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Genetic variation in *Melampsora larici-epitea* on biomass willows assessed using AFLP

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Abstract

Five pathotypes belonging to *formae speciales larici-epitea typica* (LET), *larici-retusae* (LR) and *larici-daphnoides* (LD) of *Melampsora larici-epitea* were examined using amplified fragment length polymorphism (AFLP). Of 213 AFLP markers scored, several were found to be exclusive to different *formae speciales*. The dendrogram placed the five pathotypes into distinct groups. Within pathotypes, average Nei & Li's similarity coefficients were calculated as 0.71–0.85. The similarities were 0.66–0.72 among the three pathotypes within LET and 0.34–0.44 between pathotypes belonging to different *formae speciales*. When assessed using the Shannon index, the diversity within locations was estimated as 0.55–0.59, greater than that found within pathotypes (0.24–0.42). The average per-locus diversity was 0.37 among the pathotypes and 0.11 among the locations. When the data from both LET and LR isolates were examined using AMOVA, the majority of the variation (70.85%) was attributed to among pathotypes within location. When only LET types were included, approximately half of the variation was partitioned to among pathotypes within location and the other half to among the isolates within collection. It appears that the degree of differentiation of LET4 on *S.* × *mollissima* between Loughgall and Long Ashton sites has decreased markedly since 1992, when it was first detected.

Introduction

In recent years, willows have been grown as a main crop in short rotation coppice (SRC) plantations for renewable energy in the UK and western Europe. In these plantations, rust caused by *Melampsora larici-epitea* Kleb. (the larch-alternating *M. epitea* Thüm.) is the most widespread and destructive disease (Pei et al., 1999a). Within *M. larici-epitea*, six *formae speciales* have been recognised in continental Europe (Sydow and Sydow, 1915; Gäumann, 1959). In Japan, two 'races' have now been recognised, out of four reported originally (Hiratsuka, 1932; Hiratsuka and Kaneko, 1982). In the UK, three *formae speciales, larici-epitea typica* (LET), *larici-retusae* (LR) and *larici-daphnoides* (LD), were identified within *M. larici-epitea* (Pei et al., 1996). The host range of a *forma specialis* appears largely confined to certain sections of *Salix*. For example, LET has been recorded on *S. viminalis* (Sec. *Vimen*), *S. caprea*, *S. cinerea*, and *S. aurita* (Sec. *Vetrix*), LR on *S. burjatica* Nassarov (syn. *S. dasyclados* Wimm.) (Sec. *Vetrix*) and *S. retusa* (Sect. *Retusae*), and LD on *S. daphnoides* and *S. acutifolia* (Sec. *Daphnella*). Morphologically, however, there is no clear distinction between the *formae speciales*. There is further variation in pathogenicity within a *forma specialis*. A number of pathotypes (defined according to their pathogenicity on a certain range of willow clones) were identified within LET and within LR (Pei et al., 1996; 1999a).

Melampsora larici-epitea has a complex life-cycle, producing five spore stages and alternating on larch (*Larix* spp.) (Pei et al., 1993). Rust epidemics in willow are caused by repeated cycling of urediniospores,

which contain two nuclei and function as diploid. The rust develops teliospores in the late summer and autumn and overwinters on fallen willow leaves. In spring, teliospores germinate to produce haploid basidiospores that infect larch. Fertilisation between spermagonia on larch needles results in the formation of dikaryotic aeciospores which infect willow.

Genetic relationships between pathotypes can be assessed by testing pathogenicity to host species/ clones. However, pathogenicity tests cannot provide the information on the overall genetic background and relatedness in the pathogen. The relationships between pathotypes can also be examined by determining the likelihood of gene exchange. With *M. larici-epitea,* this involves pairing spermagonia of different isolates using the alternate host larch and examining resulting progeny (Pei et al., 1999c). However, such experiments are often difficult and time-consuming. For example, a crossing experiment requires a number of steps, teliospore production, overwintering, teliospore germination, inoculation of larch, pairing of spermagonia, isolation of aeciospores and tests for pathogenicity on willow hosts. From our experience, a crossing experiment with *M. larici-epitea* takes at least eight months to complete.

Recently, amplified fragment length polymorphism (AFLP) was applied to studies of population biology of willow *Melampsora* (Pei and Ruiz, 2000; Pei et al., 2000; Samils et al., 2001). AFLP is a PCR-based DNA fingerprinting technique and has proved to be efficient in genotyping and examining genetic variation. It requires only a small sample and is therefore particularly useful for genetic studies of obligate pathogens such as rust fungi. When two forms of rust (LET and the stem infecting form) occurring on *S. viminalis* were examined using AFLP (Pei and Ruiz, 2000), the band patterns were variable (Nei & Li's similarity coefficients >69%) in LET but very similar (similarity coefficients >98.9%) in the stem-infecting form. The AFLP data suggested that LET undergoes a full sexual life-cycle while the stem-infecting form exists as an asexual population which may have a clonal lineage. AFLP profiles also revealed that a 'new' pathotype of *M. larici-epitea*, which was first detected on *S.* × *mollissima* 'Q83' in several locations in the UK in 1992, did not spread from a common source but from separate sources (Pei et al., 2000). Samils et al. (2001) examined *M. larici-epitea* collected from three *S. viminalis* clones at three sites in Sweden using AFLP. A great majority (96%) of the AFLP markers were polymorphic and the levels of genetic diversity were high among the isolates. Analysis of molecular variance attributed most of the variance (97.5%) to within locations and no significant differentiation was found among willow clones.

This study was conducted to determine the extent of genetic variation among the three *formae speciales* of *M. larici-epitea* on biomass willows in the UK using AFLP and to assess the possibility of finding potential markers which are uniquely fixed in different *formae speciales*.

Materials and methods

Rust isolates and willow clones

The majority of rust collections were obtained from *S. viminalis* 'Mullatin', *S.* × *calodendron S.* × *mollissima* 'Q83' and *S. burjatica* 'Korso' grown at Long Ashton, south west England, Craibstone, Scotland and Loughgall, Northern Ireland in September 1996 (Table 1) as part of the extended studies on the occurrence of different pathotypes in SRC willow plantations in the UK (see Pei et al., 1999a). At Long Ashton and Craibstone, over 200 leaves bearing active uredinial pustules were collected at random on a diagonal transect from a plot, which had a configuration of 5 × 5 (=25) stools at 1 m × 1 m spacing for each willow clone. At Loughgall, over 50 rust-infected leaves were randomly collected from a plot having the same plantation design as Long Ashton. The rust collections from *S. acutifolia* and *S. daphnoides* were made from the National Willow Collection (NWC) at Long Ashton and a collection at Loughgall in different years. For each rust collection, the urediniospores were collected into 2 ml polyethylene vials using a cyclone spore collector, dried at 4 °C for one week and stored at −15 °C until use. For isolation, the rust spores in the vial were mixed well with a camel hair brush and inoculated onto detached leaves of host clones placed in Petri dishes containing water-soaked filter paper. Rust isolates were made from single uredinial pustules which developed on the detached leaves. The rust isolates were tested for pathogenicity to examine their virulence/avirulence pattern on the eight willow differentials using leaf discs (Pei et al., 1996). Of the five pathotypes identified, four pathotypes – out of which three belonged to LET and one to LR, were among the most important pathotypes in UK biomass plantations (Pei et al., 1999a).

Table 1. *M. larici-epitea* samples used in the study

Rust collection	Host clone	Number of isolates	Site	Collection date	Pathotype
CCR	*S.* × *calodendron*	5	Craibstone, Scotland	Sept. 1996	LET3
CLG	*S.* × *calodendron*	5	Loughgall, N Ireland	Sept. 1996	LET3
CLA	*S.* × *calodendron*	5	Long Ashton, SW England	Sept. 1996	LET3
KCR	*S. burjatica*	5	Craibstone, Scotland	Sept. 1996	LR1
KLG	*S. burjatica*	4	Loughgall, N Ireland	Sept. 1996	LR1
KLA	*S. burjatica*	3	Long Ashton, SW England	Sept. 1996	LR1
MCR	*S. viminalis*	5	Craibstone, Scotland	Sept. 1996	LET1
MLG	*S. viminalis*	5	Loughgall, N Ireland	Sept. 1996	LET1
MLA	*S. viminalis*	5	Long Ashton, SW England	Sept. 1996	LET1
QLG	*S.* × *mollissima*	5	Loughgall, N Ireland	Sept. 1996	LET4
QLA	*S.* × *mollissima*	5	Long Ashton, SW England	Sept. 1996	LET4
DLG	*S. acutifolia*	1	Loughgall, N Ireland	Aug. 1992	LD1
DLA1	*S. daphnoides* 'Meikle'	1	NWC*, SW England	Oct. 1996	LD1
DLA2	*S. daphnoides* 'Meikle'	1	NWC, SW England	Sept. 1999	LD1
DLA3	*S. daphnoides* 'Meikle'	1	NWC, SW England	Oct. 1990	LD1

*The National Willow Collection.

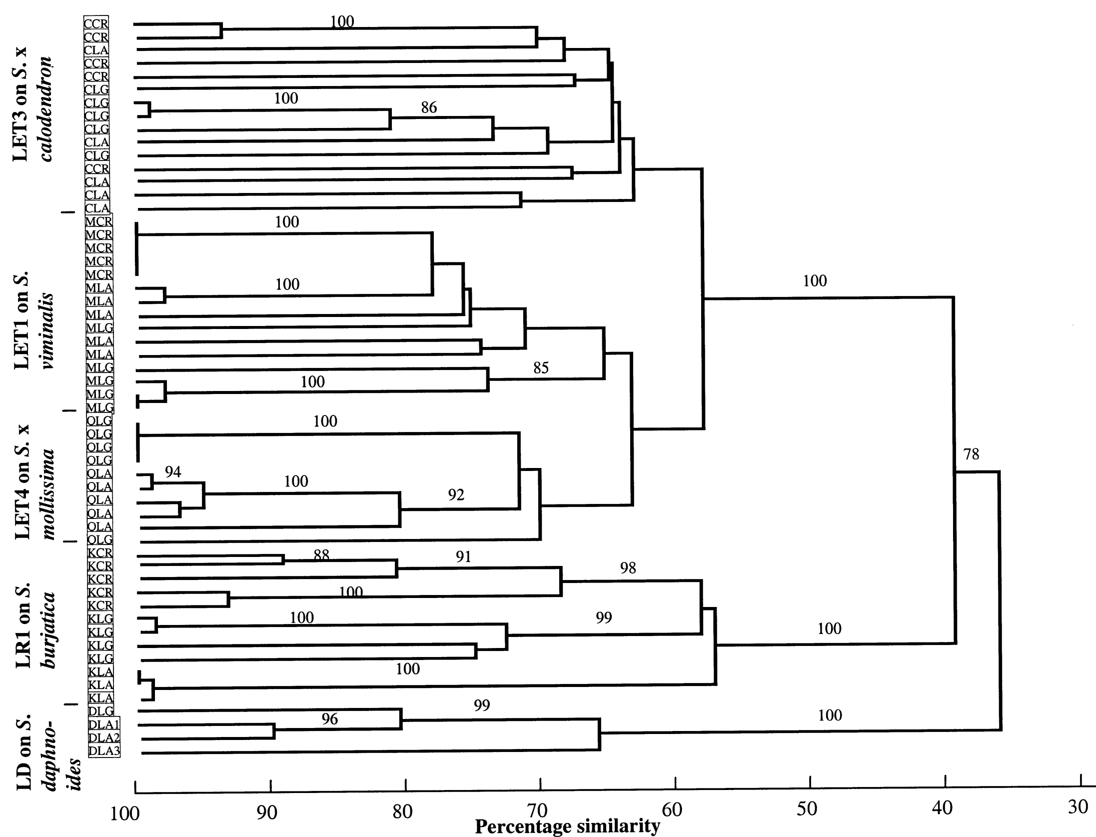

Figure 1. Dendrogram of five pathotypes belonging to f. spp. *larici-epitea typica* (LET), *larici-retusae* (LR) and *larici-daphnoides* (LD) based on 213 AFLP loci. The dendrogram is constructed using Nei & Li's similarity coefficient and bootstrap values of 75% or greater (1000 replicates) are given.

AFLP procedure

The urediniospores of the isolates were multiplied (1–2 generations) on detached leaves of host clones in 9 or 14 cm diameter Petri dishes containing tap-water soaked filter paper. The spores were collected by tapping off the leaves, dried at 4 °C for 1–2 weeks and stored at −15 °C before use. A two-tube method described by Pei and Ruiz (2000) was used to extract genomic DNA from rust urediniospores. For each isolate, approximately 5 mg spores were used. The AFLP procedure was as described by Pei and Ruiz (2000), which was modified from the two-step PCR protocol by Vos et al. (1995). In the first step (pre-amplification), both E-A and M-C primers, each having one base pair extension, were used. In the second step, *EcoR*I and *Mse*I primers having three base pair extensions were used in six combinations: E-AAA/M-CAA, E-AAA/M-CAC, E-AAA/M-CAG, E-AAA/M-CAT, E-AAA/M-CTC and E-AAA/M-CTT. The PCR products were denatured and run on a 6% polyacrylamide DNA sequencing gel and autoradiographs were obtained using Hyperfilm MP (Amersham). The second PCR amplification was repeated once.

Data analysis

The presence and absence of an unambiguous band was scored as 1 and 0 respectively. Similarities were computed between all pairs (x, y) of isolates using the formula given by Nei and Li (1979): $s = 2xy/x + y$, where xy is the number of bands shared by the pair and x + y is the total bands of the pair. All AFLP markers, both mono- and polymorphic, were included in data analysis. A dendrogram was constructed based on unweighted pair-group method using arithmetic means (UPGMA) (Sneath and Sokal, 1973). Computations were done using Genstat Release 4.2 (5th edition). The robustness of the clusters was examined by applying the bootstrap method with 1000 replicates using the WinBoot program (Yap and Nelson, 1996).

Phenotypic diversity among LET and LR types was examined using the Shannon information index (Bussell, 1999; Pei et al., 2000). Each AFLP marker was treated as a putative locus with two alleles, 1 for presence, and 0 for absence, of a band. The diversity was calculated for each single locus as:

$$H'_j = \sum p_i \log 2 p_i$$

where H'_j is the estimate of diversity within a population, p_i the frequency of either the presence ($i = 1$) or absence ($i = 2$) of an AFLP band. The average diversity over populations was calculated as:

$$H'_{\text{pop}} = \frac{1}{n} \sum H'_j$$

where n is the number of populations. The species diversity (refers to the diversity of all the isolates except for those from *S. daphnoides*) was calculated for each locus as:

$$H'_{\text{sp}} = \sum p_s \log 2 p_s$$

where p_s is the frequency of either the presence ($s = 1$) or absence ($s = 2$) of the band in all the samples. For each locus, diversity component within populations was calculated as $H'_{\text{pop}}/H'_{\text{sp}}$ and the component between populations as $(H'_{\text{sp}} - H'_{\text{pop}})/H'_{\text{sp}}$. All markers, including the monomorphic, were included in the calculation. Monomorphic markers were given zero values to allow the extent of monomorphism to be reflected in the values of per-locus diversity. The diversity was partitioned by (1) treating the host clones as populations and (2) treating the locations as populations. The isolates from *S. daphnoides* were excluded due to small sample size. The calculations were done using Microsoft Excel 97.

An analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was conducted with (1) both LET and LR collections and (2) only LET collections. An Euclidean distance matrix was constructed between all pairs of multilocus phenotypes (a multilocus phenotype refers to isolate(s) showing the same AFLP profiles over all AFLP loci examined). All markers were included in the construction of the distance matrix. AMOVA was performed using ARLEQUIN software provided by L. Excoffier. The variance components and Φ-statistics were tested by non-parametric randomisation tests using 1000 repetitions.

Results

In total, 213 AFLP markers were scored from the six primer combinations. Of those, 208 markers (97.7%) were polymorphic. Five markers were found to be exclusive to all LR isolates, four markers to all LET isolates and nine markers to all LD isolates. The dendrogram placed the five pathotypes into separate clusters (Figure 1). The three LR1 collections formed different subclusters according to collection sites (Figure 1). LET1 isolates from Craibstone, LET4

isolates from Long Ashton and Loughgall were also grouped into separate subclusters according to their sites. The same multilocus phenotypes were found among the five LET1 isolates from *S. viminalis* 'Mullatin' collected at Loughgall, two LET1 isolates from 'Mullatin' at Long Ashton, four LET4 isolates from *S.* × *mollissima* 'Q83' at Craibstone and two LR1 isolates from *S. burjatica* 'Korso' at Long Ashton (Figure 1). Within the pathotypes, average Nei & Li's similarity coefficients were 0.71–0.85 (Table 2). The similarity coefficients were 0.66–0.72 among the three pathotypes within LET and 0.34–0.44 between pathotypes belonging to different *formae speciales*.

Using the Shannon index, the diversity within locations was estimated as 0.55–0.59, greater than that within pathotypes (0.24–0.42) (Table 3). The average per-locus diversity was 0.37 among the pathotypes and 0.11 among the locations. When the data from both LET and LR isolates were examined using AMOVA, the majority of the variation (70.85%) was attributed to among pathotypes within location (Table 4). When only LET types were included, approximately half of the variation was partitioned to among pathotypes within location and half to among the isolates within collection (Table 5). In both AMOVA analyses, Φ-statistics and variance components among locations yielded

Table 2. Summary of Nei & Li's similarity coefficients of *M. larici-epitea* pathotypes*

Pathotype	LET1	LET3	LET4	LR1	LD1
LET1					
Range	0.75–0.99	—	—	—	—
Average	0.78	—	—	—	—
LET3					
Range	0.61–0.75	0.65–0.99	—	—	—
Average	0.69	0.73	—	—	—
LET4					
Range	0.56–0.74	0.65–0.81	0.75–0.99	—	—
Average	0.66	0.72	0.85	—	—
LR1					
Range	0.32–0.51	0.36–0.50	0.36–0.56	0.59–0.99	—
Average	0.43	0.42	0.44	0.71	—
LD1					
Range	0.31–0.42	0.34–0.42	0.37–0.50	0.27–0.40	0.71–0.94
Average	0.36	0.39	0.42	0.34	0.81

*The same phenotypes (showing similarities 1.0) were excluded.

Table 3. Partitioning of average per-locus diversity in LET and LR types

					H'_{pop}	H'_{sp}	H'_{pop}/H'_{sp}	$(H'_{sp} - H'_{pop})/H'_{sp}$
Grouping by pathotype	H'_{LET1}	H'_{LET3}	H'_{LET4}	H'_{LR1}				
	0.40	0.37	0.24	0.42	0.36	0.63	0.51	0.37
Grouping by location	$H'_{Craibstone}$	$H'_{Loughgall}$	$H'_{LongAshton}$					
	0.59	0.55	0.55		0.56	0.63	0.77	0.11

Table 4. AMOVA within and between LET and LR types

Source of variation	d.f.	Variance components	Φ-statistics	Percentage of variation	*P*
Among locations	2	−2.601	−0.082	−8.17	0.863
Among pathotypes within locations	8	22.559	0.627	70.85	<0.00001
Within collections	41	11.881	0.656	37.32	<0.00001

Table 5. AMOVA between pathotypes within LET of *M. larici-epitea*

Source of variation	d.f.	Variance components	Φ-statistics	Percentage of variation	*P*
Among locations	2	−0.879	−0.0372	−3.72	0.684
Among pathotypes within locations	5	12.423	0.489	52.65	<0.00001
Within collections	32	12.050	0.508	51.07	<0.00001

negative values, indicating that the rust isolates collected from different locations shared more resemblance than those collected from the same locations. The variation among the pathotypes within locations and that among the isolates within collections was highly significant ($P < 0.00001$).

Discussion

In this study, the genetic variation in five pathotypes belonging to three *formae speciales*, LET, LR and LD, of *M. larici-epitea* were studied using AFLP. Cluster analysis placed the five pathotypes into five distinct groups. The pathotypes belonging to different *formae speciales* appeared to be more distant compared to those belonging to the same *forma specialis*. Also, several AFLP markers were found to occur exclusively in different *formae speciales* and five markers were found monomorphic among all the isolates studied. An interesting question to be answered in the future is whether these markers are universally fixed in the *formae speciales* or in *M. larici-epitea.*

Between pathotypes belonging to different *formae speciales*, Nei & Li's similarity coefficients were estimated as 0.34–0.44, markedly lower than that between the pathotypes within LET (0.66–0.72). Previously, crossing experiments were carried out with LET, LR and LD to determine the likelihood of hybridisation between pathotypes (Pei et al., 1999c). When the spermagonia derived from LD isolates were paired with those derived from LET isolates, no aecia developed. Also, no aecia developed when LD were paired with LR. Between LET and LR, however, aecia formed when LET performed as the receptor. The F1 hybrids (aeciospores derived from hybridisation and subsequently producing urediniospores are regarded as F1) between LET and LR were only weakly pathogenic to parental hosts and predominantly sterile. When field rust samples collected during 1992–1994 from UK sites were tested for pathogenicity, no recombinants between LET and LR were detected (Pei et al., 1999a). The results from the crossing experiments and the studies of field rust populations suggest that the three *formae speciales* belong to different mating populations. The molecular data from this study provide clear evidence that the three *formae speciales* are genetically distant.

The three pathotypes belonging to LET were more closely related to each other than to LR or to the LD type. LET1 occurs on *S. viminalis,* currently the most important willow species in SRC plantations. Studies on pathotype composition of field rust populations (Pei et al., 1999a) showed that LET1 was predominant on *S. viminalis* clones in UK SRC plantations during 1992–1994. In this study, the similarity coefficients among LET1 isolates collected in 1996 (average similarity 0.78) were remarkably similar to those among the isolates collected across the UK in 1990–1994 (average similarity 0.805) (Pei et al., 2000). This implies that there may have been no major shift in UK LET1 populations between the early and mid 1990s. LET3 occurs on *S.* × *calodendron*, the hybrid between *S. viminalis* and *S. cinerea* in SRC plantations (Pei et al., 1999b). In the wild, LET3 was found on *S. cinerea*, the most common native willow species in the British Isles, and the rust is considered to be a well-established population in the UK (Pei et al., 1999b). From the AFLP data, it appears that there is little differentiation in the rust on *S.* × *calodendron* between different locations. LET4 was first detected in 1992 in UK SRC plantations (Pei et al., 1999a; 2000). When the rust samples collected in 1992 were examined using AFLP (Pei et al., 2000), Loughgall isolates differed greatly from Long Ashton isolates (average Nei & Li's similarity coefficient between two sites 0.36). In this study, the average coefficient in LET4 between the two sites was 0.78. It appears that the degree of differentiation of the rust on *S.* × *mollissima* between Loughgall and Long Ashton had decreased markedly since 1992, most likely due to gene flow between the regions.

The dendrogram placed LR isolates into three subclusters according to their sites. In SRC plantations, LR occurs on *S. burjatica* (*S. dasyclados*), another

important willow species for biomass. Although LR is capable of infecting *S.* × *calodendron* in leaf disc tests in the laboratory, it was not found on *S.* × *calodendron* in SRC plantations (Pei et al., 1999a) and there has been no evidence that LR occurs on *S. cinerea* in the wild. *Salix burjatica* clones are now widely grown in SRC plantations in the UK and, as the alternate host European larch is widespread in the British Isles (Bean, 1973), there may be some pockets of locations where *S. burjatica* and European larch grow together or nearby and primary inoculum (aeciospores) of LR may be spread from such sources. The present results suggest that infections on *S. burjatica* at the three sites in 1996 may have been caused by separate sources of primary inoculum.

The LD isolates collected between 1990 and 1999 appeared to be similar in their genetic background (average similarity coefficient 0.81). The host range of LD is confined to *S. daphnoides* and *S. acutifolia*, both belonging to sec. Daphnella. *Salix daphnoides* is native to central Europe and *S. acutifolia* is widely distributed in Russia (Bean, 1980; Meikle, 1984). In the British Isles, the two willow species are mainly grown as garden plants and are not considered naturalised (Meikle, 1984). In the National Willow Collection at Long Ashton, infections on *S. daphnoides* and *S. acutifolia* were not detected until mid or late summer (Pei & Hunter, unpublished observations). Such evidence suggests that only limited sources of primary inoculum of LD are available in the UK and the infections may be caused as a result of step-by-step long-distance travel of urediniospores, possibly from continental Europe.

In our preliminary studies, we included several other *Melampsora* species on *Salix* such as *M. ribesii-purpurea*, *M. capraearum*, *M. larici-pentandrae*, *M. amygdalinae*, and *M. salicis-albae* for the AFLP profiling. *Melampsora epitea* shared few common bands (less than 10%) with other *Melampsora* spp. and band-scoring became difficult because of the presence of a large number of 'odd' bands. AFLP bands are scored under the assumption that the AFLP fragments of the same size are derived from the same locus. When there is little similarity between samples, it is more likely that some of the common bands may have been derived from different loci in the genome. Therefore, other *Melampsora* species were not included in the present more detailed analyses. Further work involving more and a wider range of samples including related form species would provide opportunities to develop reliable markers for the identification of the *formae speciales* in *M. larici-epitea*. Also, the use of other markers, such as ribosomal DNA, may provide useful information on the evolutionary relationships between the *formae speciales* within *M. larici-epitea* and among the *Melampsora* species occurring on *Salicacea*.

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