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DNA Restriction Fragment Length Polymorphism and Somatic Variation in the Lettuce Downy Mildew Fungus, *Bremia lactucae*

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Twenty-five isolates of *Bremia lactucae*, representing diverse geographic origins and virulence phenotypes, were assayed for restriction fragment length polymorphisms (RFLPs) at 35 nuclear loci. Most isolates from Europe were clearly diploid. In contrast, many of the isolates from Australia, Japan, Wisconsin, and California had more than two alleles at several loci, indicating they were either polyploids or stable heterokaryons. One of these isolates (California pathotype IV) seemed to be a somatic hybrid between two California diploid isolates of the same mating type (pathotypes II and III). This is the first good evidence for natural somatic fusion in the Oomycetes. The RFLP genotypes of other polyploid or heterokaryotic populations indicated that they had also arisen by somatic hybridization. Variation between similar nondiploid isolates seemed to have been due to the somatic loss of alleles. Stable somatic changes in virulence could not be induced in a heterozygous diploid isolate. On the average, the diploid field isolates were heterozygous for 44% of their RFLP loci. In Europe, isolates exhibited great diversity, which was consistent with the frequent occurrence of the sexual cycle.

Additional key words: avirulence gene, genetic distance, heterokaryosis, *Lactuca sativa*, tetraploidy

Two of the major questions concerning the population dynamics of plant pathogens are: how much variation and potential variation is present in the populations, and what are the mechanisms by which changes in virulence occur. Addressing these questions may allow the anticipation of which changes will be most difficult for a pathogen population to achieve and therefore which control strategies may be more durable. Studies on variation in plant pathogens have been limited by the lack of genetic markers. The use of naturally occurring physiological markers such as specific virulence genes, loci controlling mating type, or vegetative incompatibility has provided valuable information on the structure of fungal populations in several pathogens (Stenlid 1985; Ilott *et al.* 1987; Michelmore and Hulbert 1987) but is limited to those for which such characters are available and well characterized. Also, assessments of population structure based on data from specific virulence genes may vary greatly from those based on phenotypically neutral markers such as isozymes (Burdon *et al.* 1983; Burdon and Roelfs 1985; Leung and Williams 1986). Isozyme polymorphisms, however, may be infrequent in many pathogen populations (Burdon *et al.* 1983; Newton 1987). When numerous polymorphisms are required, techniques to detect polymorphisms at the DNA level are more appropriate.

Techniques of molecular biology now provide the opportunity to develop large numbers of phenotypically neutral genetic markers in any organism for which DNA can be extracted (Beckmann and Soller 1983; Botstein *et al.* 1980; Landry and Michelmore 1987; Michelmore and Hulbert 1987). These polymorphisms result from specific differences in DNA sequence that alter the size of the fragments obtained after digestion of genomic DNA with a type II restriction endonuclease. Such differences have been termed restriction fragment length polymorphisms (RFLPs). The individual restriction fragments are identified by homology to cloned DNA fragments. The number of RFLP markers is effectively unlimited as any cloned, low copy number piece of DNA can be used as a probe, and several restriction enzymes can be assayed to identify RFLPs with each probe.

Analysis of specific virulence in populations of *Bremia lactucae*, the fungus causing lettuce downy mildew, has indicated that large differences exist in the structure of pathogen populations in different areas of the world (Crute 1987; Dixon and Wright 1978; Gustafasson *et al.* 1985; Ilott *et al.* 1987; Lebeda 1981; Osara and Crute 1981; Trimboli and Crute 1983; Wellving and Crute 1978). Differences exist in the amount of variation present and in how this variation is partitioned in the populations. The presence or absence of the sexual cycle seems to be correlated with the amount of variation and the ability of the populations to change in virulence (Ilott *et al.* 1987).

RFLPs have been developed as genetic markers in *B. lactucae*. More than 60 such polymorphisms have been identified between three isolates of *B. lactucae*; most of these have been demonstrated to segregate in a Mendelian fashion (Hulbert, unpublished data). This paper describes the use of 35 RFLP loci known to be distributed through the nuclear genome to investigate the worldwide variation of *B. lactucae*.

MATERIALS AND METHODS

Isolates of *B. lactucae*. The 25 isolates used in this study were selected to represent a range of geographic origins and virulence phenotypes from the collection of isolates at the University of California, Davis. The isolates represent a sample (10 isolates) of the phenotypic diversity present in

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Europe and most or all the different phenotypes collected recently from California. Other isolates originated from Wisconsin, Japan, and Australia (imported under USDA license #57-11-85). Techniques for culturing, obtaining single spores, and determining virulence phenotypes of isolates of *B. lactucae* have been described previously (Michelmore and Ingram 1982; Michelmore and Crute 1983; Michelmore *et al.* 1987).

DNA isolation. DNA of *B. lactucae* was extracted by using procedures modified from Timberlake (1978) and Hudspeth *et al.* (1980). Conidia from each isolate were harvested from 2-wk-old seedlings grown in plastic boxes (8 × 12 × 6 in.) by shaking the sporebearing seedlings in distilled water. The spores were pelleted in a bench-top centrifuge and broken by shaking with glass beads. The organelles were then collected and lysed. The DNA purification procedures were performed to allow separation of the nuclear and mitochondrial DNA. The lysate was extracted with phenol/chloroform and the DNA was precipitated with isopropanol. After the DNA was resuspended in 7 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), 9.0 g of CsCl was added and the total weight of the solution was brought to 16.6 g with TE (total volume approximately 10 ml). The DNA solution was loaded into a 13-ml quick-seal ultracentrifuge tube (Beckman) containing 200 μl of bisbenzimide (10 mg/ml). Each tube was made up to volume with a solution of CsCl (0.9 g/ml), sealed and centrifuged at 50,000 rpm, 20° C for 24–30 hr in a fixed angle rotor (70 Ti, Beckman). Bisbenzimide preferentially binds A-T rich sequences, causing the mitochondrial sequences to band as a satellite at a lower density (Hudspeth *et al.* 1980). Two bands could usually be observed in each gradient—an intense lower band containing nuclear DNA and a faint upper band, enriched for mitochondrial sequences. DNA from the two bands was recovered separately using standard procedures (Maniatis *et al.* 1982) and resuspended in TE at a final concentration of 0.25 mg/ml (nuclear DNA) or 0.05 mg/ml (mitochondrial DNA). Analysis of mitochondrial DNA will be described elsewhere.

Identification of RFLP loci. The construction and selection of low copy genomic DNA probes and cDNA probes for the detection of RFLPs will be described elsewhere (Hulbert, unpublished data); segregation of these RFLPs was studied in sexual progeny of two crosses, IMOS6b × SF5 and SF5 × C82P24 (Hulbert, unpublished data). Only those loci that had simple banding patterns and segregated in a Mendelian fashion were used in the present study. The genomic distribution of the loci was determined by analysis of cosegregation of RFLP loci and loci controlling specific virulence and mating type. The loci used in this study were scattered throughout the genome in at least 13 partial linkage groups (Hulbert, unpublished data).

Isolation of plasmid DNA inserts. Plasmids containing DNA of *B. lactucae* were isolated using either a small scale (Rodriguez and Tait 1983) or a large scale (Maniatis *et al.* 1982) alkaline lysis plasmid preparation procedure. Inserts of DNA were separated from vector sequences by digestion with the appropriate restriction enzymes followed by electrophoresis in 1% agarose-TBE gels (0.089 M Tris-borate, 0.089 M boric acid, 0.01 M EDTA, pH 8.0). The insert DNA was purified from the gel by the freeze-squeeze method (Tautz and Renz 1983).

Restriction digests, electrophoresis, blotting, nick-translation and hybridization. Genomic DNA from each isolate of *B. lactucae* was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I according to the manufacturer's recommendations (BRL). Digested nuclear DNA samples (4.0 μg per lane) were electrophoresed in 1.0% agarose TBE gels containing ethidium bromide (1.0 μg/ml) and transferred onto Zetaprobe membrane (Bio-Rad). Prehybridization and hybridization reactions were done in sealed plastic bags at 42° C with 50% deionized formamide (BRL) according to the membrane manufacturer's recommendations for single copy hybridization. Gel isolated DNA inserts from genomic or cDNA clones were labeled with ^{32}P by nick translation (1–15 × 10^7 cpm; Rigby *et al.* 1977) and added to the bags after unincorporated nucleotides had been removed by passing the reaction mixture through Bio-gel P-60 (Bio-Rad). Hybridization reactions were incubated for 48 hr. Membranes were then washed in 2× SSC (0.3 M NaCl, 0.03 M Na citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS) for 15 min followed by 0.1 × SSC, 1% SDS, and 0.1% Na-pyrophosphate at 65° C for 1 hr. The filters were exposed to XAR-5 X-ray film (Kodak) for 12 hr to 4 days at −70° C with intensifier screens to obtain a suitably intense signal. ^{32}P-labeled DNA was removed from the membranes before reprobing by washing in 0.1 × SSC, 0.1% SDS at 90° C for 20 min. Only those probe-enzyme combinations were used for which the banding pattern had been characterized in sexual progeny, as it was only in these digests that alleles could be assigned to bands. Each blot could be probed up to 15 times, allowing the analysis of multiple loci with each blot.

Selection for somatic variants. Certain isolates of *B. lactucae*, C83R19, NL6248, and SF3244D, were incompletely avirulent on the lettuce cultivar Amplus (*Dm2* + *Dm4*) but completely avirulent on the line R4T57 (*Dm4*). All of these isolates were completely virulent on lines with *Dm2*. Genetic studies demonstrated that each of the isolates was heterozygous at the locus for virulence to *Dm4* (*A4*) and that incompatibility on Amplus and R4T57 was determined by the interaction of *A4* and *Dm4* (Ilott *et al.* 1987). Sparse sporulation associated with extensive necrosis was characteristically observed on Amplus 8–11 days after inoculation with these isolates. In an attempt to select for somatic variants with complete virulence on *Dm4*, approximately 10^8 spores of each isolate were sprayed on approximately 1,000 seedlings of Amplus (7 days old) in a plastic box (8 × 12 × 6 in.). After 7–11 days, spores were collected from either all the seedlings that showed any sporulation (when sporulation was sparse) or only from seedlings on which there was profuse sporulation and no visible necrosis. The spores were concentrated to 1–5 × 10^7 spores per milliliter and reinoculated on a lawn of a further 1,000 seedlings of Amplus (7 days old). Two replicate populations from each of isolates C83R19, NL6248, and SF3244D were cultured in the above manner for 10 asexual generations. They were then assayed for their ability to sporulate on both Amplus and R4T57. Therefore, each isolate was selected on approximately 2 × 10^4 seedlings over the period of the experiment. The virulence phenotypes at unselected loci and genotypes at 17 unlinked RFLP loci were determined for the populations with altered virulence to Amplus and R4T57 to confirm their origin and to identify contaminants.

RESULTS

Genetic variation in *B. lactucae*. Genotypes were recorded for each of the 25 isolates at 35 RFLP loci (Fig. 1, Table 1).

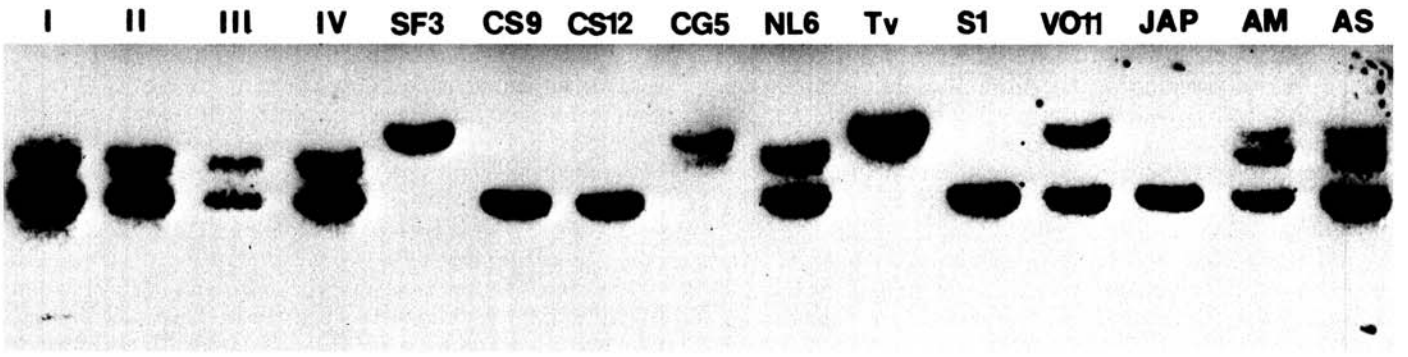

Fig. 1. Autoradiographs of a Southern blot showing restriction fragment length polymorphisms (RFLPs). Each lane contains DNA from a different isolate of *Bremia lactucae* digested with *Bam*HI and probed with the clone G091. Clone G091 was a random 600-bp fragment cloned into the *Bam*H1 site in pUC13 after digestion of genomic DNA with *Sau*3A. The genotype of each isolate is described in Table 1. The designations I, II, III and IV refer to California pathotypes I, II, III, and IV. Each band represents an allele. Three different alleles can be observed in the lanes corresponding to the Australian isolates AM and AS. Dosage effects can be observed in these two lanes; the bottom, or fastest migrating band is approximately twice as intense as the other two, corresponding to two copies of the allele. Differences in the intensity of bands between the lanes is due to the amount of DNA in each lane.

Table 1. The alleles at 35 restriction fragment length polymorphism (RFLP) loci for 19 isolates of *Bremia lactucae*

	Locus[a]																
Isolate	**G021H**	**G026P**	**G054E**	**G064E**	**G070E**	**G075H**	**G085H**	**G091B**	**G107E**	**G112E**	**G136H**	**G138H**	**G287P**	**G443B**	**G503H**	**G514E**	**G521H**	**G538H**
SF5	1, 2	2	1, 2	1, 3	2, 3	2, 3	1, 2	2, 3	2, 3	1	1, 3,	3, 4	3, 4	1, 2	2, 4	2	2	2, 3
SF3	1, 2	1, 2	1, 2	1, 3	3	1	1	1	1, 2	1	1	3	2, 3	1, 2	2, 3	2	2	3
CS9	1, 2	2	1	1, 3	2	n.s.	1, 2	3	2	2	1, 2	3, 4	3	1, 2	2	1, 2	2	3
CS12	1, 2	1, 2	1, 2	1, 3	2, 4	2	2	3	1	2	1	4	1, 3	1, 2	2	1	2	3
CG5	1	0, 1, 2	1, 2	1, 3	2	2	1, 2, 3	1, 2	1, 3	2	1, 2	3, 4	3	1, 2	2	1, 2	2	2, 3
NL6	1	1, 2	1, 2	1, 3	2	1, 2	2	2, 3	1, 3	2	1	3, 4	3	2	2, 3	1, 2	2	2, 3
Tv	1	1, 2	2	3	2	2	2, 3	1	2, 3	2	1	3, 4	3	2	3	1, 2	2	3
S1	1, 2	2	1, 2	1, 3	2	1	1, 2	3	1, 3	1, 2	0, 1	3, 4	3	1	2	1, 2	2	3
VO/11	1	1, 2	1, 2	1, 3	2	1	1, 2	1, 3	1	1	1	3	3	1	2, 4	2	2	3
JAP	1, 2	2	1	1, 3	2	1, 2	2	3	1, 2, 3	2	0, 1, 3	3, 4	2, 3	1, 2	2, 3, 4	1, 2	2	3
AM	1, 2	1, 2	1, 2	1, 3	2	1, 2	2	1, 2, 3	1, 2, 3	2	1, 3	1, 3, 4	2, 3	1, 2	2, 3	1, 2	2	2, 3
AS	1, 2	1, 2	1, 2	3	2	1, 2	2	1, 2, 3	1, 2, 3	2	1, 3	3, 4	2, 3	1, 2	2, 3	1, 2	2	2, 3
WIS	1, 2	2	1, 2	1, 3	2	1, 2	1, 2	2	1, 3	2	1, 2	3, 4	2, 3	1, 2	2, 3	2	1, 2	3
IMOS6B	1	1, 2	1, 2	1, 3	2	1	2, 3	2	1	2	1, 2	1, 4	2	1	2	1, 2	2	2, 3
C83M40	1, 2	2	1, 2	3	2, 3	1, 2	2, 3	2, 3	1, 3	2	1, 3	2, 3, 4	2, 3	1, 2	1, 2, 3	1, 2	2	2, 3
C83M42	1, 2	1, 2	1, 2	3	2, 3	1, 2	2, 3	2, 3	1, 2, 3	2	1, 2, 3	2, 3, 4	1, 2, 3	1, 2	1, 2, 3	1, 2	2	2, 3
Path. II[b]	1,2	2	1	3	2	1	2	2, 3	2	2	1	3, 4	1, 2	1	2	1, 2	1, 2	3
Path. III	1, 2	2	1	3	2, 3	1	2	2, 3	1, 3	2	1, 2	2, 3	2, 3	1	2	1	2	2, 3
Path. IV	1, 2	2	1	3	2, 3	1	2	2, 3	1, 2, 3	2	1, 2	2, 3, 4	1, 2, 3	1	2	1, 2	1, 2	2, 3
Total[c]	2	3	2	2	3	3	3	3	3	2	4	4	4	2	4	2	2	2

	G546E	**G551P**	**G555E**	**G602B**	**G610B**	**G614P**	**G642E**	**G644H**	**G723B**	**G727E**	**G813H**	**CB12B**	**CE06E**	**CE18P**	**CE40P**	**CE61B**	**G128H**
SF5	1, 2	1, 2	3	1, 2	1, 4	2	1, 3	2, 3	1	1, 2	2	1, 2	2, 3	1, 2	1	1, 2	1, 2
SF3	1, 2	1	1, 4	1, 2	4	2	1, 2	3	1	3	1, 2	1	3	2	1	1, 2	1
CS9	1, 2	1, 2	2, 5	2	4	2, 3	2, 3	3	1, 2	2, 3	2, 3	1	2, 3	2	2	3	1
CS12	2	1, 2	3, 4	1, 2	4	2	2	3	1	3	1, 3	1	3	2	1	1	1
CG5	1, 2	2, 3	2, 3	1	1, 4	2	1, 3	2, 3	1, 3	3	2, 3	1	3	1, 2	1	2, 3	1, 2
NL6	1, 2	1	3	1	1, 4	2, 3	2	2, 3	1, 3	1, 3	2	1	3	1, 2	1	2	1
Tv	1, 2	1, 3	2, 4	1	2, 4	2	2	3	1, 3	3	2	1	3	2	1	1	1, 2
S1	1, 2	1, 3	1, 4	2	1, 4	2	2, 3	3	1, 3	1, 3	2	1	3	2	1	3	1
VO/11	1	1, 3	3, 4	1, 2	4	2	2	3	1	2, 3	2	1	3	1, 2	1, 2	1, 3	1
JAP	1	2	2, 3, 4	2	2	1, 2	1	3	1, 3	2	1, 2, 3	1, 2	2, 3	1	1, 2	2, 3	1
AM	1, 2	1, 2	3, 4	1, 2	1, 4	2, 3	2, 3	2, 3	1	2, 3	2, 3	1	1, 2, 3	2	1, 2	1, 3	1, 2
AS	1, 2	1, 2	3, 4	1, 2	1, 4	2	2, 3	2, 3	1	2, 3	2, 3	1	1, 3	2	1, 2	1, 3	1
WIS	1, 2	1, 2, 3	4	1, 2	4	1, 2, 3	1, 2, 3	3	1, 3	1, 2, 3	1, 2, 3	1, 2	1, 2	1, 2	1	2, 3	1, 2
IMOS6B	1	3	1, 2	1	1	2, 3	2	3	1	3	1, 2	1	3	1, 2	1, 2	1, 2	1
C83M40	1, 2	1, 2	3	1, 2	1, 4	2, 3	2, 3	2, 3	1, 3	1, 2, 3	1, 2, 3	1	1, 3	1, 2	1	2, 3	1
C83M42	1, 2	1, 2, 3	3	1, 2	1, 4	2, 3	2, 3	2, 3	1, 3	1, 2, 3	1, 2, 3	1	1, 3	1, 2	1	1, 3	1
Path. II	1	2, 3	3	2	4	2, 3	1, 2	2, 3	3	2, 3	2, 3	1	1, 3	2	1	3	1, 2
Path. III	1	2, 3	3	1, 2	4	3	1, 2	2, 3	3	2	3	1	3	2	1	1, 3	1
Path. IV	1	2, 3	3	1, 2	4	2, 3	1, 2	2, 3	3	2, 3	2, 3	1	1, 3	2	1	1, 3	1, 2
Total	2	3	5	2	3	3	3	2	3	3	3	2	3	2	2	3	2

[a] The first letter of each locus designation refers to whether the probe was a cloned genomic fragment (G) or a cDNA (C). The last letter B, E, H, or P, refers to the restriction enzyme, *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst*I, respectively, which was used to cut the genomic DNA to observe the RFLP. For the RFLP loci in which the phenotype for each allele consists of a single band, the allelic designations with the smallest numbers (i.e. 0 or 1) correspond to the bands (alleles) with the lowest mobility. n.s. = not scoreable.

[b] Pathotypes II, II, and IV refer to groups of California isolates with identical phenotypes as described in the text and in Table 3.

[c] The total number of alleles that was observed at each locus when the isolates listed were screened with each probe-enzyme combination.

Genotypes at 13 avirulence loci had already been characterized for most of the isolates. The banding patterns of SF5, C82P24, and IMOS6B were compared to those of the other isolates to identify the different alleles. The assignment of genotypes from the banding phenotypes was, in most cases, uncomplicated; difficulties in interpretation were, however, encountered when probes identified more than one locus and novel alleles were present that had not been present in the previous segregation analyses. Probes detecting multiple loci were therefore avoided as the assignment of alleles to individual loci was impossible. New alleles, which had not been previously observed in IMOS6B, SF5, or C82P24, were observed at only nine of the 35 loci analyzed; two of these loci had two new alleles. The number of alleles at any one locus varied from two to five and averaged 2.7 for all of the loci.

Only five different genotypes were observed among the 11 California isolates tested. A previous study (Ilott *et al.* 1987) used specific virulence and mating type as markers to group 116 California isolates, collected over several years and growing areas, into three pathotypes (groups of isolates with identical or nearly identical virulence phenotypes). Most of the 11 isolates chosen for the present study fitted into one of these pathotypes (Table 2). Isolates were selected to represent potential variants within each pathotype. The two pathotype I isolates differed in virulence to *Dm15*; this difference was paralleled by differences in the alleles present at 6 of the 35 RFLP loci. The three pathotype II isolates were genetically identical at all of the 35 RFLP loci tested. Previous work suggested there may be quantitative differences in reactions to cultivars with *Dm4* and *Dm11* between the pathotype II isolates selected (Hulbert and Michelmore 1985; Ilott *et al.* 1987). No evidence of any such differences were apparent, however, when the three pathotype II isolates were examined simultaneously. Therefore, these pathotype II isolates were a homogeneous group. Two distinct virulence phenotypes were observed in the isolates previously classified as California pathotype III depending on their reaction to cultivars with the resistance genes *Dm4* and *Dm16*. The RFLP genotypes were determined for three isolates of each phenotype. Three of the isolates (C83M47, C86R3, and C86R9) were virulent on *Dm4* and *Dm16* and were retained in pathotype III. The other three isolates (C86R6, C84M4, and C86S1) were avirulent on both *Dm4* and *Dm16* and are now classified as pathotype IV. No genetic variation was detected within the three pathotype III or pathotype IV isolates at any of the 35 RFLP loci. The complete absence of any intragroup variation in pathotypes II, III, and IV supports the conclusion of Ilott *et al.* (1987) that there is a clonal origin for the isolates within each of the pathotypes.

The genetic relationships among the non-California isolates were not evident, as none of these isolates appeared phenotypically or genetically similar. To assess their genetic relationships, genetic distances were calculated from the RFLP data by the method of Nei (1975). Each isolate was considered a separate population, except for the three isolates in each of the California pathotypes II, III, and IV, which were considered as single populations. The two hybrid isolates, IMOS6B and VO/11, were not included in the analysis as they are not field isolates. A dendrogram was constructed from the pairwise genetic distances to help visualize the relationships (Fig. 2).

The two Australian isolates, AM and AS, appeared to be closely related in the genetic distance analysis. The two isolates were identical in genotype at 30 of the 35 RFLP loci. The two isolates differed in virulence to only one (*Dm1*) of the 13 *Dm* resistance genes. Because little other variation was detected in virulence phenotype in a recent survey of Australian isolates (Trimboli and Crute 1983), variation in *B. lactucae* is probably limited in Australia. Furthermore, the two Australian isolates were heterozygous at 23 of the 30 loci for which they were identical. The sexual cycle is, therefore, not responsible for generating the differences between these two isolates. It is not apparent from the two genotypes whether one of the isolates was derived asexually from the other or if both of the isolates were derived from a common progenitor.

The two California pathotype I isolates also clustered together in the genetic distance analysis. They had identical alleles at 29 of 35 RFLP loci and differed in their reaction to only one *Dm* resistance gene. As with the two Australian isolates, the differences between the two pathotype I isolates are most likely asexual in origin; they are heterozygous for 22 of the 29 loci for which they had identical alleles. Isolates C83M40 and C83M42 were, therefore, designated pathotype IA and IB respectively. Each California isolate had alleles not present in the other; therefore, they were both derived from a common progenitor rather than one being derived asexually from the other.

California pathotypes II, III, and IV appear related in the genetic distance analysis. The origin of pathotype IV is described in the next section. Pathotypes II and III are genotypically identical at 20 of the 35 RFLP loci. There is, however, no evidence of an asexual origin to the differences between pathotypes II and III; only five of the 20 loci for which the two isolates are identical are heterozygous. Two such genotypes could readily have resulted from a sexual population with limited variation.

Much diversity was detected in the European isolates. None of the European isolates seemed closely related to the California or Australian isolates nor did they seem closely related to each other. This is true even of isolates collected from the same country such as SF3 and SF5 (from Finland) or CS9 and CS12 (from Czechoslovakia). The greater diversity of the European isolates is also reflected in the diversity of different alleles. Only five alleles were found in non-European isolates that were not detected in at least one of the European isolates examined. Rare avirulence alleles conferring avirulence to *Dm12* and *Dm13* are found only in

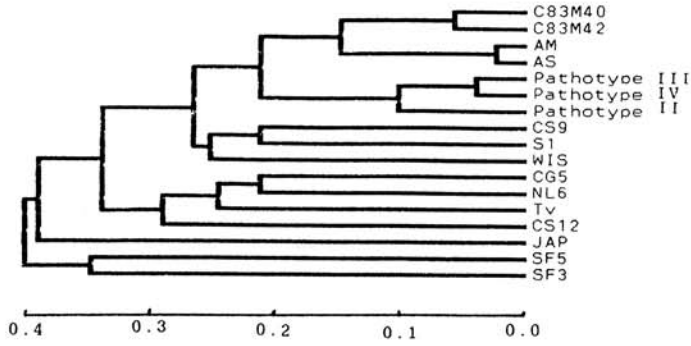

Fig. 2. Dendrogram showing the estimated genetic relationships among isolates of *Bremia lactucae*. Nei's "D" (1975) was used to calculate the 136 pairwise genetic distances between the 17 populations. The dendrogram was constructed from those distances by the unweighted group pair method (Sneath and Sokal 1973). K. Ritland (University of Toronto) provided the computer programs for these calculations. The scale shows the genetic distance; this can range from 0 (populations are identical) to infinity (all alleles at all loci are different between the two populations). Isolates AM and AS are therefore the most similar, and JAP is the isolate that is the most different from the other isolates tested.

certain European isolates and avirulence to *Dm14* is found only in certain European and Australian isolates. This extensive diversity in Europe parallels the extensive diversity in the host, *Lactuca* spp. and related genera.

Hyperploidy (tetraploidy or heterokaryosis?). Most of the individual isolates had only one or two alleles at each locus, as would be expected for a diploid species. The presence of three alleles was detected in individual isolates at some of RFLP loci; furthermore, some isolates repeatedly had three alleles at unlinked loci, indicating that these isolates carried more than two copies of these loci. Three distinct alleles were observed at eight loci in isolate C83M42, at five different loci in JAP and WIS, at four different loci in AM and C83M40, two loci in AS and CG5, and at three loci in the California pathotype IV isolates, C84M4, C86R6, and C86S1 (Fig. 3). Also, differences in gene dosage could often be observed in these isolates; at loci where only two distinct alleles were present, the band for one allele was often approximately three times as intense as the other. When three alleles were present, the band for one allele was often twice as intense as the other two, again indicating four copies of the gene were present (Figs. 1 and 3). To confirm that the isolates exhibiting multiple bands were not mixtures, cultures of these isolates were derived from single conidia. DNA from the single spore derivatives were reprobed with several of the probes that had previously detected multiple alleles. In every case, the banding patterns appeared identical to that of the previous hybridizations, indicating that these isolates were not mixtures. It is not yet known whether these isolates are tetraploids or heterokaryons; therefore they are referred to here as 'hyperploids,' although we have no evidence that the individual nuclei in these isolates have more chromosomes than diploid isolates. As conidia of *B. lactucae* are multinucleate (Sargent *et al.* 1973), the possibility of heterokaryosis cannot be ruled out simply because the isolates were derived from single conidia.

The RFLP genotype of pathotype IV was composed of the combined alleles of pathotypes II and III at all of the 35 loci examined (Fig. 3). Pathotype IV therefore seemed to be a somatic hybrid between pathotypes II and III. The virulence phenotypes of the three isolates supported this hypothesis (Table 2). At all loci where both pathotypes II and III were virulent, pathotype IV was virulent, and where both II and III were avirulent, so was pathotype IV. When pathotype II or III was avirulent while the other was virulent, as was the case at loci *A4*, *A10*, and *A16*, pathotype IV was always avirulent. This would be expected since avirulence is dominant at these loci.

Heterozygosity of isolates of *B. lactucae*. The field isolates of *B. lactucae* were highly heterozygous. Heterozygosity in the diploid isolates ranged from 74% of the loci in SF5 to 31% in Tv (Table 3). On average, isolates were heterozygous

Table 2. Virulence phenotypes of California pathotypes

	Reaction to *Dm* resistance gene[a]													
Pathotype	**1**	**2**	**3**	**4**	**5/8**	**6**	**7**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
IA	+	−	+	−	−	+	+	+	−	+	+	+	−	−
IB	+	−	+	−	−	+	+	+	−	+	+	+	+	−
II	−	+	+	(−)	+	+	+	+	(−)	+	+	+	−	−
III	−	+	+	+	+	+	+	−	−	+	+	+	−	+
IV	−	+	+	(−)	+	+	+	−	−	+	+	+	−	−

[a] + = compatible interaction, − = incompatible interaction, (−) = incompatible interaction associated with necrosis and sparse sporulation (Iliott *et al.* 1987). Pathotypes IA and IB are differentiated by their reaction to lettuce lines with *Dm15*.

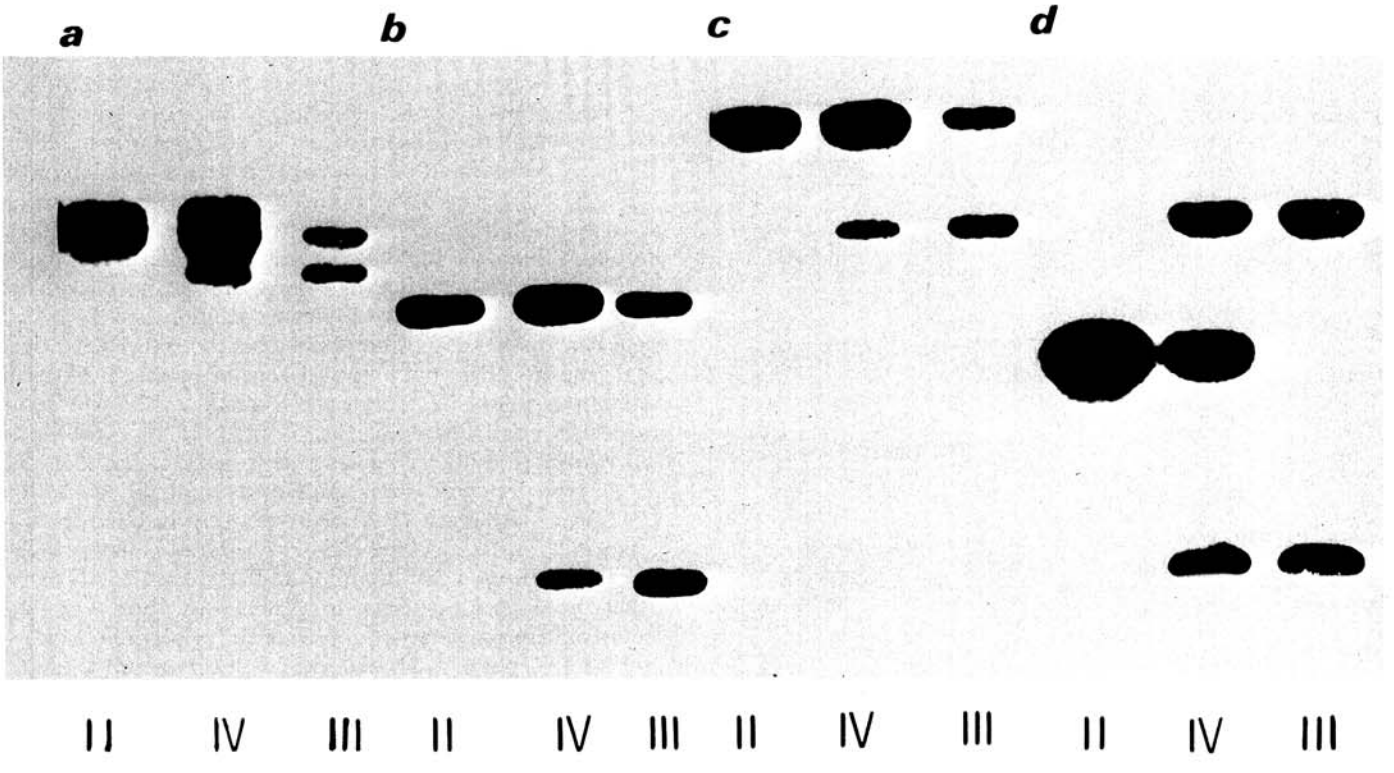

Fig. 3. Autoradiographs of four Southern blots (a–d), each hybridized with a different probe to observe a different locus: **a,** G287P; **b,** G136H; **c,** G070E; and **d,** G107E. In each case, the genotype of pathotype IV is the combined alleles of pathotypes II and III. Dosage effects can be seen in the lanes corresponding to pathotype IV. Differences in intensity of bands between the lanes is due to differences in the amount of genomic DNA that was digested and blotted.

for 44% of their RFLP loci. This was higher than either of the two hybrid isolates (IMOS6B and VO/11) included in the analysis. This was also greater than the heterozygosity at the avirulence loci. The average heterozygosity at the virulence loci in the nine diploid field isolates was 17%. The isolates analyzed, however, were not a random sample of field isolates and were maintained in the university's isolate collection for their virulence characteristics. Several of the isolates used in this study contain avirulence alleles at fewer loci than most field isolates; isolates CS9, Tv, and S1 are all homozygous recessive (virulent) at 12 of the 13 virulence loci. In the 'hyperploid' isolates, an even higher percentage of their RFLP loci were multiallelic (Table 3). On the average, the hyperploid isolates had more than one allele present at 69% of the RFLP loci.

Selection for somatic variation in a diploid isolate. After 10 generations of serial culture of each of three isolates that were heterozygous for the avirulence locus under selection (*A4*) on the incompletely resistant cultivar Amplus, four of the six populations continued to sporulate sparsely and to induce extensive host necrosis. There was, therefore, no evidence of any qualitative change in virulence. Two of the six populations, one from C83R19 and one from NL6248, did sporulate profusely on Amplus and on R4T57, which was completely resistant to the original isolates. The population from C83R19, however, also differed from the original isolate at another virulence locus (*A1*) in addition to *A4* and at seven of 17 RFLP loci tested; it was therefore assumed to be a contaminant. The population derived from NL6248 did not differ from the original isolate at any other virulence locus or at any of the 17 unlinked RFLP loci tested. No more than two alleles were detected at any one locus, and as the original isolate was a F_1 progeny from diploid parents, it was assumed to be diploid. This population, designated NL6248+4, was completely virulent on R4T57 and Amplus as it sporulated profusely on both lines for three asexual generations of culture and induced no host necrosis. This change in phenotype, however, was not stable. After NL6248+4 was cultured on cv. Cobham Green (which lacks any known *Dm* gene) for two asexual generations, the isolate did not sporulate on R4T57 and caused the original necrotic phenotype with sparse sporulation on Amplus.

Table 3. Origins and characteristics of the isolates of *Bremia lactucae*

Isolate	Origin	Supplier[a]	% Heterozygosity		Ploidy[d]
			Vir. loci[b]	RFLPs[c]	
SF5	Finland	IRC	33	74[e]	d
SF3	Finland	IRC	25	40	d
CS9	Czechoslovakia	AL via IRC	0	46	d
CS12	Czechoslovakia	AL via IRC	36	34	d
NL6	Holland	IRC	15	49	d
Tv	England	DSI via IRC	8	31	d
S1	Sweden	IRC	0	46	d
VO/11	Finland × Holland × Finland	IRC		37	d
IMOS6B	England × Switzerland	A	38	40[e]	d
CG5	Switzerland	IRC		(63)	h
JAP	Japan	JO		(51)	h
AM	Australia	DT		(80)	h
AS	Australia	DT		(71)	h
WIS	Wisconsin	LS		(74)	h
C83M40	California (Pathotype IA)	A		(77)	h
C83M42	California (Pathotype IB)	A		(80)	h
C83P24, C83M4, C83R19	California (Pathotype II)	A	31	40[e]	d
C83M47, C86R3, C86R9	California (Pathotype III)	A	8	37	d
C84M4, C86R6, C86S1	California (Pathotype IV)	A		(57)	h

[a] A = Authors, AL = A. Lebeda, DSI = D. S. Ingram, DT = D. Trimboli, IRC = I. R. Crute, JO = J. Obara, LS = L. Sequeira.

[b] Data on percent heterozygosity of virulence loci is only listed if the genotype was known for at least 11 of the 13 avirulence loci, A1–A7, A10, A12–A16. Data from Michelmore *et al.* (1984), Norwood and Crute (1984), and Ilott *et al.* (1987).

[c] Figures for percent heterozygosity of hyperploid isolates are the frequency that loci have more than one allele.

[d] Isolates are diploid (d) or hyperploid (h = heterokaryotic or polyploid).

[e] These three isolates were used to identify the restriction fragment length polymorphisms (Hulbert and Michelmore, unpublished data).

DISCUSSION

The present study detected considerable genetic variation in the populations of *B. lactucae* from Europe and California. The most variation was detected in the European isolates. This was as expected since the Mediterranean area is the center of diversity of the host genus (*Lactuca*) and therefore probably the center of diversity of *B. lactucae*. The genotypic diversity displayed by the sample of European isolates tested is consistent with the sexual cycle playing a major role in maintaining this diversity. Other populations, such as those in California, retain considerable variation, but it is partitioned into a small number of highly heterozygous individuals with no intermediate genotypes. This indicates that there is little or no sexual reproduction occurring, supporting the conclusions of Ilott *et al.* (1987).

The occurrence of isolates that had more than two alleles at individual loci, indicating polyploidy or heterokaryosis and somatic hybridization, was unexpected since these phenomena have not been reported in the Peronosporaceae. Few genetic markers have been available for other mildew species, however, to test their occurrence. The importance of such phenomena in the Peronosporales is unknown. Cytological evidence for tetraploidy has been reported in *Phytophthora* (Hansen *et al.* 1986; Sansome 1977). Heterokaryons have been recovered at low frequency in *Phytophthora megasperma* (Long and Keen 1977) using drug resistant and auxotrophic strains. There has been no definitive evidence for the occurrence of a natural somatic hybrid. Somatic variation in *P. infestans* has been reported when pairs of isolates with different specificities were grown together on a susceptible host (Leach and Rich 1969; Malcolmson 1970), but in the absence of genetic markers, it was impossible to determine the mechanisms by which the variation was generated. These phenomena should be reinvestigated using RFLPs as genetic markers. The data for *B. lactucae* suggest somatic hybrids may be common. California pathotype IV almost certainly resulted from a somatic hybridization between two diploid isolates of the same mating type (pathotypes II and III). The origins of the other hyperploid isolates are unknown; however, the frequent heterozygosity (Table 3) of the hyperploid isolates and the presence of more than two alleles at several unlinked loci suggests that they were formed by the fusion of two dissimilar isolates rather than by somatic doubling of a single isolate followed by somatic variation. Hyperploid isolates have not been detected in progeny from sexual crosses between diploid isolates (Hulbert, unpublished data).

The global distribution of diploid and hyperploid isolates invokes two questions: Why were hyperploid isolates rare in the sexual European population and why were hyperploid isolates predominant in the populations elsewhere? The rarity of hyperploid isolates in Europe may be due to their lack of maintenance in populations where the sexual cycle is common or necessary to complete the life cycle. This would be likely if most of the progeny from diploid × hyperploid crosses are diploid or if the viability or fertility of such crosses are reduced relative to that of diploid × diploid crosses. In asexual populations, however, hyperploid isolates may have increased longevity or fitness, possibly due to increased genetic plasticity. Extensive genetic variation may be maintained in a single hyperploid isolate; isolate C83M42 averaged more than two alleles at each of the 35 RFLP loci assayed. Hyperploid isolates maintained multiple alleles through many asexual generations in the laboratory and two cycles of culture through single conidia. The mechanism by which this variation was maintained is not known. Tetraploid nuclei might be stable. No mechanisms for maintaining two dissimilar nuclei are known in Oomycete fungi; heterokaryosis, however, could be maintained by the presence of balanced lethals in the two diploid nuclei. Experiments have been initiated to determine whether the hyperploid isolates are heterokaryons or polyploids and whether recessive lethals are common.

Because segregation ratios from crosses involving hyperploid isolates are different from those expected from matings between diploids, interpretations of the segregation of virulence must be made with caution unless the ploidy of the parental isolates is known. Segregation data have been observed that do not fit the Mendelian ratios expected for diploid crosses in a few small families unknowingly generated from crosses between diploid and hyperploid isolates (Ilott *et al.* 1987). Most data on the segregation of avirulence loci, however, have been derived from crosses between diploid European isolates; therefore hyperploidy has probably not complicated published genetic studies on *B. lactucae*.

The genetic variation of the California population of *B. lactucae* was probably well represented by the isolates chosen as they included most or all of the different phenotypes recorded in recent years (Ilott *et al.* 1987). The three isolates within each of pathotypes II, III, and IV were all genetically identical and probably clonal in origin. In contrast, the variation in virulence phenotype (reaction on *Dm15*) between the two pathotype I isolates was paralleled by genetic variation at 6 RFLP loci. Isolates similar to pathotype I may have been present in California since the 1930s, and further variation may exist. Variation in reaction to *Dm3* within pathotype I has been reported but could not be confirmed as the isolates recorded as avirulent to *Dm3* were lost (Ilott *et al.* 1987). The one isolate from Wisconsin was very different from any of the pathotypes in California. Analysis of isolates from other growing regions in the United States may provide insight to the origins of pathotypes II and III.

The differences between the two California pathotype I isolates and between the two Australian isolates is likely to be asexual in origin; there is no evidence of any sexual recombination in either population. The two Australian isolates were heterozygous but had identical alleles at 23 RFLP loci and the two California pathotype I isolates were heterozygous but identical for 22 loci, four of which had more than two alleles. The probability of obtaining identical but multiallelic genotypes at this many unlinked loci from a sexual cross is negligible. The differences between the pairs of isolates could usually be explained by the loss of an allele in the isolate. Novel alleles would be expected if these differences arose by mutation, but these isolates rarely carried alleles not present in other isolates. The differences between the two Australian isolates or the two California pathotype I isolates were not confined to one or two genomic locations but mapped to several linkage groups (Hulbert, unpublished data) indicating several independent events.

Knowledge of the mechanisms by which variation in asexual populations arises may allow the prediction of which changes in virulence are unlikely to occur. Specific resistance genes may provide effective resistance for extended periods in areas where the variability of the pathogen population is low. The resistance gene *Dm5/8* remained effective against *B. lactucae* in California for approximately 13 yr until pathotype II appeared. This durability was apparently due to the inability of pathotype I isolates to change to virulence at *A5/8*. Genetic analysis of limited numbers of progeny from crosses with several pathotype I isolates indicated that they were heterozygous at *A5/8* (Ilott *et al.* 1987); as these isolates have been shown to be hyperploid, the segregation ratios suggested that the allele for avirulence was probably present in more than one copy. Thus multiple changes would have been required for pathotype I isolates to overcome *Dm5/8*.

The experiments to select for somatic variants under laboratory conditions failed to detect any stable changes in virulence phenotype. Genetic events such as spontaneous mutations at *A4* or mitotic recombination therefore seem to be rare. Mitotic recombination and the generation of new genotypes have been well characterized in transient diploids formed by fusion of haploid nuclei in several ascomycete fungi (Tinline and MacNeill 1969). Such diploids, however, would not be expected to carry recessive lethal alleles as they were formed from free-living, haploid isolates. In contrast, the generation of new virulence phenotypes in naturally diploid, Oomycete fungi by mitotic crossing-over may be greatly impaired by deleterious recessive alleles. Mitotic crossing-over results in homozygosity of all loci on the chromosome arm distal to the cross-over point and would therefore be rarely detected if recessive lethals are common, particularly if the selected locus is close to the centromere. If there was a deleterious recessive allele distal and in *cis* with a recessive virulence allele, somatic recombination would be unlikely to generate virulent variants from heterozygous avirulent isolates. Three different isolates of *B. lactucae* and therefore chromosome arms carrying the recessive allele at *A4* were used in the attempts to select for somatic variants. The altered phenotype of NL6248+4 may have been epigenetic or due to a true genetic change that was unstable. If mitotic crossing-over had resulted in recombinant nuclei homozygous for *a4* and a deleterious recessive allele, the wild type nuclei would not have been completely lost from the coenocytic mycelium as they would have been required to complement the recombinant nuclei. The recombinant genotype would have been maintained during the interaction with a host carrying *Dm4*; however, when cultured on a host without *Dm4*, the recombinant nuclei would tend to have been lost. Characterization of such events requires heterozygous RFLP markers distal to the virulence gene on the chromosome arm. Despite working under aseptic conditions, one contaminant was detected; RFLP analysis, however, clearly demonstrated its extraneous origin and also confirmed the origin of the true

asexual variant. This emphasized the critical importance of neutral genetic markers in experiments that involve the selection of rare events.

In tetraploids or stable heterokaryons, a single mitotic crossing-over event would be less likely to result in a completely homozygous genotype at a lethal locus but could still eliminate the only copy of a dominant allele for avirulence. Improper disjunction during mitotic divisions would also allow whole chromosomes to be lost or replaced. Such mechanisms could be responsible for the variation within the asexual populations in Australia and California.

RFLP analysis is the most efficient method for applications that require large numbers of genetic markers, such as the development of genetic maps, or when polymorphisms are desired in populations with little variation. Their codominance and the occurrence of multiple alleles also make RFLP markers powerful tools for studying populations of plant pathogens. Much of the power of the RFLP analysis described in this study, however, resulted from knowing the genetic basis of the RFLP banding patterns. If the segregation of the RFLPs had not been previously characterized, it would have been impossible to estimate heterozygosity or to identify isolates with more than two alleles at a locus; RFLP genotypes are not always predictable from their banding patterns as one or more bands may correspond to a single allele. When well characterized, RFLPs can provide unlimited genetic markers and will lead to a detailed understanding of pathogen populations.

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