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Genetic structure of *Melampsora epitea* populations in Swedish *Salix viminalis* plantations

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Abstract

Amplified fragment length polymorphism (AFLP) was used to study the genetic structure of populations of the willow leaf rust, *Melampsora epitea*, in Swedish willow plantations. In total, 197 isolates collected from *Salix viminalis* clones in three locations in Sweden were analysed. AFLP profiles based on 83 markers were used to compute genetic distances between pairs of individuals. High levels of gene and genotypic diversity were detected in all populations, with 96% of the AFLP loci being polymorphic and with normalized Shannon's diversity indices ranging from 0.977 to 1.0. Analysis of molecular variance (AMOVA) showed small significant differences among locations, although most of the molecular variability was found within locations (97.5%). Five isolates from one willow clone in one location differed markedly from the common pattern. When these five exceptional isolates were excluded, no significant differences among willow clones were found with AMOVA. Sexual reproduction and spore migration appear to be important factors for the population genetic structure of this pathogen.

Introduction

Cultivation of willows as an energy crop started in Sweden 20 years ago, and today around 18,000 hectares are planted in the central and southern parts of the country, mostly with *Salix viminalis* L., the basket willow or common osier. *S. viminalis* was introduced into Sweden in the 18th century and was cultivated as a coppice crop for basket production in the 19th century (Larsson and Bremer, 1991). Since then, it has spread along ditches and rivers. The willow leaf rust, caused by the *Melampsora epitea* Thüm. complex (Pei et al., 1993), is today the most serious disease of willows grown in short-rotation coppice plantations. *Melampsora* infections cause premature defoliation and, when severe, may result in shoot die-back and severe production losses (Verwijst 1989; Dawson and McCracken 1994; Parker et al., 1993).

Biomass willows are propagated from stem cuttings and usually large fields are planted with the same willow clone. This puts a strong and uniform selection pressure on the pathogen, with more virulent and aggressive genotypes as a possible outcome. This occurred in the closely related poplar leaf rust (*Melampsora* spp.), which has been a severe problem in poplar cultivation in Europe for several decades. The main effort to control the disease has been breeding for resistance. However, in a number of poplar clones completely resistant to *Melampsora larici-populina*, resistance was overcome by the pathogen within a decade (e.g. Pinon et al., 1987). In this perspective it seems unwise to rely primarily on breeding for complete resistance in willows. Since the projected life of a willow plantation is at least 20 years, control strategies that provide long term stability are required. In order to make proper predictions about how the willow rust will respond to different control efforts, we need to increase our knowledge about the genetic variability and epidemiology of the pathogen and an important first step

is to determine the population genetic structure of *M. epitea*.

M. epitea is a complex of heteroecious rusts with alternate hosts of different genera (Wilson and Henderson, 1966). The predominant leaf rust on *S. viminalis* belongs to *formae speciales larici-epitea typica* Kleb., which alternates on *Larix* (Pei et al., 1996). The life cycle of this biotrophic fungus is complex, including five spore stages. The epidemic phase takes place on willows in summer and includes several cycles of asexually propagated wind-transported urediniospores. In autumn, uredinia turn into telia and the fungus survives the winter on dead willow leaves. In spring, the telia produce basidiospores which infect European larch (*Larix decidua* Mill.), the alternate host, where the spermagonial stage with sexual reproduction occurs, followed by the formation of aecia. When new willow leaves develop in early summer, they are infected by aeciospores from larch, and the cycle is completed with the uredinial stage.

The relative contribution of sexual and asexual reproduction will affect the population structure. For example, if sexually derived spores contribute mainly to the primary inoculum in a field, greater genotypic diversity is to be expected than if asexual spores serve as the main inoculum. This was demonstrated when comparing sexual and asexual populations of *Puccinia graminis* populations in the United States, using isozyme and virulence markers (Burdon and Roelfs, 1985). Genotypic diversity was very high in the sexual population, while in the asexual population there was a restricted array of isozyme genotypes and the genetic structure clearly diverged from that expected under a random mating regime.

In the Swedish climate, the sexual phase on larch is presumed to be necessary for overwintering of *M. epitea*. Consequently, recombinant aeciospores from the overwintering part of the population are assumed to be the only source of inoculum in the initial infections each growing season. In the succeeding epidemic phase, clonally propagated urediniospores will serve as inoculum. The dispersal ability of the different spore types is important in this context. Urediniospores and possibly aeciospores from other rust species are believed to be involved in long-distance dispersal (Aylor, 1990; Nagarajan and Sing, 1990; Roelfs, 1986). The range of the drought- and UV-sensitive basidiospores are limited, although under favourable conditions they can move as far as 8 km (van Arsdel, 1967). This implies, that in the case of willow rust, the distance to the alternate host (larch) may be critical in determining the proportion of the actual population which will survive to the next generation. Dispersal capacity will also be important for the genetic exchange between populations, where high rates of gene flow would reduce the likelihood of population differentiation caused by natural selection and/or genetic drift.

Several investigations of genetic variability of willow leaf rust (*Melampsora* spp.) based on morphology and pathogenicity have been made (Pei et al., 1993; Pei et al., 1996; Pei et al., 1999; Ramstedt, 1999; Spiers and Hopcroft, 1996), who reported large host adaptation in *M. epitea*. Eight distinct pathotypes were recognized in rust from different *Salix* species, but little pathogenic variation was found within rust on *S. viminalis* (Pei et al., 1996). Molecular markers (RAPD and AFLP) have been used to distinguish between stem- and leaf-infecting forms of *Melampsora* rust on willows (Pei et al., 1997; Pei and Ruiz, 2000). However, little attention has been paid to the population structure of the willow rust.

The development of AFLP (Amplified Fragment Length Polymorphism) (Vos et al., 1995), a PCR-based DNA fingerprint technique, has provided a powerful tool for the investigation of genetic diversity. AFLP is a robust and reproducible technique (Hansen et al., 1999), which requires only small amounts of template DNA and therefore is suitable for biotrophic fungi like *M. epitea*, where only small amounts of tissue are available. Generally AFLP produces large numbers of easily scored fragments, which makes differentiation among individuals (i.e. multilocus phenotypes) possible, and thus enables assessment of genotypic diversity. AFLPs are presumed to be selectively neutral, which is advantageous when examining genetic structure. A drawback, however, is the dominant nature of AFLP markers, which do not distinguish between dominant homozygotes and heterozygotes. Thus, allele frequencies and basic population genetic parameters cannot be estimated directly. Nevertheless, dominant markers (RAPDs) were shown to be informative for describing genetic structure when analysed with F-statistics and related parameters (Aagaard et al., 1998; Ross et al., 1999).

Our objectives in this investigation were (i) to develop AFLP markers that can be used to measure genetic diversity in *M. epitea*, (ii) to examine the genetic structure of *M. epitea* both among and within geographically distinct populations, and (iii) to compare the rust populations on different *S. viminalis* clones.

Materials and methods

Rust isolates

Urediniospore isolates (197) were obtained from three experimental plantations in Sweden (Figure 1, Table 1). Sampling was done in September 1996 from the same three *S. viminalis* clones in all locations. Two of the willow clones, 780112 (clone 1) and 780183 (clone 2), originate from earlier Swedish willow cultivations for wickerwork, whereas SW870083 'Rapp' (clone 3), is a commercial clone in biomass production that has been bred for rust resistance. The parents of 'Rapp' also originate from earlier Swedish willow cultivations. For the purpose of this study, rust isolates sampled from the same willow clone in each location were considered to be members of the same *M. epitea* population.

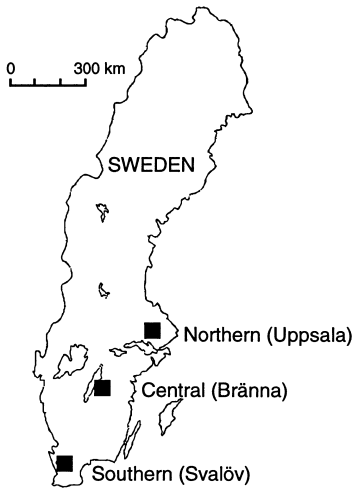

Figure 1. Map showing the location of the three experimental plantations.

Table 1. Melampsora epitea isolates

Location	*S. viminalis* clone	No. of isolates
Northern		67
	1 (780112)	22
	2 (780183)	26
	3 (SW870083*)	19
Central		63
	1 (780112)	17
	2 (780183)	26
	3 (SW870083*)	20
Southern		67
	1 (780112)	17
	2 (780183)	26
	3 (SW870083*)	24

*Commercial clone 'Rapp' from Svalöf Weibull AB.

The experimental plantations were composed of clone trials with acreages of one to two hectars. The stands were 2–9-years-old. At all locations, rust samples were collected from monoclonal willow plots (35–100 m^2) arranged in randomized complete block designs with three or four replications. About 30 infected leaves were collected from each clone and location. Distances between samples were 1–2 meters within plots and at least 15 m between replications. An exception to this sampling scheme was made for clone 3 in the southern location, where the samples were obtained from an adjacent experiment with smaller plots (6 m^2) and only two replications. The leaves were kept separately in a cooled box until cultivation of the rust the following day.

Spores from one uredinium on each collected leaf 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 12-h light period, until new uredinia developed about one week later. This isolation procedure, inoculation of spores from a single uredinium to a new leaf, was repeated twice in order to ensure that each rust isolate consisted of a single genotype. Urediniospores were then multiplied on several leaves. The spores were collected, by gently tapping the leaf and letting the spores fall off on a paper. After drying in a desiccator, the spore isolates were stored at −20 °C.

DNA extraction

DNA was extracted from urediniospores using a CTAB procedure (Chen et al., 1993) with a modification in the spore homogenization procedure (Pei et al., 1997). For each isolate, approximately 5 mg of spores were shaken vigorously (40 Hz) in a shaker (Retsch GmbH & Co. KG, Haan, Germany) for 10 min in an 1.5 ml Eppendorf tube together with three 3-mm glass beads, twenty 2-mm glass beads and 200 µl of extraction buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, and 100 mM EDTA). After addition of 12µl of 20% sodium dodecyl sulphate (SDS) the mixture was gently shaken for 1 h at room temp, mixed with 30 µl of 5 M NaCl and 26 µl of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) and kept at 65 °C for 20 min. The mixture was extracted with chloroform/isoamylalcohol (24 : 1). The top aqueous phase was transferred to a

clean tube and 0.6 volumes (approximately 150 µl) of ice-cold isopropanol was added. After 20 min of incubation at −20 °C the solution was centrifuged 10 min at 10,000 rpm to precipitate the nucleic acid. The pellet was rinsed twice with cold 70% ethanol, dried, and dissolved in 100 µl of $TE_{0.1}$ (10 mM Tris–HCl, 0.1 mM EDTA). One µl of ribonuclease at 10 mg/ml was added and the tube was kept at 4 °C overnight, or 2 h at room temperature, to completely digest the RNA. The DNA was re-precipitated, rinsed with cold ethanol, dried, dissolved in 30 µl of $TE_{0.1}$, and stored at −20 °C. The DNA extract was used undiluted in the AFLP reaction. Exact DNA concentrations were not determined for all samples, but were estimated to range between 10–25 ng/µl.

AFLP analysis

AFLP reactions were performed principally as described in the protocol from 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. Sequences of adaptors and primers used in this study are listed in Table 2.

Restriction of DNA and ligation of adaptors

Double-stranded adaptors were made by mixing the following in two Eppendorf tubes: (1) *Eco*RI adaptor mix (1 µl per reaction): 0.05 µl of 10 × OnePhorAll buffer (Pharmacia Biotechnology Inc.), 0.28 µl of adaptor E-ad1 at 100 ng/µl, 0.25 µl of adaptor E-ad2 at 100 ng/µl, and 0.42 µl of water. (2) *Mse*I adaptor mix (1 µl per reaction): 0.05 µl of 10 × OnePhorAll buffer, 0.52 µl of adaptor M-ad1 at 500 ng/µl, and 0.47 µl of adaptor M-ad2 at 500 ng/µl. The tubes were kept in 95 °C waterbath for 5 min, then cooled slowly to room temperature (about 20 min). A restriction–ligation mix (6.5 µl per reaction) was made combining the following: 1 × ligation buffer (MBI), 0.05 M NaCl, 0.05 mg/ml BSA, 1 mM ATP, 1 µl of *Eco*RI adaptor mix, 1 µl of *Mse*I adaptor mix, and 1 µl of Enzyme master mix (1 × ligation buffer, 0.05 M NaCl, 0.05 mg/ml BSA, 1 U of *Mse*I (Biolabs), 5 U of *Eco*RI (Amersham), and 1 U of T4 DNA ligase (MBI)). Restriction of DNA and ligation of adaptors were performed in the same reaction by mixing 4.5 µl of DNA extract (10–25 ng/µl), and 6.5 µl of restriction–ligation mix in an Eppendorf tube, which was incubated at room temperature overnight, or at 37 °C for 2 h, and thereafter diluted with 189 µl $TE_{0.1}$.

Preselective amplification

Preselective PCR amplification was performed in a volume of 20 µl combining the following: 1 × buffer 1 (ELT; Boehringer GmbH), 0.7 U of Expand Long Template polymerase (Boehringer GmbH), 0.2 mM of each dNTPs (Pharmacia Biotechnology Inc.), 0.44 µM primer E-00, 0.4 µM primer M-C00, and 4 µl of DNA solution from the restriction and ligation reaction. The thermocycle programme in the preselective PCR was 2 min at 94 °C; 32 cycles of 30 s at 94 °C, 1 min at 56 °C, 1 min at 72 °C; and finally 5 min at 72 °C. The amplification product was diluted 1 : 20 with $TE_{0.1}$.

Selective amplification

Selective PCR amplification was performed in a volume of 20 µl combining the following: 1 × Dynazyme buffer (Finnzymes Ltd, Finland), 0.4 U of Dynazyme polymerase (Finnzymes Ltd.), 0.2 mM of each dNTPs, 0.05 µM *Eco*RI selective primer, 0.25 µM *Mse*I selective primer, and 5 µl of diluted preselective amplification product. The thermocycle programme in the

Table 2. Sequences of AFLP adaptors and primers used in this study

*Eco*RI adaptors	E-ad1	5′-CTC GTA GAC TGC GTA CC-3′
	E-ad2	5′-AAT TGG TAC GCA GTC-3′
*Eco*RI preselective primer	E-00	5′-AGA CTG CGT ACC AAT TC-3′
*Eco*RI selective primers	E-TG	5′-AGA CTG CGT ACC AAT TCT G-3′ FAM*
	E-TA	5′-AGA CTG CGT ACC AAT TCT A-3′ HEX*
*Mse*I adaptors	M-ad1	5′-GAC GAT GAG TCC TGA G-3′
	M-ad2	5′-TAC TCA GGA CTC AT-3′
*Mse*I preselective primer	M-C00	5′-GAT GAG TCC TGA GTA A-3′
*Mse*I selective primers	M-CAA	5′-GAT GAG TCC TGA GTA ACA A-3′
	M-CAG	5′-GAT GAG TCC TGA GTA ACA G-3′

*Fluorescent label at 5′-end.

selective PCR was 2 min at 94 °C; 11 cycles of 30 s at 94 °C, 30 s at 65 °C (minus 0.7 °C per cycle), 2 min at 72 °C; then 23 cycles of 30 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C; and finally 5 min at 72 °C. The two following combinations of selective primers were used in this study: (i) E–TG and M–CAA, (ii) E–TA and M–CAG.

Electrophoresis

Prior to electrophoresis, 1.0 µl of selective amplification product was added to a loading buffer mix (0.9 µl of deionized formamide, 0.35 µl of blue loading dye, 0.25 µl of GeneScan 500 ROX internal size standard), heated at 95 °C for 3 min, placed on ice, and 1.5 µl was immediately loaded on a 4% polyacrylamide gel. Electrophoresis was performed at constant voltage (3000 V) for 2.5 h at 51 °C using an automated DNA sequencer (Model 377, PE Applied Biosystems).

Scoring procedure

GeneScan Analysis software (PE Applied Biosystems) was used to visualize and score the digital profiles. DNA fragments were accurately sized, by means of the internal size standard included in each lane, using the local southern size calling method. After inital analysis, sample files were imported into Genotyper version 2.0 (PE Applied Biosystems), and all samples were normalized to each other by scaling the sums of signals. 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 unambiguously scored 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

Considering urediniospores as dikaryons and AFLP markers as dominant, 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 (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 neighbour-joining method (Saitou and Nei, 1987) as implemented in the program Neighbor from the Phylip package (Felsenstein, 1993) and drawn with TreeView (Page, 1996). We also conducted principal component analysis (JMP software, SAS Institute Inc., Cary, North Carolina) directly on the binary data matrix, to study relationships among the isolates.

The genetic structure of *M. epitea* populations was assessed in two ways. First we used analysis of molecular variance, AMOVA (Excoffier et al., 1992), based on an Euclidean distance matrix between all pairs of multilocus phenotypes. The AMOVA was used to estimate variance components for AFLP phenotypes and for partitioning the variation among populations and within populations. Analyses were done both with regard to geographical populations (locations) and populations on different willow clones. 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 non-parametric permutation procedures. AMOVA analyses were conducted with the Arlequin software (Schneider et al., 1997). Second, differentiation was also examined using Lynch and Milligan (1994) unbiased estimate of F_{ST} for dominant markers with the program RapdFst (Apostol et al., 1996). Because AFLP markers are dominant, frequencies of null alleles were used to estimate allele frequencies. Loci with low null allele frequencies ($q^2 < 3/N$) in one or more subpopulation were excluded.

Results

The two primer combinations used identified a total of 83 AFLP fragments, that could be unambiguously scored. Three of these fragments were monomorphic for all 197 isolates in the data set. For most of the polymorphic loci (59 out of 80) the fragments were present

in all populations, evenly distributed among populations. For 11 loci, fragments were exclusive to five isolates, all sampled on willow clone 3 in the southern location. These five exceptional isolates, hereafter referred to as the B-isolates, also lacked 19 of the fragments that were common in all the populations (those fragments present in at least 50% of all isolates), and in total they shared only 15 fragments with other isolates. For the remaining 10 loci, fragments were infrequent (present in 0.5–7% of all isolates) and absent in, or exclusive to, one location or willow clone.

The genotypic diversity, quantified by a normalized Shannon's diversity index (H_S) was high in all populations, ranging from 0.977 to 1.0 with an overall value of 0.996 for all isolates. Out of the 197 isolates, 191 unique AFLP multilocus phenotypes were identified. Three pairs of identical phenotypes were found: two pairs on willow clone 3 (in the central and southern location, respectively) and one pair on different clones (clones 1 and 3 in the southern location). Distances between twin phenotypes were 18–60 m.

Since the B-isolates appeared to be genetically separated from the other rust isolates, they were excluded from the AMOVA and the analysis of F_{ST}. The AMOVA revealed that most of the molecular variability was attributable to differences within locations (94.9–98.4%) and within willow clones (98.9–100%) (Table 3). Nevertheless, low but statistically significant levels of genetic differentiation among locations were found for all clones (Φ_{ST} values of 0.051, 0.016, and 0.022). In pairwise comparisons between locations (with clones pooled) the southern location was significantly different from both the northern location ($\Phi_{ST} = 0.041$; $P < 0.001$; data not shown in Table 3) and the

Table 3. Analysis of molecular variance (AMOVA) and F-statistics

Source of variation	AMOVA					F-statistics[b]	
	df	Variance components	Percentage of total variance	Φ-statistics	Probability[a]	F_{ST}	Std
Within willow clone 1:							
Among locations	2	0.36	5.1	$\Phi_{ST} = 0.051$	$P < 0.001$	0.028	(0.051)
Within locations	53	6.68	94.9				
Within willow clone 2:							
Among locations	2	0.11	1.6	$\Phi_{ST} = 0.016$	$P = 0.019$	0.010	(0.024)
Within locations	75	6.58	98.4				
Within willow clone 3:							
Among locations	2	0.15	2.2	$\Phi_{ST} = 0.022$	$P = 0.017$	0.023	(0.047)
Within locations	55	6.58	97.8				
All willow clones[c]:							
Among locations	2	0.17	2.5	$\Phi_{ST} = 0.025$	$P < 0.001$	0.015	(0.021)
Within locations	189	6.62	97.5				
Within northern location:							
Among willow clones	2	0.05	0.7	$\Phi_{ST} = 0.007$	$P = 0.155$	0.010	(0.027)
Within willow clones	64	6.25	99.3				
Within central location:							
Among willow clones	2	−0.04	−0.7	$\Phi_{ST} = -0.007$	$P = 0.802$	−0.004	(0.018)
Within willow clones	60	6.40	100.7				
Within southern location[c]:							
Among willow clones	2	0.07	1.1	$\Phi_{ST} = 0.011$	$P = 0.109$	0.002	(0.037)
Within willow clones	59	7.21	98.9				
All locations[c]:							
Among willow clones	2	0.00	0.1	$\Phi_{ST} = 0.001$	$P = 0.410$	0.000	(0.009)
Within willow clones	189	6.74	99.9				
Hierarchical[c]:							
Among locations	2	0.16	2.4	$\Phi_{CT} = 0.024$	$P = 0.027$		
Among willow clones within location	6	0.03	0.4	$\Phi_{SC} = 0.004$	$P = 0.196$		
Within willow clones	183	6.61	97.2				

[a]Probability of a larger value obtained by chance, determined by 1000 randomizations of the data set.
[b]F_{ST} values calculated according to Lynch and Milligan (1994). Number in parenthesis is standard deviation.
[c]B-isolates were excluded from the analyses.

central location ($\Phi_{ST} = 0.035$; $P < 0.001$), while the northern and central locations did not differ ($\Phi_{ST} = -0.002$; $P = 0.713$). No significant differences among willow clones were found with the AMOVA (Table 3).

After excluding the loci with low frequency of null alleles, 57 out of 80 polymorphic markers were retained for the unbiased F_{ST} analysis. Estimates of F_{ST} averaged over the 57 loci are shown in Table 3. They are of the same order of magnitude as estimates of Φ_{ST}, though always somewhat lower (Table 3). As with AMOVA, genetic differentiation among locations was low and differentiation among willow clones was negligible.

Most isolates, 192 out of 197, in the neighbour-joining analysis form a large dispersed cluster (A) with no apparent grouping, neither with regard to location

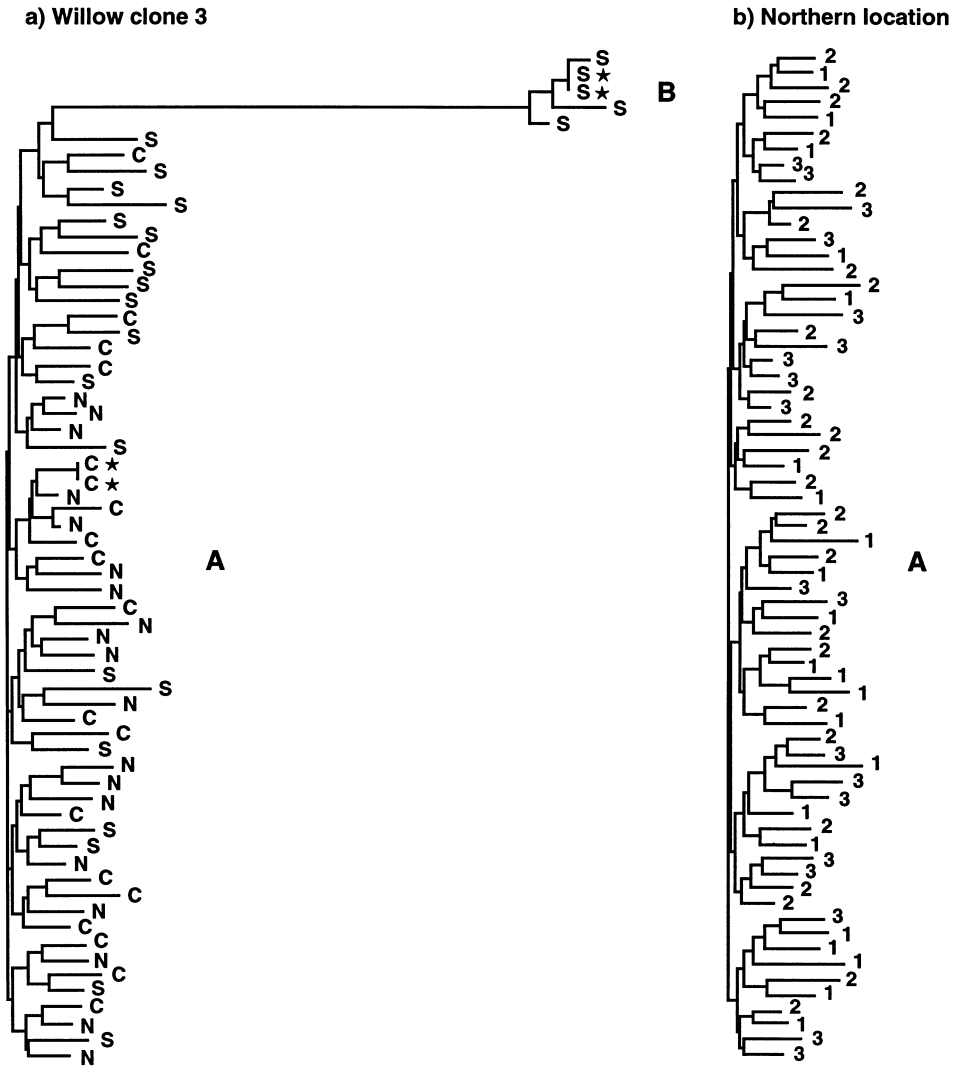

Figure 2. Neighbour-joining phenograms showing two subsets of the *Melampsora epitea* isolates. Clusters are designated A and B. Identical phenotypes are indicated with asterixs (*). (a) Comparison of 63 isolates from the three locations on willow clone 3. Locations are denoted with letters: N = northern, C = central, and S = southern. (b) Comparison of 67 isolates from the three willow clones in the northern location. Willow clones are denoted with their numbers (1, 2, and 3). Remarks: Phenograms for willow clones 1 and 2 (not shown) do not show any clustering with regard to locations. Phenogram for central location (not shown) does not show any clustering with regard to willow clones (i.e. it looks similar to phenogram for northern location). In phenogram for southern location (not shown) the B-isolates cluster in the same way as in phenogram for willow clone 3.

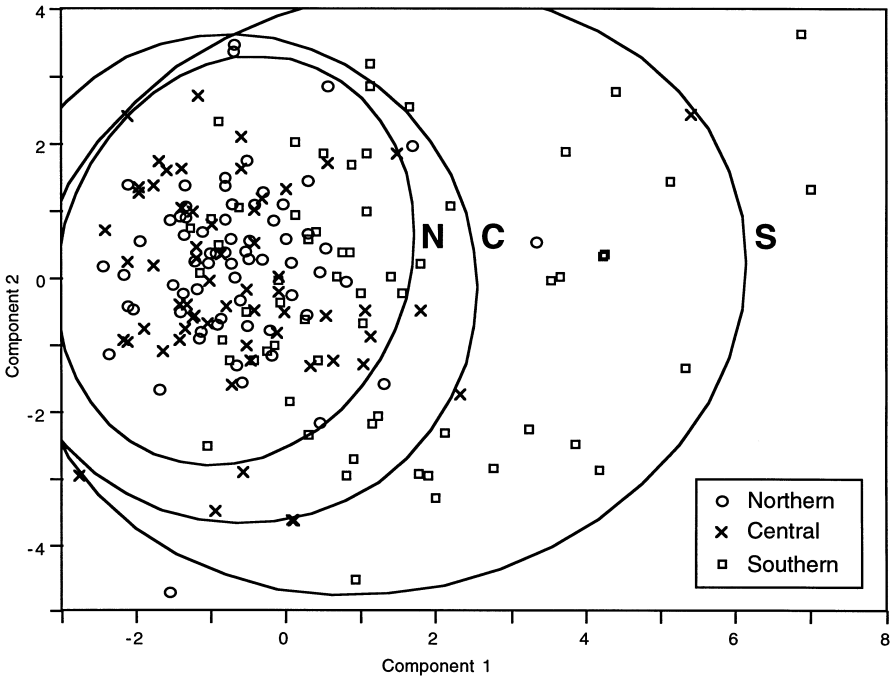

Figure 3. Principal component analysis of *Melampsora epitea* isolates (B-isolates excluded). Locations are indicated with symbols. Bivariate normal ellipses of $P = 0.95$ for the three locations (N, C, and S) are indicated.

(Figure 2a) nor with regard to willow clone (Figure 2b). An exception are the five B-isolates, sampled from clone 3 in the southern location, which form a small cluster (B) distant from the A-cluster (Figure 2a). The five B-isolates were excluded in the principal component analysis, in order to visualize the relationship among other isolates more clearly. The first two axes explained only 8.9% of the total variation in the material. Isolates from the northern and central locations are scattered mainly within the same area, while isolates from the southern location are more dispersed (Figure 3). There was a significant difference between the southern and northern location for the variation of the first principal component. No clustering with regard to willow clones was detected (not denoted in Figure 3).

Discussion

Our data shows high levels of genetic diversity within *M. epitea*, 96% of the AFLP loci being polymorphic and a very high genotype diversity. Most of the genetic variation was found within populations. In fact, most isolates (97%) had unique multilocus phenotypes and only a few clonemates were detected. These results suggest that sexual reproduction is an important mechanism to create and maintain genetic variation in Swedish leaf rust populations.

As reported in other sexually reproducing rust species – a G_{ST} of 0.0084 was calculated for DNA fingerprints of *Puccinia striiformis* among provinces in China (Shan et al., 1998) and a F_{ST} of 0.061 was determined for RAPD haplotypes of *Cronartium ribicola* f.sp. *fusiforme* among regions in southern US (Hamelin et al., 1994) – the level of geographic differentiation was low.

Low values of F_{ST}-related measures can be a consequence of extremely high genetic diversity values since F_{ST} depends on the within-population variability (Charlesworth, 1998) and/or gene flow between populations. This can be illustrated by assuming a Wright's infinite island model. Then F_{ST} is given by

$$F_{ST} = \frac{1}{1 + 4N(m + \mu)},$$

where N is the population size, m is the migration rate and μ is the mutation rate. If the mutation rate is negligible compared to the migration rate, we have

$$F_{ST} = \frac{1}{1 + 4Nm}.$$

In an equivalent way, F_{ST} will reflect the rate of mutation if migration is neglible. Typically, however, rates of migration are much greater than rates of mutation (Hartl and Clark, 1989). In the case of *M. epitea*, there is no information on mutation rates and hence we cannot rule out completely that mutation contributes to the low geographic differentiation.

Most likely, the low geographic differentiation results from gene flow between populations, since long-distance migration is well documented in rust species (Aylor, 1990; Nagarajan and Singh, 1990; Roelfs, 1986). The migration needs not be restricted to Swedish populations: occasional wind-transported immigration of rust spores from more distant areas is also likely as long-distance dispersion occuring along specific routes, favoured by certain weather patterns, have been documented in other species (Nagarajan and Singh, 1990). Immigration of willow rust might occur from central and eastern Europe, where *S. viminalis* has its natural (or naturalized) distribution, along river banks (Larsson and Bremer, 1991), or from European countries where willows are grown in biomass plantations, for instance the United Kingdom.

Although natural spore dispersal could explain high levels of gene flow, it cannot be excluded that spores have been introduced into new areas along with the willow cuttings at planting. However, there is no obvious way for stem cuttings to transmit spores, since harvest of cuttings are done in winter, after leaf fall.

The small, but significant, differences among locations, with the southern population genetically differentiated from the others and also with the largest genetic variation, could have various explanations. One possibility is that the differentiation is due to climatic adaptation, since the climate is milder and the vegetation period is longer in southern Sweden. Another explanation could be that the southern location is closer to other European countries, from where spores might migrate.

In general, rust populations on different *S. viminalis* clones were similar. Willow clone 3 has been bred for rust resistance and the rust infestation of this clone, observed during a 7-year period, was 42% compared to a standard clone (clone 2 in this study) (Larsson, 1998). Thus, the rust infecting clone 3 could be expected to differ in virulence genes. However, considering the tremendous array of unique genotypes present in *M. epitea* populations, created by sexual recombination, differences in virulence alone may not be detected unless the virulence alleles are closely linked to AFLP loci. The only distinct difference among clones detected in this investigation was the occurence of B-isolates exclusively on clone 3. However, in another study (Samils, unpublished) the B-type was found on clone 1 and clone 2 in southern Sweden, which suggests that this rust type is adapted to southern Sweden rather than to a specific clone. Alternatively, the B-type might be a recent migrant. At any rate, the cultivation of willows in extensive monoclonal fields is a recent system, and specialization to willow clones may develop with time. In this context, it should be noted that the sexual phase occurs on the alternate host (larch), which facilitates mating of rust from different willow hosts, and thus may slow down adaptation to specific willow clones.

There are substantial differences in DNA-pattern between the A- and the B-isolates. In fact, only 19% of the AFLP fragments were shared between the two groups. This suggest the presence of a reproduction barrier between the two groups. Morphologically, the B-isolates appear similar to other isolates in the uredinial stage. No differences were detected in appearance on inoculated leaves or when urediniospores were examined under a light microscope. In addition to *M. epitea*, two *Melampsora* rusts that occur on *S. viminalis* in Europe are described. These are *M. ribesii-viminalis* Kleb., which occasionally has been reported in biomass willow plantations (Pei et al., 1993; Pei et al., 1995) and an asexual 'stem-infecting form' (SIF) of uncertain taxonomic rank (Pei et al., 1995; Pei and Ruiz, 2000). When the B-type was compared to the AFLP-pattern of defined isolates of SIF (provided by Dr Pei), they appeared to be clearly different. Regarding *M. ribesii-viminalis*, it cannot be determined without further studies whether the B-type belongs to this species, since *M. epitea* and *M. ribesii-viminalis* are morphologically similar in the uredinial stage and no AFLP characterization of *M. ribesii-viminalis* is available.

In conclusion, the genetic structure observed in *M. epitea*, i.e. high levels of both gene and genotypic variation, most of which is found within populations, is consistent with a sexually reproducing species with the ability of long-distance dispersal. A few isolates showed AFLP fingerprints that differed markedly from the common pattern, and their relationship to *M. epitea* needs further investigation. In general, the observed genetic pattern suggests that this fungus, through high rates of both genetic recombination and migration, has the potential of rapid adaptation to changing

environments, for instance to new resistances in the willows. This should be carefully considered in the development of control strategies for the willow rust.

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References

Aagaard JE, Krutovskii KV and Strauss SH (1998) RAPDs and allozymes exhibit similar levels of diversity and differentiation among populations and races of Douglas-fir. Heredity 81: 69–78

Apostol BL, Black IV WC, Reiter P and Miller BR (1996) Population genetics with RAPD-PCR markers: *Aedes aegypti* in Puerto Rico. Heredity 76: 325–334

Armstrong J, Gibbs A, Peakall R and Weiller G (1996) The Rapdistance Package, Version 1.04. Australian National University, Canberra

Aylor DE (1990) The role of intermittant wind in the dispersal of fungal pathogens. Annual Review of Phytopathology 28: 73–92

Burdon JJ and Roelfs AP (1985) The effect of sexual and asexual reproduction on the isozyme structure of *Puccinia graminis*. Phytopathology 75: 1068–1073

Charlesworth B (1998) Measures of divergence between populations and the effect of forces that reduce variability. Molecular Biology and Evolution 15: 538–543

Chen XM, Line RF and Leung H (1993) Relationship between virulence variation and DNA polymorphism in *Puccinia striiformis*. Phytopathology 83: 1489–1497

Dawson WM and McCracken AR (1994) Effect of *Melampsora* rust on the growth and development of *Salix burjatica* 'Korso' in Northern Ireland. European Journal of Forest Pathology 24: 32–39

Excoffier L, Smouse PE and Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitocondrial DNA restriction sites. Genetics 131: 479–491

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) Version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle

Goodwin SB, Spielman L, Matuszak JM, Bergeron SN and Fry WE (1992) Clonal diversity and genetic differentiation of *Phytophtora infestans* populations in northern and central Mexico. Phytopathology 82: 955–961

Hamelin R, Doudrick RL and Nance WL (1994) Genetic diversity in *Cronartium quercuum* f.sp. *fusiforme* on loblolly pines in southern U.S. Current Genetics 26: 359–363

Hansen M, Kraft T, Christiansson M and Nilsson N-O (1999) Evaluation of AFLP in *Beta*. Theoretical and Applied Genetics 98: 845–852

Hartl DL and Clark AG (1989) Principles of population genetics, 2nd edn. Sinauer Associates, Sunderland, Massachusetts.

Larsson G and Bremer B (1991) Korgviden – nyttoväxter förr och nu. (The *Salix viminalis* group – useful plants then and now.) Svensk Botanisk Tidskrift 85: 185–200

Larsson S (1998) Genetic improvement of willow for short-rotation coppice. Biomass and Bioenergy 15: 23–26

Lynch M and Milligan BG (1994) Analysis of population genetic structure with RAPD markers. Molecular Ecology 3: 91–99

Nagarajan S and Singh DV (1990) Long-distance dispersion of rust pathogens. Annual Review of Phytopathology 28: 139–153

Nei M and Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences of the USA 76: 5269–5273

Page RDM (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357–358

Parker SR, Royle DJ and Hunter T (1993) Impact of *Melampsora* rust on yield of biomass willows. In Abstracts of the 6th International Congress of Plant Pathology, Montreal, Canada, 28 July–6 August 1993, p. 117

Pei MH, Royle DJ and Hunter T (1993) Identity and host alternation of some willow rusts (*Melampsora* spp.) in England. Mycological Research 97: 845–851

Pei MH, Royle DJ and Hunter TA (1995) A comparative study of stem- and leaf-infecting forms of *Melampsora* rust on *Salix viminalis* in the U.K. Mycological Research 99: 357–363

Pei MH, Royle DJ and Hunter T (1996) Pathogenic specialization in *Melampsora epitea* var. *epitea* on *Salix*. Plant Pathology 45: 679–690

Pei MH, Whelan MJ, Halford NG and Royle DJ (1997) Distinction between stem- and leaf-infecting forms of *Melampsora* rust on *Salix viminalis* using RAPD markers. Mycological Research 101: 7–10

Pei MH, Royle DJ and Hunter T (1999) Hybridization in larch-alternating *Melampsora epitea* (*M. larici-epitea*). Mycological Research 103: 1440–1446

Pei MH and Ruiz C (2000) AFLP evidence of the distinctive patterns of life-cycle in two forms of *Melampsora* rust on *Salix viminalis*. Mycological Research 104: 937–942

Pinon J, van Dam C, Genetet I and de Kam M (1987) Two pathogenic races of *Melampsora larici-populina* in northwestern Europe. European Journal of Forest Pathology 17: 47–53

Ramstedt M (1999) Rust disease on willows – virulence variation and resistance breeding strategies. Forest Ecology and Management 121: 101–111

Roelfs AP (1986) Development and impact of regional cereal rust epidemics. In: Leonard KJ, Fry WE (eds) Plant

Disease Epidemiology: Population Dynamics and Management (pp 129-150), Macmillan, New York

Ross KG, Shoemaker DD, Krieger MJB, DeHeer CJ and Keller L (1999) Assessing genetic structure with multiple classes of molecular markers: a case study involving the introduced fire ant *Solenopsis invicta*. Molecular Biology and Evolution 16: 525–543

Saitou N and Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406–425

Shan W, Chen S, Kang Z, Wu L and Li Z (1998) Genetic diversity in *Puccinia striiformis* Westend. f.sp. *tritici* revealed by pathogen genome-specific repetitive sequence. Canadian Journal of Botany 76: 587–595

Sheldon AL (1969) Equitability indices: dependance on the species count. Ecology 50: 466–467

Schneider S, Kueffer JM, Roessli D and Excoffier L (1997) Arlequin Version 1.1: a software for population genetic data analysis. Genetic and Biometry Laboratory, University of Geneva, Switzerland

Spiers AG and Hopcroft DH (1996) Morphological and host range studies of *Melampsora* rusts attacking *Salix* species in New Zealand. Mycological Research 100: 1163–1175

Van Arsdel EP (1967) The nocturnal diffusion and transport of spores. Phytopathology 57: 1221–1229

Vos P, Hogers MB, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407–4414

Verwijst T (1989) Clonal differences in the structure of a mixed stand of *Salix viminalis* in response to *Melampsora* and frost. Canadian Journal of Forest Research 20: 602–605

Wilson M and Henderson DM (1966) The British Rust Fungi. Cambridge University Press, Cambridge, UK