Genetic Analysis of the Fungus, *Bremia lactucae*, Using Restriction Fragment Length Polymorphisms

Scot H. Hulbert,^{*1} Terrence W. Ilott,^{*2} Erik J. Legg,^{*} Stephen E. Lincoln,[†] Eric S. Lander,[†] and Richard W. Michelmore^{*}

^{*}Department of Vegetable Crops, University of California, Davis, California 95616, and [†]Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142

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ABSTRACT

Restriction fragment length polymorphisms (RFLPs) were developed as genetic markers for *Bremia lactucae*, the biotrophic Oomycete fungus which causes lettuce downy mildew. By using 55 genomic and cDNA probes, a total of 61 RFLP loci were identified among three heterothallic isolates of *B. lactucae*. Of these 61 RFLP loci, 53 were heterozygous in at least one of the three strains and thus were informative for linkage analysis in at least one of two F₁ crosses that were performed. Analysis of the cosegregation of these 53 RFLPs, eight avirulence loci and the mating type locus allowed the construction of a preliminary genetic linkage map consisting of 13 small linkage groups. Based on the extent of linkage detected among probes, the genome of *B. lactucae* can be estimated to be approximately 2000 cM. Linkage was detected between a RFLP locus and an avirulence gene, providing a potential starting point for chromosome walking to clone an avirulence gene. The high frequency of DNA polymorphism in naturally occurring isolates and the proper Mendelian segregation of loci detected by low copy number probes indicates that it will be possible to construct a detailed genetic map of *B. lactucae* using RFLPs as markers. The method of analysis employed here should be applicable to many other outbreeding, heterozygous species for which defined inbred lines are not available.

GENETIC markers have numerous applications in studies of plant pathogenic fungi (MICHELMORE and HULBERT 1987). They allow identification of laboratory strains as well as field isolates for epidemiological studies (STENLID 1985; ILOTT, DURGAN and MICHELMORE 1987). Genetic markers can be used to dissect the life cycle of different species (OLD, MORGAN and BELL 1984; BURDON and ROELFS 1985) and to study the mechanisms by which variation in virulence is generated and maintained in pathogen populations (FLOR 1964; ERSELIUS and SHAW 1982; HULBERT and MICHELMORE 1988). They may also be used to elucidate evolutionary relationships among different isolates or taxa (HANSEN *et al.* 1986). If sufficient numbers of markers can be identified, they can be used to develop detailed genetic maps which are powerful tools for studying variation and cloning genes of interest, such as avirulence (*Avr*) genes.

Most phytopathogenic fungi lack well defined genetic markers. Morphological markers are generally rare. Specific virulences have not proved useful as genetic markers except in a few biotrophic fungi when the interactions with their host were genetically well characterized (SAMBORSKI and DYCK 1976; LAW-

RENCE, MAYO and SHEPHERD 1981; CRUTE 1986). Drug resistance markers and genes which complement auxotrophic mutations are generally absent in natural populations; such markers would also be difficult or impossible to use in biotrophic fungi.

Restriction fragment length polymorphisms (RFLPs) can potentially provide a large number of genetic markers (BOTSTEIN *et al.* 1980; MICHELMORE and HULBERT 1987). Such molecular markers have several advantages over classical genetic markers for many applications, including the development of genetic maps: (1) hundreds of markers can be analyzed in a single cross, since most RFLPs seem to be neutral variations, without deleterious or strong epistatic effects; (2) genotypes can often be determined from any tissue, since only a DNA sample is required; (3) numerous alleles may often be found, at least for some RFLP loci; and, (4) RFLPs are generally codominant, allowing the identification of all possible genotypes. Codominant markers provide maximum linkage information from segregation data; this is particularly important when progeny sizes are small. Although isozyme polymorphisms share some of these advantages, they are less well suited for general genetic mapping: since each requires a unique assay, identifying new isozyme polymorphisms is not routine; and, they appear to be infrequent in at least several path-

¹ Present address: Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907.

² Present address: Plant Breeding Institute, Cambridge, England.

ogen populations (BURDON, LUIG and MARSHAL 1983; NEWTON 1987). RFLPs are providing large numbers of genetic markers in an increasing number of species (reviewed in LANDRY and MICHELMORE 1987).

This paper describes the characterization of RFLPs in *Bremia lactucae*, an obligate biotrophic fungus which causes lettuce downy mildew. *B. lactucae* is an Oomycete fungus with a diploid vegetative phase. The Oomycotina contains many important plant pathogens such as *Phytophthora* spp., *Pythium* spp. and the downy mildews. Lettuce downy mildew is genetically the best characterized of these diseases. Specificity between host and pathogen is determined by a gene-for-gene interaction (FLOR 1956) with at least 13 matching gene pairs (NORWOOD *et al.* 1983; MICHELMORE *et al.* 1984; NORWOOD and CRUTE 1984; HULBERT and MICHELMORE 1985; FARRARA, ILOTT and MICHELMORE 1988).

The development of RFLPs as genetic markers in any organism involves three phases: (1) development of probes; generally either random low copy number genomic fragments, cDNA clones or characterized sequences such as heterologous genes are used; (2) identification of RFLPs among parental lines; and (3) analysis of segregation of RFLP loci to construct genetic linkage maps. RFLP projects are currently underway in several higher plants for which inbred lines are available (BERNATZKY and TANKSLEY 1986; HELENTJARIS *et al.* 1986; LANDRY *et al.* 1987). However, a fungus such as *B. lactucae* posed a number of additional problems, which may be typical of RFLP mapping projects in several species (see, *e.g.*, SHAW 1983; SHATTOCK *et al.* 1987): (1) The species is outbreeding and highly heterozygous. Since inbred strains are not available, mapping must be carried out in crosses between individuals taken from natural populations, in which the parental phase of marker alleles is initially unknown. In this respect, the project is similar to the recent construction of an RFLP linkage map for the human genome (DONIS-KELLER *et al.* 1987). (2) The genetic length of the *B. lactucae* genome was unknown, as was the number of chromosomes. (3) Only a small percentage (often less than 0.1%) of sexual spores germinate and those that do must subsequently establish themselves on the host before the isolate can be characterized; therefore, differential viability among the various segregating genotypes could potentially give rise to pseudolinkage between unlinked chromosome segments carrying deleterious alleles. (4) Some isolates display difference in ploidy level or aneuploidy (HULBERT and MICHELMORE 1988). (5) Progeny from outcrosses must be distinguished from potential contaminating progeny resulting from self-fertilization. Small percentages of progeny derived from self-fertilization have been detected in crosses between isolates of *Phytophthora infestans* (SHATTOCK

et al. 1987), another heterothallic Oomycete fungus.

We report here the identification of 61 RFLPs among three isolates of *B. lactucae*. For 53 of these RFLP loci, at least one of the isolates was heterozygous. The segregation of these 53 RFLPs, 8 virulence genes and the mating type locus was analyzed in two crosses to verify the Mendelian nature of the markers and to determine whether segregation ratios would be severely biased. A preliminary genetic map, consisting of several small linkage groups, was constructed based on the analysis of cosegregation. Based on this analysis, it was possible to estimate the genetic length of *B. lactucae* at approximately 2000 centiMorgans (cM) and to estimate that approximately 190 RFLPs will be needed in order to construct a nearly complete linkage map, with 95% of the genome within 20 cM of a marker.

MATERIALS AND METHODS

Isolates of *B. lactucae*: Isolates of *B. lactucae* were vegetatively propagated by inoculating susceptible lettuce seedlings with asexual spores (conidia). Sexual spores (oospores) were generated by pairing isolates of opposite mating type on susceptible lettuce cotyledons; Oospores form following the fusion of the meiotic products from the gametangia of the two isolates. Sexual progeny were obtained following systemic infection of lettuce seedlings by individual Oospores.

Three parental isolates of *B. lactucae* were used to identify polymorphisms and to generate the sexual progeny for the segregation analysis. Isolate SF5 was a field isolate from Finland (OSARA and CRUTE 1981). Isolate C82P24 was a field isolate from California (ILOTT, DURGAN and MICHELMORE 1987). Isolate IMOS6b was an F₁ hybrid obtained from a cross between isolate IM25R7, from England, and isolate CG1, from Switzerland (MICHELMORE *et al.* 1984); such a hybrid was used to increase the chance of heterozygosity at many loci. Isolate SF5 was the B₁ mating type while isolates IMOS6b and C82P24 were the complementary B₂ mating type. This enabled sexual progeny to be generated from two different crosses among the three parents: IMOS6b × SF5 and C82P24 × SF5. Fertility of the parents appeared normal in both crosses as large numbers of viable oospores were obtained. The progeny isolates were generally fit, sporulating six to eight days after inoculation of susceptible cultivars. Procedures for maintaining and storing isolates, obtaining sexual progeny from crosses and determining virulence phenotypes and mating types of isolates have been described elsewhere (MICHELMORE and INGRAM 1980; MICHELMORE and CRUTE 1982; MICHELMORE *et al.* 1988).

DNA isolation: DNA from *B. lactucae* was extracted using a procedure modified from TIMBERLAKE (1978). Asexual spores (conidia) from each isolate were harvested from 2-week-old, spore-bearing seedlings grown in plastic boxes (8 × 12 × 6") by shaking in distilled water. The spores were washed by pelleting at 200 × g for 5 min at 4°, twice in distilled water and once in SSE0.5 (4 mM spermidine, 1 mM spermine, 10 mM EDTA, 10 mM Tris, 80 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 500 mM sucrose, 0.2% 2-mercaptoethanol, pH 7.5). The pellet of spores (approx 1 g) was then resuspended with 5 ml cold SSE0.5 in a 50-ml centrifuge tube. After the addition of glass beads (approx-

mately 2/3 volume; 450–500 μm diameter; Sigma), the spore suspension was shaken for 2 to 4 min; this treatment broke more than 95% of the spores. The suspension of organelles and broken spores was filtered through one layer of pre-treated Miracloth (Calbiochem; pretreated by boiling for 5 min in 10 mM EDTA twice, and then equilibrating with SSE0.5) and collected in a 50-ml centrifuge tube. The glass beads were rinsed three times with 5 ml SSE0.5 to recover most of the organelles. Organelles were pelleted by centrifugation for 10 min at $10,000 \times g$ at 4° . They were then gently resuspended in 4 ml cold SSE0.5. The organelles were lysed at 65° for 30 min after the addition of 4 ml of EDTA solution (2 M, pH 9.0) and 1 ml 10% Na-Sarcosyl. Following lysis, 9.6 g CsCl were added and the total weight of the solution was brought to 19.0 g with TE. After the CsCl had dissolved, the tubes were centrifuged at $20,000 \times g$, for 30 min. Each supernatant was filtered through two layers of cheesecloth into a 13 ml quick-seal ultracentrifuge tube (Beckman) containing 500 μl of ethidium bromide (10 mg/ml). Each tube was made up to volume with a solution of CsCl (97 g CsCl in 100 ml TE), sealed, and centrifuged at 50,000 rpm for 20 hr in a fixed angle rotor (70.1 Ti Beckman). DNA was recovered using standard procedures (MANIATIS, FRITSCH and SAMBROOK 1982) and resuspended in TE at a final concentration of 0.25 mg/ml.

Construction of probes: Clones of genomic DNA were constructed for RFLP analysis and those carrying inserts homologous to low copy number sequences were selected by the method of LANDRY and MICHELMORE (1985). Genomic DNA of *B. lactucae* was digested with *Mbo*I, *Bam*HI or *Eco*RI and small (0.4–1.0 or 1.0–5.0 kb) fragments were cloned into the appropriate sites of pUC13. DNA samples ($<0.1 \mu\text{g}$) from plasmid miniscreens of positive clones were denatured and placed on Zetaprobe membrane (Bio-Rad) using a dot-blot apparatus (Bio-Rad). Genomic DNA from *B. lactucae* was labeled with ^{32}P and hybridized to the membranes using $1\text{--}5 \times 10^7$ cpm (specific activity $2\text{--}5 \times 10^8$ cpm/ μg DNA). Clones showing detectable hybridization were assumed to carry repetitive DNA sequences and were therefore discarded. When screening libraries with large inserts (1.0–5.0 kb), the clones were preselected for low copy number by colony hybridization (MANIATIS, FRITSCH and SAMBROOK 1982) before the individual plasmid clones were screened.

A cDNA library was also constructed for use as probes. Total RNA was isolated from the asexual spores of *B. lactucae* using the method of CATHALA *et al.* (1983). Polyadenylated RNA was selected on oligo(dT)-cellulose columns (MANIATIS, FRITSCH and SAMBROOK 1982). The cDNA library was constructed using the procedure of ALEXANDER, MCKNIGHT and WILLIAMS (1983).

Restriction digests, electrophoresis, blotting, nick-translation and hybridization: DNA samples from *B. lactucae* were individually digested with the restriction endonucleases, *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I, according to the manufacturer's (BRL) recommendations. Digested DNA samples (4.0 μg /lane) were electrophoresed in 1.0% TBE agarose gels containing ethidium bromide (1.0 μg /ml). The DNA was depurinated (0.25 M HCl, 20 min), denatured (0.4 M NaOH, 0.8 M NaCl, 30 min), neutralized (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.6, 30 min) and transferred from the gel onto Zetaprobe membrane (Bio-Rad) using $3\times$ SSC as the transfer buffer (SOUTHERN 1975). After transfer (8–20 hr), the membranes were washed in $3\times$ SSC and baked under vacuum at 80° for 2 hr. DNA inserts from genomic or cDNA clones were excised, gel isolated, labeled, and hybridized to the Southern blots as described previously (HULBERT and MICHELMORE 1988).

Identification of RFLP loci: In order to identify combinations of probes and endonuclease digestions which detected RFLPs, Southern blots carrying DNA from the three parental isolates digested with each of the four restriction enzymes were hybridized with low copy number genomic, or cDNA, probes (Figure 1a) to see whether the different isolates produced different patterns of hybridization. For each such probe-enzyme combination detecting a polymorphism, it was necessary to determine whether the various strains were heterozygous or homozygous at the RFLP locus, since segregation could only be followed at heterozygous loci. For this purpose, DNA samples from 8 to 10 progeny were digested and probed to determine whether RFLP alleles segregated in the gametes of at least one parent (Figure 1b). When possible, the enzyme for digestion of the progeny DNAs was selected so that segregation for RFLP could be observed in both crosses with the same enzyme digest. This ensured that the same locus was analyzed in both crosses for each individual probe. Blots of progeny DNAs could be hybridized up to 15 times with different probes. Labeled DNA was removed from the blots prior to reprobing by washing in $0.1\times$ SSC, 0.1% SDS at 95° for 15 min.

Analysis of segregation: Segregation of 53 RFLP loci, eight avirulence genes and the mating type locus was analyzed in progeny of two crosses. Thirty eight progeny were analyzed from the cross SF5 $\times$ C82P24 and 32 from the cross SF5 $\times$ IMOS6B. χ^2 goodness-of-fit values were calculated for segregation ratios of the individual loci. Maximum likelihood estimates of recombination frequencies between linked loci were calculated on a HP9000 computer with an extension of the computer program MAPMAKER (LANDER *et al.* 1987; LANDER and GREEN 1987). Although originally developed for analyzing human linkage maps, the program was useful for *B. lactucae* because of the similarities in the mating system used (*i.e.*, outcrosses between highly heterozygous individuals). The program allowed the calculation of linkage distances and the determination of gene order via multipoint linkage analysis.

Estimation of genome size: The total number of centimorgans in the genome of any organism can be estimated from partial linkage data as follows. Let G denote the length of the genome in cM, let $P(T, G)$ denote the probability that the LOD score for a random pair of markers will be greater than or equal to T , let N denote the number of markers typed and let M denote the number of informative meioses. The expected number of pairs of markers having LOD score $\geq T$ is then $1/2N(N-1)P(T, G)$. If the actual number of such pairs is K , then an estimate of G is given by the equation $P(T, G) = K/1/2N(N-1)$. It only remains to determine the function $P(T, G)$.

The precise formula for $P(T, G)$ involves double integration first over the distance X between the loci, and then over the probability that the LOD score exceeds T conditional on X . However, an adequate first-order approximation is provided by the formula $P(T, G) = 2X/G$, where $X = X(T)$ is the distance between two markers for which the expected value of the LOD score is T . Accordingly, the genome size is given by

$$G = N(N-1)X/K,$$

where X and K depend on the LOD and threshold T . (Different thresholds T could be tried to obtain various estimates of G .)

It only remains to determine $X = X(T)$. For a single meiosis, the expected LOD score (LANDER and BOTSTEIN 1986) for a pair of markers linked at distance X is

$$E(X) = \theta \log 2\theta + (1 - \theta) \log 2(1 - \theta),$$

FIGURE 1.—Southern blots showing parental and progeny genotypes at the locus *G727*. a, Southern blot showing RFLP between isolates of *B. lactucae* at the locus *G727*. Each lane carried DNA from one of three isolates digested with one of four different restriction enzymes. Parent 1 is isolate IMOS6b, parent 2 is isolate C82P24, and parent 3 is isolate SF5. b, Southern blots showing segregation at the locus *G727* in sexual progeny from two crosses of *B. lactucae*. Each lane carried DNA from an individual parental or progeny isolate digested with *EcoRI*. The genotype of P1 (IMOS6b), P2 (C82P24) and P3 (SF5) was 3/3, 2/3, and 1/2, respectively. The genotypes of the nine progeny shown from the cross P1 $\times$ P3 were 2/3, 2/3, 2/3, 2/3, 2/3, 1/3, 2/3, 1/3 and 2/3. The genotypes for the progeny shown from cross 2 $\times$ P3 were 1/3, 1/2, 1/2, 1/2, 1/3, 1/2, 2/2 and 1/2. Segregation ratios from larger numbers of progeny and expected segregation ratios are listed in Tables 1 and 2.

where θ is the recombination fraction corresponding to distance X . For a total of M meioses, the value of X is then given implicitly by the equation $T = M E(X)$.

This discussion above applies directly when the markers are all informative in the same number of meioses. In the case of outbreeding species, such as *B. lactucae*, it is slightly more complicated in that different pairs of markers are informative in different numbers of meioses. The approach was therefore modified by summing the effects of the 1891 individual meioses (rather than simply multiplying by $1/2N(N-1)$).

RESULTS

Sources of probes: Both cDNA and genomic clones were used as probes to detect polymorphisms; there

were advantages and disadvantages to using each type of probe. Clones of genomic DNA of *B. lactucae* frequently carried repetitive (high copy number) sequences. Such clones were generally not useful as genetic markers as they did not identify unique bands. The cDNA clones rarely detected repetitive sequences. The complexity of the cDNA library made from spore mRNA, however, was much less than that of the genomic libraries: several different cDNA clones identified identical banding patterns on Southern blots, indicating that the clones carried inserts originating from the same locus. In several cases this was verified by demonstrating absolute cosegregation of the different clones in sexual progeny. Of 39 cDNA

probes tested, five were homologous to the same fragment; one group of three and three pairs of identical clones were also found.

The size of the genomic clones was important because such clones frequently carried high copy number sequences. Although larger clones should detect more frequent RFLP, they are also more likely to contain repeated sequences if such sequences have a short period interspersed pattern. This seemed to be the case with *B. lactucae* as the frequency of clones containing repetitive genomic sequences increased significantly when larger DNA fragments were cloned. Of the small fragments (0.4 to 1.0 kb) cloned into pUC13, approximately 30% (27/97) carried repetitive sequences. For larger fragments (1.0–5.0 kb), this frequency increased to approximately 90% or higher. This effect was observed in libraries made from both *Bam*HI and *Eco*RI fragments. It was more efficient to use a two-step procedure for eliminating clones carrying high copy number sequences: clones were first screened by colony hybridization, then the clones that did not show hybridization to labeled genomic DNA were screened by dot blot hybridization. Screening by colony hybridization alone was not reliable enough to screen out all of the inserts homologous to high copy genomic sequences; however, most clones that showed no detectable hybridization in both screens were homologous to single or low copy genomic sequences. For future studies, libraries made from genomic fragments digested with methylation sensitive enzymes may provide a higher frequency of low copy number clones (YOUNG, MILLER and TANKSLEY 1987; HELENTJARIS, WEBER and WRIGHT 1988); this possibility has yet to be investigated in *B. lactucae*.

Identification of polymorphism, heterozygosity in the parental isolates and segregation in crosses: Overall, a total of 201 low copy number genomic clones and 30 distinct cDNA clones were screened for their ability to detect polymorphism. Both the cDNAs and the genomic probes frequently detected polymorphism between the three parental isolates. Approximately 20% (40/201) of the low copy number genomic probes tested were polymorphic and had simple banding patterns; another 18% of the genomic probes were polymorphic but had banding patterns that were too complex to use to detect the segregation of single loci. Most of the low copy number polymorphic probes detected polymorphism in more than one, and sometimes all four, of the enzyme digests. Of the 30 unique cDNA probes, 21 (70%) detected polymorphism in at least one of the four digests of the three parental isolates. All of these RFLPs had simple banding patterns.

Each of the 40 genomic probes which detected polymorphism and 15 of the cDNA probes were tested to verify the Mendelian nature of the polymorphisms,

to determine if any of the probes detected more than one locus and whether each of the three parents were heterozygous or homozygous at the loci. A total of 61 loci were identified by the 55 probes as one of the cDNAs and five of the genomic probes identified two distinct loci. The F₁ isolate IMOS6b, and the two field isolates SF5 and C82P24 were heterozygous at 38%, 67%, and 37% of the loci, respectively. A total of 53 RFLP loci were heterozygous in at least one of the isolates.

Segregation could thus be analyzed for 53 of the 61 RFLP loci in F₁ progeny in at least one of the two crosses, SF5 × C82P24 and SF5 × IMOS6b, among the three parents. Only five RFLP loci detected by genomic probes and three detected by cDNAs did not segregate in either cross. Forty RFLP loci segregated in both crosses; most of these were detected by genomic probes as only five of sixteen cDNA loci for which segregation was analyzed segregated in both crosses.

Segregation of the individual RFLP loci usually fit the ratios expected for Mendelian loci (Tables 1 and 2). Segregation of only two of 44 RFLP loci deviated significantly ($P < 0.05$) from the expected ratios in the SF5 × C82P24 cross and only six of 49 loci deviated in the IMOS6b × SF5 cross. No locus deviated significantly from the expected segregation ratios in both crosses.

In addition to the RFLP loci, eight specific avirulence loci segregated. Five segregated in both crosses (*Avr3*, *Avr5/8*, *Avr6*, *Avr7* and *Avr11*), two segregated only in SF5 × C82P24 (*Avr4* and *Avr15*) and one segregated only in IMOS6b × SF5 (*Avr14*). As expected, both crosses segregated for mating type. The mating type and virulence loci all segregated closely to the expected ratios in both crosses.

Linkage analyses: Linkage analysis was performed on the 53 RFLP loci, eight virulence genes and the mating type locus in the sexual progeny of the two crosses. Thirty-eight progeny were analyzed from the cross SF5 × C82P24 and 32 from the cross SF5 × IMOS6b. The mating design was not ideal for developing a linkage map as sufficient data to demonstrate linkage could usually be generated only for those loci which segregated in at least two of the parents. To generate a more extensive genetic map, more progeny from a single cross (SF5 × C82P24) are currently being analyzed. Cosegregation data did, however, allow the construction of a preliminary map (Figure 2) consisting of 13 tentative linkage groups and comprising some 230 cM within the linked loci. Seven of the groups carried three or more loci and six consisted of pairs of loci. Using the same thresholds as employed in human genetics, loci were considered tentatively linked if the LOD score exceeded 2.0 and probably linked if the LOD score exceeded 3.0. In brief, the

TABLE 1
Parental genotypes, segregation and goodness-of-fit tests for RFLP loci in the cross SF5 × C82P24

Locus-enzyme ^a	Genotype		Expected ratio	Observed ratio	χ^2
	SF5	C82P24			
<i>G021-H</i>	1/2	1/2	1:2:1	9(1/1):16(1/2):12(2/2)	1.16
<i>G054-E</i>	1/2	1/1	1:1	11(1/1):27(1/2)	6.74**
<i>G064a-H</i>	1/3	3/3	1:1	22(1/3):14(3/3)	1.78
<i>G064b-H</i>	2/3	3/3	1:1	22(2/3):15(3/3)	1.32
<i>G070-E</i>	2/3	2/2	1:1	18(2/2):20(2/3)	0.11
<i>G075-H</i>	1/2	3/3	1:1	18(1/3):18(2/3)	0.00
<i>G085-H</i>	1/2	1/1	1:1	20(1/1):18(1/2)	0.11
<i>G091-B</i>	2/3	2/3	1:2:1	11(2/2):20(2/3):5(3/3)	2.44
<i>G107a-E</i>	2/3	2/2	1:1	19(2/2):18(2/3)	0.03
<i>G128-H</i>	1/2	1/2	1:2:1	8(1/1):19(1/2):10(2/2)	0.24
<i>G136-P</i>	1/3	1/1	1:1	21(1/1):16(1/3)	0.68
<i>G138-H</i>	2/3	2/3	1:2:1	12(2/2):19(2/3):7(3/3)	1.32
<i>G201a-E</i>	1/2	1/2	3:1	32(2-):6(3/3)	1.72
<i>G201b-E</i>	1/3	1/3	3:1	30(3-):7(4/4)	0.73
<i>G242a-E</i>	1/3	1/3	3:1	27(1-):11(3/3)	0.32
<i>G287-P</i>	3/4	1/2	1:1:1:1	9(1/3):14(1/4):5(2/3):10(2/4)	4.32
<i>G443-B</i>	1/2	1/1	1:1	17(1/1):20(1/2)	0.24
<i>G503-H</i>	1/2	1/1	1:1	21(1/1):17(1/2)	0.42
<i>G514-E</i>	2/2	1/2	1:1	21(1/2):15(2/2)	1.00
<i>G521-H</i>	2/2	1/2	1:1	18(1/2):19(2/2)	0.03
<i>G538-H</i>	2/3	3/3	1:1	24(2/3):14(3/3)	2.63
<i>G546-H</i>	1/2	1/1	1:1	13(1/1):24(1/2)	3.27
<i>G551a-P</i>	1/2	2/3	1:1:1:1	10(1/2):10(1/3):10(2/2):7(2/3)	0.73
<i>G551b-P</i>	1/2	1/1	1:1	13(1/1):23(1/2)	2.78
<i>G602-B</i>	1/2	2/2	1:1	20(1/2):15(2/2)	0.71
<i>G610-B</i>	1/2	2/2	1:1	22(1/2):14(2/2)	1.78
<i>G614-P</i>	1/1	1/2	1:1	19(1/1):19(1/2)	0.00
<i>G634-E</i>	1/2	2/2	1:1	28(1/2):10(2/2)	8.53**
<i>G636-H</i>	1/3	1/3	1:2:1	10(1/1):20(1/3):7(3/3)	0.73
<i>G640-B</i>	1/2	3/3	1:1	13(1/3):21(2/3)	1.88
<i>G642-E</i>	1/3	1/2	1:1:1:1	4(1/1):7(1/2):10(1/3):12(2/3)	4.45
<i>G644-H</i>	2/3	2/3	1:2:1	10(2/2):20(2/3):8(3/3)	0.32
<i>G710-E</i>	1/2	1/1	1:1	21(1/1):16(1/2)	0.68
<i>G727-E</i>	1/2	2/3	1:1:1:1	8(1/2):10(1/3):9(2/2):11(2/3)	0.53
<i>G813-H</i>	1/1	1/2	1:1	14(1/1):23(1/2)	2.19
<i>G854-H</i>	3/4	1/2	1:1:1:1	8(1/3):10(1/4):7(2/3):12(2/4)	1.59
<i>CE40a-P</i>	1/2	2/2	1:1	15(1/2):22(2/2)	1.32
<i>CB12-B</i>	1/2	1/1	1:1	15(1/1):21(1/2)	1.00
<i>CC01-H</i>	1/1	1/2	1:1	20(1/1):15(1/2)	0.71
<i>CE06-E</i>	2/3	1/3	1:1:1:1	5(1/2):10(1/3):9(2/3):10(3/3)	2.00
<i>CE33-E</i>	2/3	1/1	1:1	10(1/2):19(1/3)	2.80
<i>CE44-E</i>	2/2	1/2	1:1	21(1/2):15(2/2)	1.00
<i>CE56-E</i>	2/3	1/3	1:1:1:1	6(1/2):12(1/3):9(2/3):8(3/3)	2.14
<i>CE61-B</i>	1/2	2/2	1:1	18(1/2):17(2/2)	0.03

^a Italicized letters and numbers in the first column are the locus designation. The suffixes *a* and *b* were used when more than one locus was detected by a single probe. The first letter of the locus-enzyme designation refers to whether the probe was a genomic clone (*G*) or a cDNA clone (*C*). The last letter refers to the restriction enzyme used to digest the genomic DNA. B = *Bam*HI, E = *Eco*RI, H = *Hind*III, P = *Pst*I.

* $P < 0.05$; ** $P < 0.01$.

LOD score for a pair of loci is the $\log_{10}$ of the ratio of the probability that the data would have arisen if the loci are linked to the probability that the data would have arisen if the loci are unlinked (LANDER and BOTSTEIN 1986).

The loci identified by cDNAs did not appear to be strongly clustered in the genome, although 2 of 13 such loci were tightly linked. More cDNA loci will have to be mapped to determine if mRNA present in

spores are transcribed from genes which are nonrandomly distributed in the genome.

In the majority of cases, the genomic and cDNA probes each identified polymorphisms at a single locus. Only one of the 12 cDNA probes (*CE40*) for which segregation was analyzed identified two loci. These two loci are represented as *CE40a* and *CE40b*, on linkage groups D and L, respectively. Two of 35 genomic probes (*G107* and *G551*) each recognized

TABLE 2

Parental genotypes, segregation and goodness-of-fit tests for RFLP loci in the cross IMOS6b × SF5

Locus-enzyme ^a	Genotype		Expected ratio	Observed ratio	χ^2
	IMOS6b	SF5			
<i>G021-H</i>	1/1	1/2	1:1	11(1/1):21(1/2)	3.13
<i>G026-P</i>	1/2	2/2	1:1	16(1/2):13(2/2)	0.31
<i>G054-E</i>	1/2	1/2	1:2:1	9(1/1):18(1/2):4(2/2)	2.42
<i>G064a-H</i>	1/3	1/3	3:1	25(1-):6(3/3)	0.53
<i>G064b-H</i>	3/3	2/3	1:1	18(2/3):13(3/3)	0.80
<i>G070-E</i>	2/2	2/3	1:1	16(2/2):15(2/3)	0.03
<i>G075-H</i>	3/3	1/2	1:1	13(1/3):19(2/3)	1.13
<i>G085-H</i>	2/3	1/2	1:1:1:1	6(1/2):11(1/3):5(2/2):10(2/3)	3.25
<i>G091-B</i>	2/2	2/3	1:1	12(2/2):20(2/3)	2.00
<i>G107a-E</i>	1/1	2/3	1:1	16(1/2):15(1/3)	0.03
<i>G107b-E</i>	1/1	1/2	1:1	13(1/1):14(1/2)	0.04
<i>G128-H</i>	1/1	1/2	1:1	17(1/1):15(1/2)	0.13
<i>G136-P</i>	1/2	1/3	1:1:1:1	3(1/1):8(1/2):10(1/3):6(2/3)	3.96
<i>G138-H</i>	1/3	2/3	1:1:1:1	11(1/2):12(1/3):5(2/3):4(3/3)	6.25
<i>G201a-E</i>	1/2	1/2	3:1	28(2-):4(3/3)	2.67
<i>G201b-E</i>	1/1	1/3	1:1	16(1/1):16(1/3)	0.00
<i>G242a-E</i>	3/3	1/3	1:1	15(1/3):15(3/3)	0.00
<i>G242b-E</i>	2/3	3/3	1:1	14(2/3):16(3/3)	0.13
<i>G287-P</i>	2/2	3/4	1:1	11(2/3):17(2/4)	1.29
<i>G443-B</i>	1/1	1/2	1:1	12(1/1):20(1/2)	2.00
<i>G503-H</i>	1/1	1/2	1:1	19(1/1):13(1/2)	1.13
<i>G514-E</i>	1/2	2/2	1:1	18(1/2):12(2/2)	1.20
<i>G538-H</i>	2/3	2/3	1:2:1	9(2/2):22(2/3):1(3/3)	8.50*
<i>G546-H</i>	1/1	1/2	1:1	11(1/1):19(1/2)	2.13
<i>G551a-P</i>	3/3	1/2	1:1	14(1/3):12(2/3)	0.15
<i>G551b-P</i>	1/1	1/2	1:1	18(1/1):6(1/2)	6.0*
<i>G555-E</i>	1/2	3/3	1:1	14(1/3):16(2/3)	0.13
<i>G602-B</i>	1/1	1/2	1:1	19(1/1):13(1/2)	1.13
<i>G610-B</i>	2/2	1/2	1:1	19(1/2):13(2/2)	1.13
<i>G614-P</i>	1/2	1/1	1:1	11(1/1):20(1/2)	2.61
<i>G634-E</i>	1/2	1/2	1:2:1	7(1/1):15(1/2):9(2/2)	0.29
<i>G636-H</i>	3/3	1/3	1:1	22(1/3):10(3/3)	4.50*
<i>G640-B</i>	1/2	1/2	1:2:1	6(1/1):12(1/2):11(2/2)	2.59
<i>G642-E</i>	2/2	1/3	1:1	11(1/2):19(2/3)	2.13
<i>G644-H</i>	3/3	2/3	1:1	18(2/3):14(3/3)	0.50
<i>G710-E</i>	1/1	1/2	1:1	17(1/1):14(1/2)	0.29
<i>G727-E</i>	3/3	1/2	1:1	15(1/3):16(2/3)	0.03
<i>G813-P</i>	1/2	2/2	1:1	13(1/2):15(2/2)	0.14
<i>G854-H</i>	2/5	3/4	1:1:1:1	8(2/3):8(2/4):8(3/5):7(4/5)	0.10
<i>CE40a-P</i>	1/2	1/2	1:2:1	8(1/1):11(1/2):8(2/2)	0.93
<i>CB12-B</i>	1/1	1/2	1:1	12(1/1):18(1/2)	0.12
<i>CC05-P</i>	1/2	2/2	1:1	14(1/2):13(2/2)	0.85
<i>CE04-P</i>	1/2	1/1	1:1	20(1/1):8(1/2)	5.14*
<i>CE06-E</i>	3/3	2/3	1:1	18(2/3):13(3/3)	0.81
<i>CE40b-P</i>	1/2	1/1	1:1	18(1/1):8(1/2)	3.85*
<i>CE42-P</i>	1/2	2/2	1:1	18(1/2):8(2/2)	3.85*
<i>CE43-P</i>	1/1	1/2	1:1	20(1/1):8(1/2)	5.14*
<i>CE56-E</i>	2/3	2/3	1:2:1	2(2/2):17(2/3):11(3/3)	5.93
<i>CE61-B</i>	1/2	1/2	1:2:1	7(1/1):13(1/2):11(2/2)	1.84

^a Italicized letters and numbers in the first column are the locus designation. The suffixes *a* and *b* were used when more than one locus was detected by a single probe. The first letter of the locus-enzyme designation refers to whether the probe was a genomic clone (G) or a cDNA clone (C). The last letter refers to the restriction enzyme used to digest the genomic DNA. B = *Bam*HI, E = *Eco*RI, H = *Hind*III, P = *Pst*I.

* $P < 0.05$.

two unlinked loci. Locus *G107b* mapped to linkage group F while *G107a* was not significantly linked to any other locus. Locus *G551a* mapped to linkage group B but it was not possible to map *G551b*. The probe G551 detected numerous bands; the banding

pattern was suggestive of a multigene family in which two of several loci were polymorphic. Three genomic probes, G064, G201 and G242, identified two linked loci, on linkage groups J, E, and B, respectively. There is no evidence as yet of a large tandem duplication

FIGURE 2.—Linkage relationships among RFLP loci and avirulence genes. Avirulence loci are designated *AvrX*; loci detected by cDNA probes are prefixed by *C*; loci detected by genomic probes are prefixed by *G*. Estimated map distances (in cM) are shown to the left of the vertical line. Solid lines indicate that the LOD score for linkage between two loci is equal to three or greater; dotted lines indicates the LOD score was between two and three. The loci *CC05* and *G242b* map to linkage group B (with LOD scores of 3 or greater) but the data contain too few coinformative meioses to determine their most likely order with respect to the other genes on the linkage group. Loci for which linkages were not detected are listed at the bottom of the figure.

Linkages not detected: *Avr3*, *Avr4*, *Avr5*, *Avr7*, *Avr11*, *Avr14*, *CC01*, *CE04*, *CE33*, *CE56*, *CE42*, *CE61*, *GO26*, *GO54*, *GO70*, *GO91*, *G107a*, *G551b*, *G555*, *G610*, *G614*, *G634*, *G640*, *G854*, Mating type

event as the duplicated loci are dispersed and not localized in linkage blocks.

No linkage was detected between the avirulence loci. This agrees with previous data (NORWOOD *et al.* 1983; MICHELMORE *et al.* 1984), that the avirulence loci are not tightly linked.

DISCUSSION

A detailed linkage map will be a powerful tool that has not yet been available for any plant pathogenic fungus. An effectively unlimited supply of RFLPs can readily be identified in *B. lactucae* by screening low copy-number genomic clones. Over 20% of the low copy-number genomic clones used as probes revealed simply inherited RFLPs. Moreover, the chance that an isolate was heterozygous at an RFLP locus was 38%, 67% and 37% in IMOS6B, SF5 and C82P24, respectively. It should now be straightforward to construct a complete RFLP linkage map of the genome of *B. lactucae* and to utilize the map to analyze varia-

tion in naturally occurring isolates (HUBERT and MICHELMORE 1988).

Sources of RFLPs: For *B. lactucae*, it is necessary to prescreen random genomic fragments to eliminate those containing repetitive DNA sequences. Such screenings may not be necessary for most other fungi, since *B. lactucae* seems to have significantly more dispersed repetitive DNA (D. FRANCIS, S. HULBERT, R. MICHELMORE, unpublished data) than most other fungal genomes characterized to date (TIMBERLAKE 1978; KRUMLAUF and MARZLUF 1979; ULLRICH, KOBURN and SPRECHT 1980). It may thus be possible to use large genomic phage clones (RAEDER and BRODA 1986) as probes in other fungi.

The cDNAs proved to be less useful than the library of random genomic clones, due to the low complexity of the cDNA library prepared from spores. *B. lactucae* being a biotrophic fungus, spores were chosen as a source for the library because they can be harvested without significant contamination from plant mate-

rial. cDNA libraries from nonbiotrophic fungi could be made from more complex populations of mRNAs such as from vegetative hyphae.

Patterns of segregation: Despite our initial concerns that the low viability of sexual spores might reflect differential viability of segregating genotypes, the segregation of individual RFLP loci largely fit the expected ratios. There was, therefore, no evidence that the progeny studied constituted a heavily biased sample of the possible genotypes. Since *B. lactucae* is an obligately outcrossing, predominantly diploid species that frequently reproduces asexually, most isolates probably carry numerous deleterious recessive alleles. If some form of inbreeding had been used to generate the sexual progeny (such as an F_2 or backcross family), biased segregation ratios might have been frequent.

All of the progeny appeared to be normal diploids with alleles from both parents. Thus, there was no evidence of self-fertilization, apomixis or haplospory. The only meiotic anomaly concerned one individual, A1, from the cross SF5 $\times$ C82P24 which seemed homozygous when it should have been heterozygous at two RFLP loci; SF5 had alleles 2 and 3 at locus *G075* and C82P24 was homozygous for allele 1, but the progeny isolate A1 had only allele 1. Similarly, at locus *CE56*, SF5 had alleles 2 and 3 and C82P24 had alleles 1 and 3 but A1 had only allele 1. This result can not be explained by aneuploidy as two alleles were present at locus *G636*, which is linked to *G075* (Figure 2). It is not known whether this isolate had one or two copies of the loci *CE56* and *G075*. Homozygosity at both loci could possibly have resulted from a single mitotic crossing over event; linkage between these two loci was not detected, however, it is possible that they are loosely linked on the same chromosome arm.

Design of crosses: RFLP mapping in plant species has so far only employed inbred lines as parents and RFLP analyses have been made of F_2 or backcross families (BERNATZKY and TANKSLEY 1986; HELENTJARIS *et al.* 1986; LANDRY *et al.* 1987). This is the most efficient method for developing genetic maps because the phase of linked alleles is always known, all polymorphisms segregate, and all of the polymorphisms provide information on linkage. Unfortunately, the development of homozygous inbreds to use as initial parents may not be practical for many species. It may be difficult or impossible for outcrossing species, due to inbreeding depression, or for plants with self-incompatibility systems that can not be overcome. In addition, it will be tedious in bisexual species such as many animals, some plants or diploid heterothallic fungi and it will be impractical for organisms with long generation times, such as most trees.

For many species then, it will be necessary to carry out genetic mapping by using crosses between outbred individuals from natural populations. *B. lactucae* rep-

resents a prototype for such a mapping project, the only other reported example being the recent construction of an RFLP linkage map for humans (DONIS-KELLER *et al.* 1987). In the case of humans, linkage analysis is performed in a collection of three-generation families, with four grandparents, two parents and about 10 children; given the relatively small size of human families, such three-generation crosses are preferable for linkage analysis, since they usually reveal the linkage phase (*i.e.*, *cis-trans*) of alleles at different loci. Three-generation crosses offer no special advantage over F_1 crosses for linkage analysis in organisms such as *B. lactucae* in which many progeny can be generated from controlled crosses; the same degree of efficiency provided by the availability of grandparents can be achieved by simply scoring a few additional progeny in an F_1 cross. The abundance of RFLPs and high levels of heterozygosity in *B. lactucae* demonstrate that a single F_1 cross between naturally occurring isolates will suffice for construction of a genetic map.

Two separate crosses were studied here for the following reasons: (1) In species like *B. lactucae* where sexual progeny are derived from a small percentage (often less than 0.1%) of the sexual spores, there is a risk that selection upon the progeny will give rise to associations between unlinked alleles (*i.e.*, pseudolinkage) that would be interpreted as linkage. The analysis of two crosses might detect such anomalies. (2) Using two crosses with one parent in common generally enabled the parental alleles to be determined in each cross with only two generations (parents and F_1). This was not always evident with one cross and was particularly helpful when RFLP phenotypes had multiple bands. (3) Since no previous estimates of heterozygosity of neutral markers were available for any isolate of *B. lactucae*, it was not known to what extent the markers would segregate in the F_1 generation. Therefore, one of the crosses employed the parental isolate IMOS6B which was an F_1 between two field isolates (MICHELMORE *et al.* 1984). This appeared to be unnecessary, however, because the two field isolates used as the other two parents were also frequently heterozygous for the RFLPs. Although the use of two crosses was advantageous for these initial investigations, the eventual construction of a complete linkage map for *B. lactucae* is probably best performed by concentrating on a single large cross, with approximately 100 progeny.

Linkage analysis assumes that there is no chromosomal structural heterozygosity between the parents. This may not be the case when utilizing naturally occurring isolates. The problems caused by structural heterozygosity would be minimized by developing the initial map from a single cross (indeed, separate maps for each parent could be derived) and by avoiding the

use of multigeneration families (such as are used in human linkage analysis; WHITE *et al.* 1985).

Different considerations for developing RFLP mapping strategies might apply to other diploid fungi. In diploid species that are homothallic and naturally inbreeding, naturally occurring isolates would be expected to be relatively homozygous; therefore, the most efficient approach is likely to be to select for rare outcrossed F_1 progeny and then to use traditional F_2 analysis. Haploid fungi should be especially favorable for RFLP mapping; since linkage phase is unambiguous, the meiotic products can be readily scored as recombinant or nonrecombinant with respect to any pair of loci (RAEDER and BRODA 1986).

Genetic linkage map: A partial linkage map of the *B. lactucae* genome can be constructed from the segregation data, which includes 37 of the 62 markers and spans approximately 230 cM within the linked markers. Based on this data, it is possible to derive a rough estimate of the total genetic length of the *B. lactucae* genome.

Supposing that the length of the *B. lactucae* genome is G cM, the chance that a randomly chosen pair of loci lie within x cM is approximately $2x/G$, where x is assumed to be small compared to the mean genetic length of chromosomes. Provided that the two loci are both informative in at least one of the three parents studied, one can show that (1) if $x \leq 15$ cM, the maximum LOD score between them will almost surely exceed 3.0 and (2) if $x \leq 20$ cM, the maximum LOD score will almost surely exceed 2.0. Since 1521 of the 1891 pairs of loci studied are coinformative in at least one parent, one would expect (1) to find (1521) (30/ G) pairs of loci within a distance of at most 15 cM, with a maximum LOD score of at least 3.0, and (2) to find (1521) (40/ G) pairs of loci within a distance of at most 20 cM, with a LOD score of at least 2.0. The actual number of such pairs is 23 and 29, respectively. This corresponds to the estimates $G = 1984$ cM and 2098 cM. Thus, the linkage data suggests a genetic length of approximately 2000 cM. (For a more general treatment of the problem of estimating genetic length of an organism given partial linkage data, see the MATERIALS AND METHODS.) Given this genetic length, some 190 RFLPs would be expected to be needed in order that at least 95% of the genome lie with 20 cM of a marker.

It is not known to what extent the linkage groups identified correspond to different chromosomes. *B. lactucae* is thought to have a haploid chromosome number of at least seven, and probably more, chromosomes (MICHELMORE and SANSOME 1982), but the small sizes of the chromosomes make cytological investigations difficult. Recent technological advances in separation of fungal chromosomes by pulsed-field gel electrophoresis (SCHWARTZ and CANTOR 1984;

CARLE and OLSON 1985) may allow a molecular karyotype to be developed in the future. Hybridization of RFLP probes to Southern blots of electrophoretically separated chromosomes would allow the rapid assignment of loci defined by segregation analysis to their physical linkage groups (CARLE and OLSON 1985).

RFLP loci may be used as the starting points for chromosome walking experiments to clone closely linked genes. The present linkage analysis estimated a distance of 6.5 cM between the RFLP locus *G538* and the avirulence locus, *Avr6*. It is difficult to predict the physical distance between the two loci; relationships between physical and genetic distance vary among species as well as among different areas of a single genome (MORTIMER and SCHILD 1984; DVORAK and APPELS 1986; LAWRENCE *et al.* 1987). In the yeast *Saccharomyces cerevisiae*, 6 cM would correspond to approximately 20 kb. In *B. lactucae*, assuming a genome size of 2000 cM and a haploid genome size of 5×10^7 bp (D. FRANCIS and R. MICHELMORE, unpublished data), 6 cM corresponds on average to 150 kb. Characterization of further RFLP loci should identify closer linkages to *Avr6* and other avirulence loci.

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