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Genetic diversity in relation to sexual and asexual reproduction in populations of *Melampsora larici-epitea*

Berit Samils[1], Valérie Stepien[2], Ulf Lagercrantz[1], Martin Lascoux[3] and Urban Gullberg[1]
[1]*Department of Plant Biology, Swedish University of Agricultural Sciences (SLU), Box 7080, S-750 07 Uppsala, Sweden (Phone: +46 18673352; Fax: +46 18673279; E-mail: Berit.Samils@vbiol.slu.se);* [2]*Unité de Phytopathologie, Faculté Universitaire des Sciences Agronomiques, Passage des Déportés 2, B-5030 Gembloux, Belgium;* [3]*Department of Conservation Biology and Genetics, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18D, S-752 36 Uppsala, Sweden*

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Abstract

Genetic diversity of *Melampsora larici-epitea* leaf rust from three cultivated stands of the willow *Salix viminalis* was studied using AFLP polymorphisms at 60 loci. One population was located in Northern Ireland and two in Sweden. Analysis of molecular variance (AMOVA) showed that most of the genetic variation was distributed on a fine scale within the field in all populations. Both Swedish populations displayed a very high genotypic diversity (normalized Shannon's indices of 0.95 and 1.00) and random association among loci. These results suggested that sexual reproduction had an important influence on the Swedish populations. The occurence of the alternate host (larch) adjacent to one of the Swedish rust populations did not affect the genetic diversity. However, severe rust attacks started earlier in the season in this population. The *M. larici-epitea* population in Northern Ireland was characterized by a low genotypic diversity (normalized Shannon's index = 0.54) and non-random association among loci was indicated by test of multilocus association and by pairwise tests among loci. These results suggested that asexual reproduction had a major effect on the genetic structure of this population, probably because of the overwintering of asexual spores and/or a population bottleneck associated with the annual sexual phase.

Introduction

Recombination of genes and creation of new genotypes during the sexual process undoubtedly enhances the ability of a pathogen to adapt to the deployment of new, resistant hosts. Asexual reproduction also serves a purpose in that it enables specific well-adapted genotypes to rapidly increase to high frequencies in a population. It is therefore important, in order to make adequate decisions in the management of the disease, to gather information on the reproductive strategy of the pathogen. Whilst direct observations of the occurence of different reproduction modes are difficult for many pathogens because of their small size and variable life forms, information can be obtained indirectly through selectively neutral molecular markers. Generally, sexual populations can be distinguished from asexual ones by the following two characteristics: (a) relatively high levels of genotypic diversity, and (b) random association between alleles at different loci, that would be expected to result from random mating (Milgroom, 1996). Deviation from random association between alleles at different loci, which is expected in populations where clonal reproduction is dominating, is referred to as linkage disequilibrium. It should be emphasized that other population processes, like random drift, selection, and migration (resulting in population admixtures), can also cause linkage disequilibrium, irrespective of the mode of reproduction (Hartl and Clark, 1989). Differences in genotypic diversity between sexual and asexual populations was demonstrated in a study of *Puccinia graminis* in the

United States, using isozyme and virulence markers (Burdon and Roelfs, 1985). Genotypic diversity was very high in the sexual population, while the asexual population (where the sexual phase was absent due to the eradication of the alternate host, *Berberis vulgaris*), was composed of only nine isozyme multilocus phenotypes.

Leaf rust, caused by *Melampsora* spp., is an economically important disease on willows (*Salix* spp.) cultivated as an energy crop in Sweden (Verwijst, 1990) and other parts of Europe (Dawson and McCracken, 1994; Parker et al., 1993). The predominant leaf rust in biomass plantations, *M. larici-epitea* Kleb. (the larch-alternating group in the collective species *M. epitea* Thüm.) (Pei et al., 1993) has an annual sexual cycle with an asexual epidemic phase. The epidemic phase includes several cycles of clonal propagation and spread of dikaryotic urediniospores on willows. The fungus overwinters as telia on fallen willow leaves. In the spring, telia germinate and produce basidiospores. The basidiospores infect larch (preferably *Larix decidua* Mill.) (Pei et al., 1993), the alternate host, where sexual reproduction, i.e., mating between spermatia and receptive hyphae, takes place. The sexual phase results in the formation of recombined aeciospores, which infect new willow leaves early in the summer, whereafter the asexual uredinial phase follows and the cycle is completed. The wind-transported urediniospores and aeciospores are believed to be capable of middle- to long-distance spread in other rust species (Aylor, 1990; Nagarajan and Sing, 1990; Roelfs, 1986). The dispersal range of the drought- and UV-sensitive basidiospores is limited, probably to a few hundred meters (Roelfs, 1985), although under favourable conditions basidiospores have been reported to move as far as 8 km (van Arsdel, 1967). This implies that, in the case of *M. larici-epitea*, the distance between willow plantations and larch stands may be critical in determining the proportion of the actual population which will be able to participate in sexual reproduction and contribute to recombined inoculum in the following epidemic phase. In addition, the sexual phase on larch is presumed to be crucial for winter survival of *M. larici-epitea*, since evidence of overwintering in the uredinial form is lacking. This means that field populations without larches in the vicinity may go extinct in the winter.

Amplified fragment length polymorphism (AFLP) is a PCR-based DNA fingerprinting technique, which has recently been applied to willow rust (Pei and Ruiz, 2000; Samils et al., 2001). AFLP has proven to be highly efficient in the assessment of genetic diversity. Since the technique requires only minute quantities of DNA, it is suitable for biotrophic fungi like *M. larici-epitea*, where only small amounts of tissue are available.

The objective of this study was to examine the importance of sexual and asexual reproduction for the genetic structure of three different populations of *M. larici-epitea* by estimating genetic diversity and non-random association among loci. Two of the populations are in central Sweden, roughly at the same latitude, and one is in Northern Ireland, i.e., under very different climatic conditions. Furthermore, one of the Swedish populations is adjacent to a larch stand (the alternate host) while no larches are present within 1.5 km of the other Swedish population.

Materials and methods

Sites

Rust populations at two sites in Sweden and one site in Northern Ireland were studied. The Swedish sites are both farmers' plantations and are located 45 km apart in the province of Uppland, central Sweden. At one of the sites, Djurby, a larch stand is present less than 100 m from the willow field. Aecial infections on the larches in spring are common in Djurby, and the rust infections on the willow has been observed to start earlier in the summer in Djurby compared to other plantations (Ramstedt M, personal communication). At the other site, Sätuna, the closest larch is situated 1.5 km from the plantation. Aecial infections have been observed on this larch. The site in Northern Ireland, Castlearchdale, is an experimental field with both monoclonal and polyclonal stands of willow. There are larches in the neighborhood (less than 500 m from the willow fields). Aecial infections have been observed also on these larches (McCracken AR and Dawson WM, pers. comm.).

Disease assessment

Records of disease severity of *Salix viminalis* L. clone '78183' were made once a week in the two Swedish fields during the growing season of 1995. At each site, data from the same 10 marked shoots on a transect of the plantation were recorded, beginning on

June 29 and ending November 9. Infection levels were classified on a 5-step scale: 0 = no rust, 1 = 1–10 uredinia per leaf, 2 = 11–100 uredinia per leaf, 3 = 101–300 uredinia per leaf, 4 = more than 300 uredinia per leaf. A rust score for each shoot was calculated as the average infection level on 10 leaves in the middle of the leafy part of the shoot.

Rust isolates

All rust samples in this study were collected from *Salix viminalis* clone '78183'. Both Swedish fields were 5-year-old monoclonal plantations and sampling was done within an area of 60 × 300 m in Sätuna and within an area of 60 × 100 m in Djurby. Sampling in the Swedish plantations was done on two occasions during the autumn of 1995; the first sampling was done on September 4, when infection levels were still increasing. Forty leaves from each plantation, at randomly selected locations, were collected. The second sampling was done on October 3, when rust attacks had started to decline. This sampling was hierarchical; samples were taken from 18 locations evenly distributed in the plantation and at least 16 m apart. Two leaves, one from each of two neighboring plants, were picked at each location. From each leaf two uredinia were picked for isolation of spores, resulting in 72 samples collected from each plantation. The leaves were kept separately in a cooled box until cultivation of the rust the following day.

In Northern Ireland, sampling was done on September 9–10, 1997, in a mixture trial. The trial consists of two blocks, located about 500 m apart and established three and four years earlier, respectively. Samples were collected in 4 plots: one monoclonal (10 × 10 m) and one polyclonal plot (30 × 55 m) in each of the two blocks. The collection was hierarchical, but somewhat different from the Swedish collection; samples were taken from 8 locations in each plot, at least 2 m apart. Three leaves from the same plant were picked at each location and a single uredinium from each leaf was cultivated, resulting in 96 collected samples. The leaves were kept separately in a cooled box for two to three 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. This isolation procedure, 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 then multiplied on several leaves. They were collected by gently tapping the leaf and letting the spores fall off on a paper. After drying in a dessicator, spores were stored at −20 °C.

DNA extraction and marker development

DNA was extracted from urediniospores using a CTAB procedure (Chen et al., 1993) with modifications as described elsewhere (Samils et al., 2001). 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 previously (Samils et al., 2001). Two primer combinations that gave distinct banding patterns were used: (i) E-TG and M-CAA, and (ii) E-TA and M-CAG.

Data analysis

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 fragments with clearly separated size ranges and overall high signals (fragments with scaled peak heights mainly above 100), that could be scored unambigously 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.

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. The multiple occurence of genotypes in an epidemic population may complicate the analysis of the genetic structure (Milgroom, 1996), and for this reason clone-corrected

samples were used in the analyses of molecular variance and non-random association among loci, i.e., only one representative of each AFLP multilocus phenotype per population was included.

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).

The genetic structure of *M. larici-epitea* populations was assessed by analysis of molecular variance, AMOVA (Excoffier et al., 1992), based on an Euclidean distance matrix between all pairs of AFLP multilocus phenotypes. The AMOVA was used to estimate variance components for AFLP phenotypes and for partitioning the variation within populations and among populations. Hierarchical analysis of the variation within each field was performed. Comparison was also made between the Northern Ireland population and the Swedish populations. In the Swedish populations, pairwise comparisons were made between samples from different sites and sampling dates. 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).

Non-random association of AFLP phenotypes at different loci was assessed in two ways. Firstly, a chi-square statistic was used to test the significance of pairwise linkage disequilibrium among AFLP loci. Calculations were made using the program POPGENE (Version 1.31, Yeh et al., 1997). Secondly, we used the program Multilocus 1.2 (Agapow and Burt, 2000) to calculate the index of association (I_A), an estimate of multilocus association (Maynard Smith et al., 1993). We also calculated I_A^S, a standardized index of association that removes the dependence on the number of loci and thus makes it comparable between studies ($I_A^S = I_A/(r-1)$ where r is the number of loci analyzed) (Hudson, 1994). The statistic I_A is equal to zero if there is random association among loci. A randomization procedure (1000 randomizations) was used to test for complete panmixia. In this procedure, the observed dataset is compared to datasets in which an infinite amount of sex and recombination has been imposed by randomly shuffling the alleles among individuals, independently for each locus.

Results

Disease progress in the Swedish plantations

Rust infections were present on only a few leaves in Sätuna at the start of the assessments (June 29), while in Djurby the average rust score was already 1.0 (Figure 1). Disease levels were significantly higher in Djurby than in Sätuna until August 17. In September there was a massive increase in disease levels in both Djurby and Sätuna. Disease levels decreased rapidly by the end of September when the uredinia turned into telia.

AFLP analysis

After some losses in the procedure of isolation and bulking-up of rust samples, 217 isolates out of the collected 320 were finally obtained for analysis (Table 1). A total of 70 distinct AFLP fragments was identified with the two primer combinations used in this study. Ten of the fragments were monomorphic for all isolates in the data set. Four fragments were exclusive to the Northern Ireland population and nine fragments were exclusive to the Swedish populations. In total, 171 unique AFLP multilocus phenotypes were distinguished: 156 in the Swedish samples and 15 in the Northern Ireland sample. When the two primer pairs were analyzed separately, one primer pair (41 markers) could distinguish 161 unique phenotypes, and the other

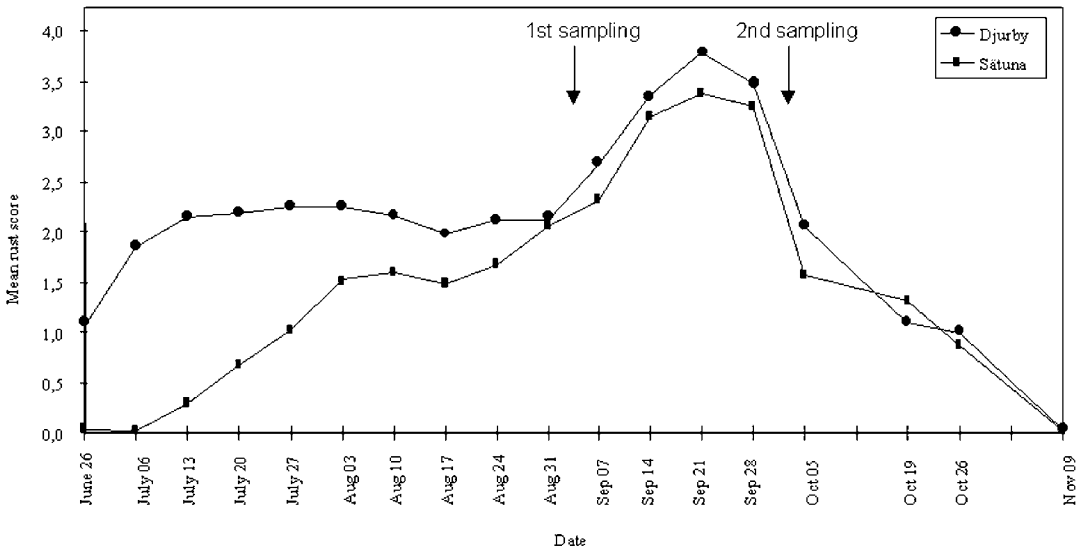

Figure 1. Disease progress in Djurby and Sätuna. The two sampling occasions are indicated.

Table 1. Sample information and estimates on genotypic diversity for *M. larici-epitea* populations in this study

Category	Site		
	Djurby	Sätuna	Castlearchdale
Year of sampling	1995	1995	1997
Region	Uppland, Sweden	Uppland, Sweden	Fermanagh, N. Ireland
Willow host	*S. viminalis* '78183'	*S. viminalis* '78183'	*S. viminalis* '78183'
Alternate host (larch) in neighborhood	Yes	No	Yes
Sample size (*n*)	89(24 + 65)[a]	81(21 + 60)	47(47 + 0)
Number of AFLP multilocus phenotypes	81	79	15
Frequencies of multiple AFLP phenotype[b]	A: 10%; B: 1%; C: 1%; D: 1%	A: 4%; B: 1%; C: 1%; D: 1%	E: 32%; F: 23%; G: 15%; H: 6%
Genotypic diversity[c]	0.95	1.00	0.54

[a]Numbers in parenthesis denote sample sizes in September and October, respectively.
[b]A, B, C, D, E, F, G, and H denote different AFLP multilocus phenotypes.
[c] Genotypic diversity was calculated by a normalized Shannon's diversity index (H_S) as described by Goodwin et al. (1992).

primer pair (29 markers) could distinguish 136 unique phenotypes.

Genotypic diversity

Genotypic diversity was very high in both Swedish populations. The normalized Shannon's index was 0.95 in Djurby and close to 1.00 (0.996) in Sätuna (Table 1). Four AFLP multilocus phenotypes were found in multiple copies at the Swedish locations, and all of them occurred at both locations. One phenotype was found in three copies in Sätuna in September, eight copies in Djurby in September, and one copy in Djurby in October. The other three phenotypes were all found in two copies, one of each in Sätuna and Djurby in October. Identical phenotypes were never found on the same leaf. Among 56 pairs of uredinia isolated from the same leaves (30 pairs in Djurby and 26 pairs in Sätuna), no pair was composed of clonemates. The smallest distance between identical phenotypes was approximately 10 m.

Since we did not detect any differences in gene or genotypic diversity between monoclonal and

Table 2. Analysis of molecular variance (AMOVA) for *M. larici-epitea* isolates within the three fields. Analyses were performed on entire samples

Source of variation	df	Variance components	Percentage of total variation	ϕ-statistics	Probability[a]
Castlearchdale, N. Ireland					
Between blocks	1	0.07	1.2	0.012	0.25
Among locations within blocks	25	−0.44	−7.2	−0.073	0.63
Between leaves within locations	20	6.53	106.0	−0.060	
Djurby, Sweden					
Among locations within field	17	0.09	1.5	0.015	0.31
Between leaves within locations	17	−0.09	−1.5	−0.015	0.59
Between uredinia within leaves	30	5.92	100.0	0.000	
Sätuna, Sweden					
Among locations within field	17	−0.18	−3.0	−0.030	0.87
Between leaves within locations	16	0.28	4.8	0.048	0.06
Between uredinia within leaves	26	5.73	98.2	0.018	

[a]Probability of a larger value obtained by chance, determined by 1000 randomizations of the data set.

Table 3a. Analysis of molecular variance (AMOVA) for the three *M. larici-epitea* populations. Analyses were performed on clone-corrected samples

Source of variation	df	Variance components	Percentage of total variation	ϕ-statistics	Probability[a]
Between Sweden – N. Ireland	1	0.580	8.9	0.089	<0.001
Within countries	173	5.92	91.1		
Between Swedish sites	1	0.016	−0.3	−0.003	0.82
Within Swedish sites	159	5.79	100.3		

[a]Probability of a larger value obtained by chance, determined by 1000 randomizations of the data set.

Table 3b. Population pairwise ϕ-statistics (AMOVA) for the Swedish *M. larici-epitea* subpopulations (clone-corrected samples)

	Djurby September	Djurby October	Sätuna September
Djurby October	0.032**		
Sätuna September	−0.004	0.022**	
Sätuna October	0.018*	−0.002	0.017*

$^{**}P < 0.01$; $^{*}P < 0.05$ (determined by 1000 randomizations of the data set).

polyclonal plots in the trial in Castlearchdale (data not shown), all Northern Ireland samples were pooled and regarded as comparable to the Swedish monoclonal plantations. Genotypic diversity was considerably lower in the Northern Ireland population with a normalized Shannon's index of 0.54 (Table 1). Four multilocus phenotypes occured in high frequencies, and constituted 32%, 23%, 15%, and 6% of the total sample in Northern Ireland. Isolates of the three most frequent putative clones were found in both blocks (500 m apart) roughly equally distributed between the blocks (data not shown). Only in two cases, out of 21 possible, were clonemates found on the same plant.

Genetic differentiation

AMOVA was performed separately for the three populations to study the spatial distribution of genetic variation within the fields. In this initial analysis, entire samples (i.e., with all copies of a multilocus phenotype included) were used in order to detect within-field variation due to clustering of clonal isolates. No significant differences among locations within the fields were detected in any of the three populations (Table 2), not even between the two blocks located 500 m apart in the Castlearchdale plantation. Most of the molecular variation (98–100%) was present on a

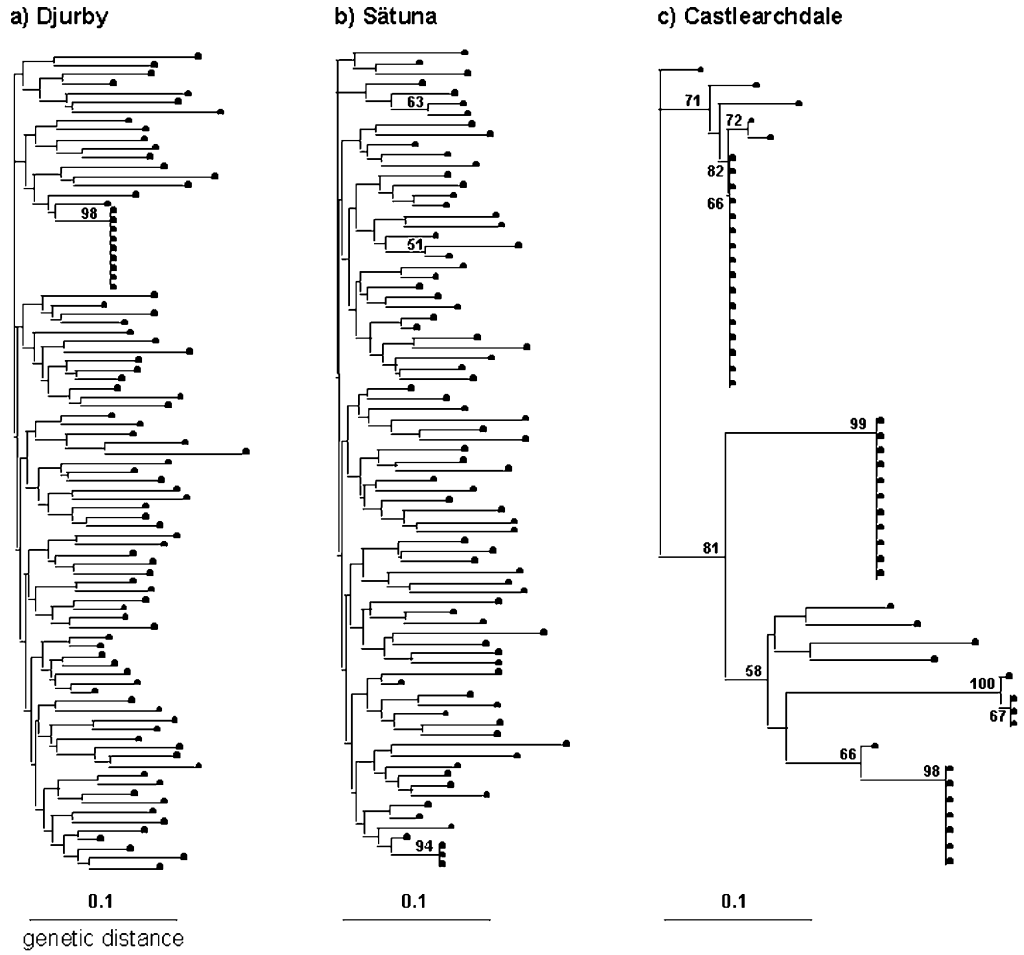

Figure 2. Neighbor-joining phenograms of the isolates from the three *M. larici-epitea* populations: (a) Djurby, (b) Sätuna, and (c) Castlearchdale. Numbers above branches indicate the percentage of bootstrap values that support the branch. Bootstrap values are only provided for those branches with >50% support.

very fine spatial scale (among leaves within locations or among uredinia within single leaves). Small negative variance components in the AMOVA can arise by chance alone in the absence of genetic structure. However, large negative variance values, as those obtained in this analysis (Table 2), should be interpreted as individuals from different subpopulations (i.e., from different locations or leaves) being genetically more related than individuals from the same subpopulation. This is most likely a result of identical AFLP phenotypes (clones) distributed throughout the fields. Since the results from the AMOVA indicated that genetic variation was randomly distributed within all three fields and no spatial clustering of clonal isolates was indicated, the sample from each field was considered to be representative of the entire population, although it was obtained in a hierarchical fashion. In the following analyses, the samples were treated as random samples.

When the populations were compared, the AMOVA revealed a significant genetic differentiation between the Northern Ireland population and the Swedish populations ($\Phi_{ST} = 0.089$; $P < 0.001$; Table 3a). However, most of the molecular variability was attributable to differences within locations (91%). When comparing the two Swedish locations, no differentiation was detected ($\Phi_{ST} = -0.003$, $P = 0.82$). However, significant differences between the two sampling occasions were found at both locations ($\Phi_{ST} = 0.032$ and 0.017 respectively, $P < 0.001$; Table 3b).

Cluster analyses

In the neighbor-joining phenograms, no distinct groups were indicated in the two Swedish populations except for the clusters of identical multilocus phenotypes (Figure 2a,b), while the Northern Ireland population was more structured (Figure 2c). In a phenogram with the Swedish and Northern Ireland isolates joined (not shown due to large size), the isolates and subgroups from Northern Ireland intermixed with the Swedish isolates rather than forming a separate cluster.

Non-random associations among loci

Non-random association between pairs of AFLP phenotypes was evaluated for each of the three populations. Since the statistical power depends on allele frequencies (Brown, 1975), only the AFLP loci with estimated allele frequencies between 0.1 and 0.9 (31 loci) were used in the pairwise tests. Table 4 reports the result for both clone-corrected and entire samples. In both Swedish populations (Table 4), only a low number of the pairwise tests for linkage disequilibrium were significant (6.2% in Djurby and 4.3% in Sätuna for clone-corrected samples). These rates are almost within the range expected with a type I error rate of $\alpha = 0.05$. In the Northern Ireland clone-corrected sample, 17.6% of the tests were significant. The results on pairwise tests were supported by the analysis of multilocus associations; substantial multilocus associations were only detected in Northern Ireland ($I_A = 2.34$; $I_A^S = 0.040$) and these were significantly larger than in a panmictic population at $P = 0.001$ (Table 4). The very low degree of multilocus associations observed in the Sätuna population in Sweden ($I_A = 0.13$; $I_A^S = 0.002$) was only marginally significant (probability of panmixis = 0.046).

Discussion

The earlier development and higher levels of disease in Djurby compared to Sätuna was probably caused by the proximity of a larch stand, the alternate host of *M. larici-epitea*, from which the initial aeciospores are dispersed early in summer. As one asexual propagation cycle is only about two weeks, a few weeks earlier start of disease development could be important for the increase of inoculum and enhance the possibility to reach high disease levels earlier in the summer.

The proximity of the alternate host did not have any appearant effect on the genetic structure of the Swedish rust populations. The genotypic diversity was equally high, even at a fine scale (i.e., individual leaves), in both populations. The importance of sexual reproduction

Table 4. Measures of associations among AFLP loci in *M. larici-epitea* populations

Sample	Sample size (n)	Pairwise comparisons % of significant comparisions[a]	Multilocus associations		
			I_A	I_A^S	Probability of panmixia[b]
Sweden					
Djurby					
Entire[c]	89	9.1% (496)[e]			
Clone-corrected[d]	81	6.2% (465)	0.00	0.000	0.482
Sätuna					
Entire	81	5.6% (465)			
Clone-corrected	79	4.3% (465)	0.13	0.002	0.046
Northern Ireland					
Castlearcdale					
Entire	47	60.1% (378)			
Clone-corrected	15	17.6% (465)	2.34	0.040	<0.001

[a]Percentage of pairs of AFLP loci in significant nonrandom association at $P = 0.05$.
[b]Probability of a null hypothesis of complete panmixia, determined by 1000 randomizations of the data set.
[c]Entire sample includes all isolates analyzed.
[d]Clone-corrected sample includes one representative of each AFLP multilocus phenotype.
[e]Total number of pairs of loci tested.

was supported by the tests of non-random association among loci where both Swedish populations showed only insignificant departures from panmixia. In this study, the rust within a willow plantation was regarded as a population. In reality, populations might extend over larger areas, which could explain the absence of differences in diversity between the two locations. During the epidemics, considerable amounts of urediniospores might be spread by the wind among willow fields within a wide area, where repeated cycles of asexual propagation would allow for a continuous exchange of genotypes among willow fields. The limited range of basidiospore dispersal may restrict sexual reproduction to occur only at those locations, within this widespread population, where willow and larch grow in proximity to each other. But the recombined aeciospores from the larches in these locations, and the subsequently clonally produced urediniospores, may then again be dispersed throughout the range of the population during the next epidemic. Hence, the genetic diversity of a rust population might not be dependant on the proximity of larch to single fields, but rather to the rate of sexual reproduction on a larger scale. In this perspective, the rust in Sätuna and Djurby (located 45 km apart) could perhaps be considered as parts of the same population instead of separate populations. Indeed, no genetic differentiation between the two sites was detected by AMOVA. Long-distance dispersal of aecio- and urediniospores has already been suggested to explain low geographic differentiation of the willow rust in Sweden in a previous study (Samils et al., 2001). In the present study, a small but significant difference between sampling occasions was detected by AMOVA at both Swedish sites. In fact, rust samples collected at the same time but from different sites were more similar than rust from the same site at different sampling occasions, suggesting a simultaneous change in the composition of the two rust populations. A possible explanation might be temporal spore dispersal from more distant, genetically different, rust populations. Spore dispersal is likely to increase successively in the epidemics as spore production increases.

The differentiation between the Swedish rust populations and the Northern Ireland population that was detected by AMOVA ($\Phi_{ST} = 0.089$) could be explained either by restricted gene flow between the countries, by climatic adaptation, or by the difference in time between sampling occassions (i.e., two years later in Northern Ireland). This result could be compared to a small differentiation ($\Phi_{ST} = 0.028$) among *M. larici-epitea* populations in central and southern Sweden in a previous study (Samils et al., 2001), where isolation by distance, climatic adaptation or the location of southern populations close to continental Europe was suggested to explain the differences.

The genetic structure of the Castlearchdale population in Northern Ireland differed markedly from that of the two Swedish populations; the level of clonality was much higher and the genetic structure more pronounced, and non-random association among loci was indicated. Three hypotheses are considered to explain these differences. One hypothesis states that the effects of natural selection are more pronounced in Northern Ireland, because of an earlier start of the growing season and a favourable humid climate which allows for a larger number of asexual propagation cycles during each epidemic phase. Selection on genotypes with varying fitness will decrease genotypic diversity and this process could be more advanced in Northern Ireland at the time of sampling. Selection during clonal reproduction will also cause non-random associations among loci. If a specific advantageous genotype increases in frequency, alleles at all loci in that genotype will also increase in frequency through 'hitchhiking' (Milgroom, 1996). When clone-corrected samples are used for analysis of linkage disequilibrium, estimates will not reflect associations among loci produced by clonal propagation in the actual epidemic phase. However, part of the associations may persist between years, since one cycle of recombination will not break down associations completely. At maximum, the rate of decay is a decrease by a half per sexual generation (or, more precisely, $1 - r$, where r is the rate of recombination) (Hedrick, 1987). Although the effects of natural selection might be greater in Northern Ireland than in Sweden, there is little reason to believe that selection acts on a few specific genotypes, unless a new allele with high fitness has appeared recently in the population (e.g., a new virulence allele matching a resistance gene of the host). More likely, in a population with annual recombination, alleles with varying fitness are present in many genotypes in random association with other loci.

Our second hypothesis proposes that the rust population in Castlearchdale in Northern Ireland has gone through one or more seasonal population bottleneck(s), i.e., severe temporary reductions in size. The effect of a severe population bottleneck in the overwintering phase, including the sexual spore stages, will be a small and possibly fragmented breeding population, where

non-random mating among a limited number of individuals might result in non-random association among loci. Another effect would be that a limited amount of initial aeciospore inoculum is produced, which could explain a low genotypic diversity. The specific cause of such a seasonal bottleneck is not obvious. One possibility is that the mild winters in Northern Ireland are unfavourable for overwintering of telia. For instance, in the stem rust of wheat (*P. graminis* f.sp. *tritici*) teliospores germinate better when they are produced under cool temperatures, and thus, sexual populations normally have been present at cooler latitudes (Roelfs, 1985). Another possible bottleneck could be the spread of basidiospores to larch and the finding of a mating partner in the spermagonial stage. When compared to the situation in Sätuna in Sweden, the occurence of larch in Castlearchdale would not seem to be the limiting factor. The closest larches are situated only a few hundred meters from the willow plantation in Castlearchdale and the coexistence of *S. viminalis* and *Larix* is common within the region (McCracken AR, personal communication). Earlier observations have indicated that sexual reproduction in Northern Ireland is rare despite the presence of the alternate host. At sites where larch (*L. decidua*) and biomass willow grow in close proximity, the sexual stage has only been detected infrequently, despite intensive searching for a number of years (McCracken et al., 2000).

Our third hypothesis postulates that willow rust has the ability to overwinter asexually, i.e., as urediniospores, in Northern Ireland but not (or to a less degree) in Sweden. This has never been reported in *M. larici-epitea*, and was suggested to be unlikely in England (Pei and Ruiz, 2000). However, asexual overwintering occurs in a stem-infecting form of *Melampsora* (with uncertain taxonomic status) (Pei and Ruiz, 2000), which overwinters in buds or cankered stems of willows. If a limited number of clonal lineages are carried over from year to year, the chances for certain genotypes to amplify to high frequencies, either by selection or by chance, would be improved greatly. In the absence of sexual recombination, non-random associations among loci (caused by selection or drift) would be promoted, with linkage disequilibrium as a result. If asexual overwintering is possible in *M. larici-epitea*, the mild winters in Northern Ireland are likely to be more favourable for urediniospore survival than the cold Swedish winters, which could explain the differences between rust populations in the two countries. High levels of clonality, in combination with a high degree of associations among loci, are typically found in fungal populations where sexual recombination is absent or infrequent. For example, in populations of *Sclerotinia sclerotiorum*, a fungus which is suggested to be predominantly clonal, the clonal fraction of populations was on average 66%, and the index of association I_A was on average 0.54 (Kohli and Kohn, 1998). Hsiang et al. (2000) reported a clonal fraction of 26% and an I_A of 1.37 in a population of *Kabatina juniperi* where asexual reproduction is suggested to be the major form of reproduction (no sexual structures are known).

In conclusion, asexual reproduction was indicated to play a major role for the genetic structure of the *M. larici-epitea* population in Castlearchdale. Whether this is a result of the presence of asexual overwintering, or the result of a population bottleneck associated with the annual sexual reproduction, or maybe a combination of both, has yet to be determined. In the Swedish willow rust populations on the other hand, sexual reproduction is suggested to have the greatest impact on genetic structure.

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