representative of each multilocus phenotype was included (Table 2). The results from pairwise comparison of the different stand types are shown in Table 3. No significant difference between the two blocks was detected (data not shown).

Discussion

All population characteristics used in this study indicate a host-specific genetic composition of *M. larici-epitea* populations on the two *S. viminalis* varieties. Differences in genotypic composition were indicated by the non-random distribution of one *M. larici-epitea* genotype (C2). The fact that all the clones that occurred in low frequencies (C4–C11) were found exclusively on either of the two varieties, provides further support for a host-specific genotypic composition. Thus, it seems that the pathogen population is composed both of genotypes that are more adapted to one or the other host variety and those (like C1) that are well adapted to both varieties.

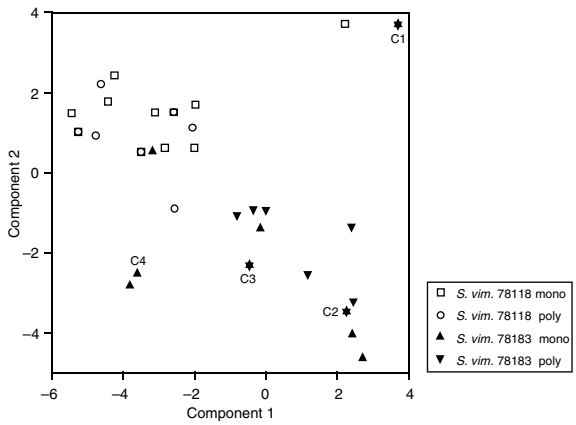

Figure 2. Principal component analysis of *M. larici-epitea* isolates. The clones C1, C2, C3, and C4 are indicated.

The genetic differentiation between the two willow varieties detected by AMOVA was relatively large ($\Phi_{ST} = 0.20$). The present result contrasts with a study of *M. larici-epitea* populations on *S. viminalis* in Sweden, where no genetic differences between three different varieties were found (Samils et al., 2001a). However, molecular markers do not necessarily detect virulence differences. When sexual reproduction is significant, which has been suggested for Swedish *M. larici-epitea* populations (Samils et al., 2001b), loci are usually in linkage equilibrium. Thus, associations between selectively neutral markers (such as AFLPs) and loci under selection (such as virulence loci) are not likely to be found (McDonald et al., 1995; Milgroom, 1996). In populations that are largely or exclusively asexual, on the other hand, associations between anonymous DNA markers and virulence loci are more likely to occur. For example, associations between virulence and DNA markers (RAPDs) were stronger and more frequent in an asexual field populations of *Puccinia triticina* (wheat leaf rust) than in a sexually derived population (Liu and Kolmer, 1998). In asexual populations of *P. graminis* f.sp. *tritici* (wheat stem rust) isozyme and virulence phenotypes were highly

Table 2. Analysis of molecular variance (AMOVA) for *M. larici-epitea* isolates

Source of variation	df	Variance components	Percentage of total variation	Φ-statistics	Probability[a]
Entire sample[b]					
Between willow clones	1	1.58	19.8	0.198	<0.001
Between stand type[c] within willow clone	2	0.11	1.4	0.018	0.17
Within stand type	83	6.26	78.7		
Clone-corrected sample[d]					
Between willow clones	1	1.63	19.7	0.197	<0.001
Between stand type within willow clone	2	−0.23	−2.8	−0.035	0.85
Within stand type	36	6.90	83.1		

[a]Probability of a larger value obtained by chance, determined by 1000 randomizations of the data set.
[b]Entire sample includes all isolates analyzed.
[c]Stand type refer to monoclonal and polyclonal plots.
[d]Clone-corrected sample includes one representative of each AFLP multilocus phenotype.

Table 3. Population pairwise Φ-statistics (AMOVA) for *M. larici-epitea* isolates from different *S. viminalis* varietes and stand types. Analysis was performed on clone-corrected samples

	'78183' mono[a]	'78183' poly[b]	'78118' mono
'78183' poly	−0.041		
'78118' mono	0.185**	0.245***	
'78118' poly	0.069	0.135*	−0.052

[a]Monoclonal plots. [b]Polyclonal plots.
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ (determined by 1000 randomizations of the data set).

associated, while no association was detected between isozyme alleles and virulence genes in a sexual population (Burdon and Roelfs, 1985). In asexual populations of *M. lini* (flax rust), variation among virulence phenotypes was detected by RFLP markers (Burdon and Roberts, 1995). The actual *M. larici-epitea* population has previously been described to have characteristics typical of populations in which asexual reproduction is predominant, i.e., a high level of clonality and nonrandom association among loci (Samils et al., 2001b). The results were based on the same rust collection from *S. viminalis* '78183' as used in the present study and similar results have been obtained for the rust collection from *S. viminalis* '78118' (unpublished data). This study has suggested that sexual reproduction was relatively infrequent and that asexual reproduction dominated in the Castlearchdale population. It can be hypothesized that annual sexual recombination is not obligatory, i.e., clonal lineages persist across years. In this case, a possible explanation of the differentiation between host varieties could be that the *M. larici-epitea* population in Castlearchdale originates from different genetically diverged source populations. These populations also differ for the frequencies of genes involved in adaptation to host varieties. If sexual recombination is absent or infrequent, a genetically subdivided population could be maintained.

The use of virulence markers, i.e., pathotyping, for characterization of host specificity in *M. larici-epitea* to *S. viminalis* has been restricted because specific gene-for-gene interactions have not been found for this pathosystem. Thus, the standard differential for *M. larici-epitea* on *Salix* is composed mainly of different *Salix* species and species hybrids (Pei et al., 1996; Ramstedt, 1999), and is designed to characterize between-host-species variation rather than within-species variation. Only two *S. viminalis* varieties (out of nine components) are included in the differential and the possibilities to distinguish different virulence phenotypes on *S. viminalis* by the current pathotyping system is therefore limited. To characterize *S. viminalis*/*M. larici-epitea* interactions, quantification of pathogenicity may be more informative than the all-or-none virulence reaction used in pathotyping (Lascoux et al., 1996).

No differences in genotypic diversity of the pathogen populations in mixed and monoclonal stands were found for either of the two *S. viminalis* varieties. Moreover, no significant differences in disease levels were detected between monoculture and mixture for the two willow varieties during the growing season in which samples were obtained (unpublished data). However, in both the year preceeding and following the present experiments there was a tendency (although not significant) for lower disease levels in mixture than in monoculture for both varieties (unpublished data). It should also be remembered that data for only two of the 20 components of the mixture were included in this study, meaning that the result need not be representative for the total mixture. In a recent study, greater pathotype diversity and lower disease levels were indicated in mixed stands of *Salix* compared to monoculture (McCracken et al., 2000).

There is no obvious reason for the higher genotypic diversity in block II compared to block I. One possibility could be that the different locations of the two blocks (0.5 km apart) had an effect. For instance, the amount of recombined aeciospore inoculum dispersed from neighboring larches, the alternate host, might have been larger in block II. Or, the diversity of the rust populations in adjacent willow stands might differ between the two blocks. Another possibility might be that the difference in time of establishment (block I being planted one year before block II) had an effect on the genotypic diversity. In the asexual reproduction cycles during the epidemics, host selection may result in increased frequencies of those genotypes with a high fitness on the willow varieties in the actual stand and decreased frequencies of those with a low fitness. As a consequence, genotypic diversity may decrease. If asexual urediniospores are able to overwinter in the willow stands, such a selection process might have been more advanced in block I than in block II.

In this experiment, genetic differences between *M. larici-epitea* populations on two different *S. viminalis* varieties were revealed with neutral DNA markers. The logical next step would be to make pathogenicity studies of this rust. If the indications of host-specific composition of *M. larici-epitea* populations on *S. viminalis* varieties are confirmed by further

experiments, which should be performed on more varieties and preferably for a succession of years, the use of mixtures composed to a large extent of *S. viminalis* varieties would be a promising approach to disease control.

Acknowledgements

We thank Hanna Thorsén for assistance at the rust sampling in Castlearchdale. This work was supported by grants from the Swedish National Energy Administration (Energimyndigheten).

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