

## Genetic Variation in Powdery Mildew of Barley: Development of RAPD, SCAR, and VNTR Markers

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### ABSTRACT

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Isolates of *Erysiphe graminis* f. sp. *hordei* derived from airborne conidia and progeny of specific crosses were screened for random amplified polymorphic DNA (RAPD) variation detected with 10-base primers. Using two DNA samples, 58 of 76 primers yielded good amplification products. Twenty-seven primers were screened further on a test set of 16 *E. g. hordei* isolates collected from throughout Europe. Approximately 119

resolvable bands were reproducibly amplified, 56 bands were variable, and each primer yielded at least one polymorphism. A subset of 10 of these primers detected 30 polymorphisms in the European test set but only 19 variable bands in 48 isolates collected with a stationary spore trap. In addition to the RAPD markers, we developed six sets of specific primers that detected variation, five of which detected multiple alleles, one detected the presence and absence of a band, and a seventh was monomorphic. These polymerase chain reaction markers, in conjunction with virulence and fungicide sensitivity, are being used to investigate evolutionary processes and genetic linkage in the barley powdery mildew pathogen.

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The interaction between barley (*Hordeum vulgare* L.) and the powdery mildew pathogen, *Erysiphe graminis* DC. f. sp. *hordei* Ém. Marchal, is genetically well characterized. Physiologic spe-

cialization in this pathosystem was first observed in 1930 by Mains and Dietz, and research since has shown that the host-pathogen interaction is consistent with Flor's (7) gene-for-gene concept. Virulence gene and fungicide sensitivity analyses have been used to investigate individual isolates, populations, and evolutionary biology. However, these characters are under strong selection,

limited in number, laborious to assay, and may be sensitive to environmental variation. To avoid some of these limitations, restriction fragment length polymorphism analysis (24) has been used to provide genetic markers for population genetic studies (2,3) and linkage analysis (5).

Here we report the use of polymerase chain reaction (PCR) to detect molecular markers in *E. g. hordei*. Random amplified polymorphic DNAs (RAPDs; 33,35) have proven useful in identifying genetic variation in a wide range of organisms (11,15,16,26). RAPDs have been useful in detecting variation in regional samples throughout Europe and in local populations and in showing that these characters segregate as Mendelian factors in progeny of *E. g. hordei*.

In addition, we report the development of specific pairs of primers to amplify and detect variation at genetically defined loci that have advantages over RAPD markers. These markers are sequence characterized amplified regions (SCARs) (26). SCARs that are well-optimized tend to be less sensitive than RAPDs to varying reaction conditions. SCARs can be selected for codominance, amplifying more than one allele, which helps greatly in population and mapping studies. A set of standard SCARs is more amenable than RAPDs to comparative work among different laboratories.

## MATERIALS AND METHODS

The *E. g. hordei* isolates used in this study were single-colony isolates: 1) 16 isolates were chosen from a 1990 systematic European survey from Austria (1), Czechoslovakia (1), France (3), Germany (2), Great Britain (4), Poland (1), and Spain (4) (22); 2) 48 single-colony isolates were collected with a stationary spore trap during April 1991 in Zürich; and 3) single-ascospore progeny were collected from a cross, K5, between isolates from France and Czechoslovakia.

Ascospore colonies were collected by washing leaf segments containing cleistothecia for 1 s in 70% ethanol (EtOH), 20 s in 1% sodium hypochlorite solution, rinsed in sterile distilled water, blotted on filter paper, and placed on damp cotton wool in a clear plastic container sealed with plastic film for 3 days at 17 C. The leaf segments were transferred to inverted 9-cm-high clear-plastic drinking cups with filter paper on the bottom that was kept damp by two 1-cm-wide strips of filter paper that ran down the sides of the cups into petri-dish water reservoirs. Under this "settling tower" was placed a 5.5-cm petri plate containing 0.5% water agar with 30 ppm of benzimidazole overlaid completely with cuttings of 12-day-old primary leaves of the barley cultivar Igrí. These plates were changed twice daily for 4 days. Colonies were allowed to grow for 8 days at 17 C under constant illumination (900 lx). After the appearance of conidia, single colonies were transferred via a sterile toothpick to individual leaf plates prepared as above.

Conidia were inoculated onto a 84-cm<sup>2</sup> petri dish. Colonies were allowed to grow for 12–14 days at 17 C under constant illumination (900 lx). Conidia were collected by dislodging them onto a glass plate and scraping the conidia into an Eppendorf tube with a clean razor blade. Sterile distilled water (200  $\mu$ l) was added, the tubes were centrifuged at 13,000 g for 2 min and immediately frozen for storage. The samples were subsequently freeze-dried for 48 h.

**DNA extraction.** DNA was extracted by a simplified method of Murray and Thompson (23) for cetyltri-methylammonium bromide (CTAB) preparation: 50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 0.7 M NaCl; 1% CTAB (w/v); and 1% 2-mercaptoethanol. Freeze-dried tissue (5–10 mg), bead-beaten with a mini-beadbeater (Biospec Products, Bartlesville, OK) set on high with 0.5-mm glass beads for 80 s, was subsequently incubated in a 1.5-ml Eppendorf tube with 400  $\mu$ l of CTAB buffer at 60 C for 30 min. After the first 10 min, the tubes were vortexed for 5 s to completely disperse the conidia. The mixture was centrifuged at 13,000 g for 3 min, and the aqueous phase was discarded. Fresh CTAB extraction buffer was added to the pellet of glass beads and cell debris, to a final volume of 500  $\mu$ l, and mixed and incubated

for 10 min. The mixture was extracted for 30 min at approximately 60 rpm, with tubes in a horizontal position, using an equal volume of chloroform/isoamyl alcohol (24:1 [v/v]) and centrifuged at 13,000 g for 10 min. The aqueous phase was transferred to a new tube, followed by a second chloroform extraction performed as above. The aqueous phase was transferred to a new tube, and the DNA was precipitated with the addition of 0.5 volume of isopropanol and collected by centrifugation at 1,3000 g for 10 min. The pellet was washed in 70% EtOH, dried, and dissolved in Tris-EDTA (TE) buffer, pH 7.6.

**PCR-RAPD analysis.** Amplification reactions were carried out in volumes of 20  $\mu$ l containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2 mM MgCl<sub>2</sub>; 100 nM each of dATP, dCTP, dGTP, and TTP; 0.5  $\mu$ M primer; 1–5 ng of DNA; and 0.8 units of *Taq* DNA polymerase (Super TAQ, Stehelin Basel, Switzerland). The primers used were either those synthesized by Operon Technologies (Alameda, CA) or custom synthesized by Microsynth AG (Zürich). Amplification was performed in a Perkin Elmer Cetus Gene Amp PCR system 9600 (Norwalk, CT) programmed for two cycles of 30 s at 94 C, 30 s at 36 C, 1 min ramp to 120 s at 72 C, 33 or 38 cycles of 20 s at 94 C, 15 s at 36 C, 15 s at 45 C, and 120 s at 72 C, followed by 10 min at 72 C. Reaction products were resolved by electrophoresis on 1.5% agarose gels in 0.5 $\times$  Tris-borate-EDTA (TBE) buffer for 450 V-h, stained with ethidium bromide, and visualized with UV light (302 nm).

Primers were first screened on two isolates to determine which yielded strong amplifications. The strongly amplifying primers were screened on a set of 16 European isolates with diverse virulence haplotypes to detect polymorphisms. Where possible, the segregation of RAPD polymorphisms was tested with the progeny of crosses developed in our lab. The example RAPD primers demonstrated here are PJ02 5'ACGAGGGACT and OPE10 5'CACCAGGTGA (Figs. 1 and 2).

**Cloning of RAPD fragments for SCARs.** Six RAPD amplification products from five primers (OPE7 5'AGATGCAGCC, PJ01 5'AGGAGGACCC, OPV2 5'AGTCATCC, OPD15 5'CATCCGTGCT, and OPM18 5'CACCATCCGT; primers were synthesized by Operon Technologies, Alameda, CA, except PJ01 which was synthesized in-house) were purified from gel slices by Magic PCR Preps (Promega, Madison, WI). The protruding 3' termini of the fragments were polished with T4-polymerase before blunt-end ligation in pSK-Bluescript *Eco*RV (29). Transformation of competent *Escherichia coli* DH5 $\alpha$  was carried out following Sambrook et al (29). The cloned inserts were tested by amplification of miniprep plasmid DNA with the original RAPD primers. Double-stranded sequencing of clones was carried out as described in the USB sequenase kit (USB, Cleveland). The remainder of the sequencing reactions was used as a probe to hybridize to a Southern-blot of the original RAPD amplifications to check if the correct fragment was sequenced (data not shown). For six loci, specific primer sequences of 19–21 bases were chosen to minimize primer-pair annealing, beginning on the RAPD primer sequence or within 100 bp of the primer sequence. The pairs of specific primers were: SEGH7A.L 5'ATGTAGTGGAGAG-GGTAC, SEGH7A.R 5'AACGATGGCCTCAACGAA, SEGH-7B.L 5'CTAGTTCAGAGCCCAATG, SEGH7B.R 5'ATGCAG-CCTCACTAGCAA, SEGH1.L 5'AGTCATAGACTCAGGCAG, SEGH1.R 5'GTCCGTTATCTGAGCTTAT, SEGHV2.L 5'AGT CAC TCC CAT CAC CTT CAA, SEGHV2.R 5'AGT CAC TCC CCT CGA CCA GCC, SEGHM18.L 5'CAC CAT CCG TAA AAT AAG GTT, SEGHM18.R 5'CAC CAT CCG TTA TAA GCT CTG, SEGH15.L 5'CAT CCG TGC TGT AGC TTG CCC, and SEGH15.R 5'CAT CCG TGC TTC ATG CCA TTT. The S is for SCAR, EGH is for *E. g. hordei*, and the final numbers and letters differentiate primers. In addition, we designed extended primers from the existing primer and sequence data (CNS1EXT 5'ATATTTGAGACAAGCATATGACTACTG and CNL12EXT 5'GCTGAACGCCTCTAAGTCAGAATCC) to amplify the intergenic spacer, IGS, of nuclear ribosomal DNA, rDNA. An alignment of rDNA sequences of the following species was used to extend complements of the original two primers, NL12 and NS1(34): *Sinapsis alba* (4), *Mucor racemosus* (12), *Schizosac-*

*Saccharomyces pombe* (GenEMBL:s25rrna.em—fun) for the 25S-rDNA, and *Saccharomyces cerevisiae* (19) and *Gigaspora magerita* (30) for the 18S rDNA. These primers flank the non-transcribed spacer region of rDNA that is length-variable in many species due to subrepeating elements (14).

**PCR protocols.** For SCARs, 25  $\mu$ l of PCR solutions was prepared using 1 unit of *Taq* polymerase, 1–5 ng of template DNA, 0.1 mM dNTP, 3 mM MgCl<sub>2</sub> (for SEGH7A, SEGH7B, and SEGH1), and 1.5 mM MgCl<sub>2</sub> (for SEGHV2, SEGHM18, SEGH15, and rDNA-IGS); for 50 mM KCl, 0.01% (w/v) gelatin, 0.1% Triton X-100, and 10 mM Tris-HCl, pH 9.0. Oligonucleotide SCAR primers synthesized by Operon Technologies were used at a final concentration of 0.5  $\mu$ M for all primer pairs, except for SEGH7A, which was 0.2  $\mu$ M. A touchdown cycling profile was used for SEGH7A: 1 cycle of 94 C for 40 s, 62 C for 20 s, and 72 C for 2 min, followed by 5 cycles of 94 C for 20 s, 60 C for 20 s, and 72 C for 2 min with a decrease of 2 C per cycle for the annealing temperature and afterward 25 cycles of 94 C for 20 s, 52 C for 20 s, and 72 C for 2 min (33 cycles for SEGHV2, SEGHM18, SEGH15, and rDNA-IGS), followed by a final extension time of 5 min and 12 C until removed from the cycler.

For SEGH1 and SEGH7B, the cycling was the same, except the touchdown was from 58 to 50 degrees and there were 35 cycles of the three temperature PCR. The samples were stored at 4 C. Touchdown cycling was set up so the annealing temperature

was very high initially and was decreased in steps toward the optimum temperature so the specific products are favored in the critical initial cycles (6). Reaction products were resolved by electrophoresis on 1.5% agarose gels in 0.5 $\times$  TBE for 450 V·h, stained with ethidium bromide, and visualized with UV light (302 nm), except for SEGH7A and SEGHM18, which were run on 2% agarose gels for 750 V·h or longer.

## RESULTS

The initial screening of two DNA samples with 76 10-base primers yielded 58 that produced good amplification products under the initial reaction conditions. Twenty-seven primers were further tested on a set of 16 *E. g. hordei* isolates collected from throughout Europe. Approximately 119 resolvable bands were reproducibly amplified in total (4.4 per primer). There were 56 variable bands, each primer yielding at least one polymorphism. A subset of 10 of these primers detected 30 polymorphisms in the European test set but only 19 variable bands in 48 isolates collected with a stationary spore trap on the roof of a high building in Zürich. Primers OPE01, OPE03, OPF10, and PJ02 all produced at least three easily detectable polymorphic bands in the samples tested. We describe the variation detected with two RAPD primers and two sets of SCAR primers. A list of primers that yield variable bands in *E. g. hordei* is available on request.

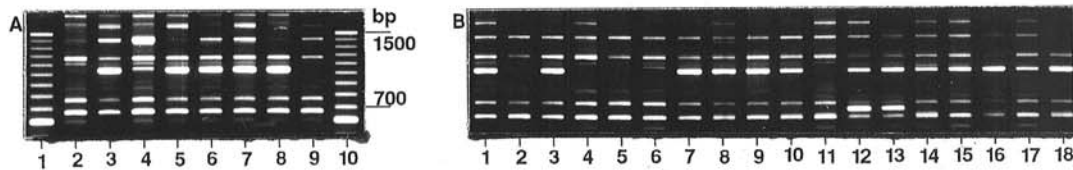


Fig. 1. Randomly amplified DNA products for primer PJ02. A, shows the segregation of variants for the parents (lanes 2 and 3) of six random progeny from cross K5, with size standards indicated on the outside lanes. B, shows 18 random isolates collected throughout Europe.

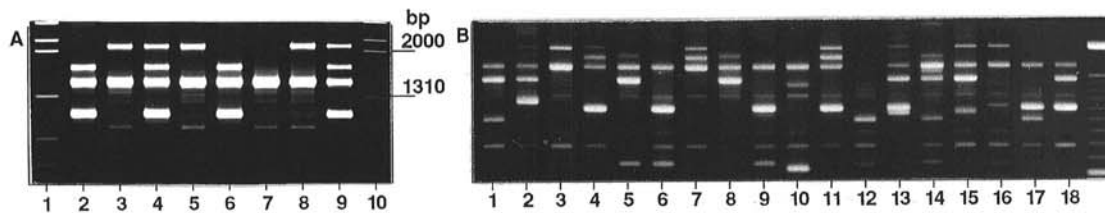


Fig. 2. Randomly amplified DNA products for primer OPF10. A, shows the segregation variants for the parents (lanes 2 and 3) of six random progeny from cross K5, with size standards indicated on the outside lanes. B, shows 18 random isolates collected throughout Europe.

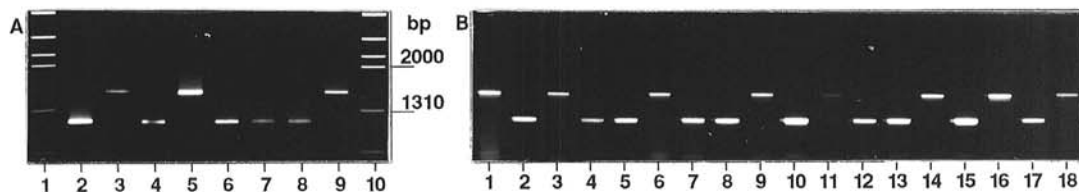


Fig. 3. Sequence characterized amplified region DNA products for the SEGH1 locus. A, shows the segregation of two alleles for the parents (lanes 2 and 3) of six random progeny from cross K5, with size standards indicated on the outside lanes. B, shows 18 random isolates collected throughout Europe.

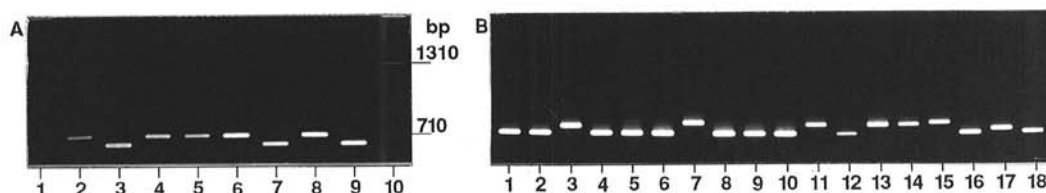


Fig. 4. Sequence characterized amplified regions DNA products for the SEGH7A locus. A, shows the segregation of two alleles for the parents (lanes 2 and 3) of six random progeny from cross K5, with size standards indicated on the outside lanes. B, shows four alleles in 18 random isolates collected throughout Europe.



Figures 1-4 have two parts: parts A show the markers in the two parents and their segregation in six random progeny of cross K5, and parts B show variation in isolates collected throughout Europe. These markers segregated in a ratio not significantly different from the expected, 1:1 (data not shown). The figures show results for: Figure 1, RAPD primer PJ02; Figure 2, RAPD primer OPF10; Figure 3, SCAR SEGH1; and Figure 4, SCAR SEGH7A. There was no length polymorphism detected with SCAR SEGH7B.

For Figure 1A (RAPD primer PJ02), three variable bands were detected with PJ02 in the progeny and designated PJ02<sub>1620</sub>, PJ02<sub>1400</sub>, and PJ02<sub>1025</sub>, where the subscripts refer to the approximate fragment size in base pairs. An additional variable band, PJ02<sub>720</sub>, was observed in random European isolates in Figure 1, lanes 12 and 13.

For OPF10, Figure 2, three bands segregated, OPF10<sub>2100</sub>, OPF10<sub>1700</sub>, and OPF10<sub>1020</sub>, the latter two showing no recombinants in 160 progeny. However, considerably more variation was detected with this primer in the European sample (Figure 2B), and, as can be seen, OPF10<sub>1700</sub> and OPF10<sub>1020</sub> were not strictly associated in random population samples, indicating that they are tightly linked but separate loci.

Figure 3A shows the segregation of the two alleles detected with SCAR primers SEGH1, SEGH1<sub>1670</sub>, and SEGH1<sub>1230</sub>. Only two alleles were detected in a broad European sample (Figure 3B).

Figure 4A shows the segregation of the two VNTR (variable number of tandem repeats) alleles, SEGH7A<sub>680</sub> and SEGH7A<sub>730</sub>, detected with SCAR SEGH7A in the cross K5. Seven alleles were detected at this locus in a broad European sample, and some of these variants are shown in Figure 4B. We determined by sequencing that the differences in these alleles are due to a variable number of tandem repeats of the 13-bp motif 5'GATTTATGTTTT. The original RAPD (OPE-07) marker from which this specific VNTR locus was produced is shown in Figure 5; the arrow indicates the region of the gel where there are five allelic bands in a set of European isolates. This highly polymorphic locus detects variation from the single-field level up to the European level. In the Zürich population of 48 isolates, only four alleles were detected.

SCARs SEGHV2, SEGHM18, and SEGH15 and IGS are not presented in the figures. SCAR SEGHV2 has yielded a 2,100-bp product, which is present or absent from individual isolates. SCAR SEGHM18 to date has detected five alleles between 680 and 750 bp, which require long gel runs on 2% agarose to resolve. SCAR SEGH15 has detected two alleles at approximately 720 and 800 bp. *Msp*I digests of the approximately 2.1-kb amplified IGS fragment yields a length variable fragment around 1,280 bp with three variants detected thus far in our samples. There were four conserved digestion products: one at approximately 600 bp and three small and difficult to resolve products.

The short-term stability of asexual lineages and consistency of DNA extractions are illustrated in Figure 6. DNA extractions were performed on conidia collected from eleven successive asexual generations of one isolate. Figure 6 shows RAPD reaction products for two primers (PJ02 and OPF10) with DNA from generations 1-4, 6, 8, and 11.

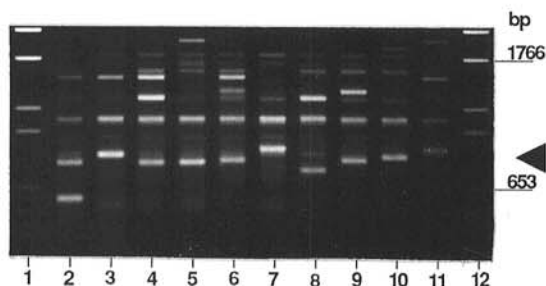


Fig. 5. Random amplified polymorphic DNA products for the primer OPE07. The arrow indicates the region where alleles were detected, which was cloned and sequenced, to produce the specific primers for the SEGH7A locus shown in Figure 4.

Our research program focuses on the population biology of *E. g. hordei* at several levels of organization, from the European scale to individual colonies (21,36). Because of the long history of work on this model pathosystem, it is well suited for detailed genetic investigations. Early studies at the national level indicated the importance of extending population surveys to a European scale. Large-scale virulence and fungicide surveys from Italy to Denmark and from Northern England to Poland and Austria have become routine (18,22,37). These studies have resolved some details of the spatial distribution of virulence and fungicide resistance and have indicated regional differentiation of populations. They also suggest large-scale movement of "clonal" lines on a European scale (2,8,17), based on observations limited to periods of 2 yr. Our interest was to further complement the phenotypic data collected from surveys and field experiments with genotypic DNA markers that can be determined for large sample sizes.

Using DNA variation, the degree of genetic relatedness can be assessed with greater accuracy than with virulence markers, which requires the assumption that the same phenotypic response has the same genetic basis. PCR-based technology is ideally suited to detect genetic variation in populations of obligate fungal pathogens where the difficulty of collecting tissue imposes constraints on the number of individuals sampled. RAPD (33,35) markers have been used recently in a wide range of studies, including mapping of disease-resistance genes in tomatoes (20), rice (28), oats (27), and lettuce (25) or race determination of *Leptosphaeria* (9), *Fusarium* spp. (10), or *Cochliobolus* (13). Several groups (32) have shown that genes segregate in a Mendelian fashion. Here we have used RAPD markers to detect genetic variation in European samples and local populations of *E. g. hordei* and to demonstrate that these markers segregate in specific crosses. It is critically important to demonstrate that DNA variants behave as Mendelian factors if they are to be used in population genetic analysis, because the majority of analyses are based on knowing allelic states of individuals.

In developing RAPD markers, our approach was first to screen a large number of primers to detect a subset that has high-yield amplification products and then to test these primers on a diverse set of isolates that would detect more than one variable band per reaction in population studies. As the number of polymorphisms and amplification consistency of arbitrary primers varies considerably for a given species, an initial screening of a large number of primers provides a safe strategy for selecting primers that will be used routinely in large studies. The stability of RAPD reactions is demonstrated in Figure 6, in which DNA was extracted from the same isolate over 11 asexual generations. The banding patterns are reproducible and do not appear to be overly sensitive to different DNA extractions. Of the several to many bands produced in a RAPD reaction, we do not use the "apparently polymorphic" low-yield bands observed on our gels; if more polymorphisms are needed, it is better to run more primers and use the strongly amplifying bands.

Concerning contamination of DNA samples in obligate fungal pathogens, such as mixtures of isolates (DNA or mixed individuals) in RAPD reactions, we have found that it often takes

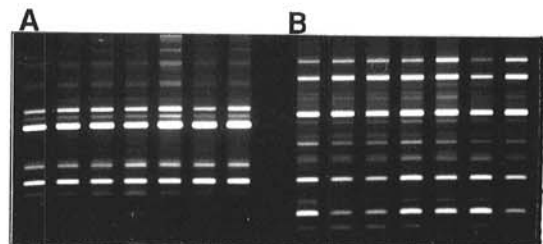


Fig. 6. Random amplified polymorphic DNA polymerase chain reaction products from seven separate DNA extractions collected over 11 successive asexual generations of one isolate. Products are shown for two primers (A, PJ02 and B, OPF10) with DNA from generations 1-4, 6, 8, and 11.

more than 5% mixture before the contaminant DNA is detectable. Also, one of the benefits of the multiallelic SCARs, especially when used on haploid genomes such as *E. g. hordei*, is that they efficiently detect mixtures because of the amplification of more than one allele per reaction. Estimates of the mixture detection rate can be roughly estimated. With even allele frequencies within loci, the likelihood of drawing two genotypes with the same alleles at all loci is 0.0071 using SCARs SEG7A, SEG18, SEG1, and SEG15, which detect 7, 5, 2, and 2 alleles, respectively. Including the four variants from the multigene tandem repeat rDNA-IGS *Msp*I digests, this number reduces to 0.0019. Also, for SEG7A, when two-way mixtures of DNA from isolates with different alleles are deliberately produced, the reactions show a characteristic ladder of more than two bands (gels not shown) presumably by heteroduplex formation (1) during PCR. These factors do not eliminate all the potential problems associated with working on obligate plant pathogens such as *E. g. hordei* but do allow for a reduction in the error rate. The final error detection rate depends on the number of multiallelic SCAR loci, the number of alleles per locus, and their frequencies in the sampled populations.

**Molecular variation.** Fifty-eight of the 76 primers tested in this study yielded good product amplification without any optimization. Twenty-seven primers detected useful polymorphisms when tested on a European set of 16 isolates. Of the 119 easily resolvable bands, 56 putative loci (47%) were polymorphic in the limited sample reported here. This represents considerable molecular variation detectable in *E. g. hordei* at the European level, consistent with the maintenance over time of large pathogen populations (38). Given this limited initial screening, it is likely that a higher proportion of the RAPD primers would detect additional genetic variation (rare bands) when tested on a large collection. We would not use these rare bands without first determining that they were indeed amplified from the *Erysiphe* genome and not the result of contamination of the particular DNA sample with foreign DNA.

Some of the variation detected in the test set of European isolates is demonstrated in Figures 1–4 parts B for RAPD primers PJ02 and OPF10 and SCAR SEG1 and VNTR SEG7A. For SEG7A and SEG18, we are developing an allelic ladder from amplified products to be run within lanes as size standards. This will allow for the accurate determination of the allelic states when screening large populations. Multiallelic markers such as SEG7A and SEG18 provide considerable resolving power to distinguish individuals in populations, relative to the presence or absence of a band for RAPD loci. When specific primers are tested on large numbers of *E. g. hordei* isolates, some additional rare alleles are likely to be detected. These rare alleles have considerable potential for directly and indirectly detecting gene flow and geographic subdivision among populations (31).

**Genetic analysis.** Figures 1–4 parts A demonstrate the segregation of some of the variants detected by RAPD primers PJ02 and OPF10 and SCAR SEG1 and VNTR SEG7A. The parents are presented in the first two sample lanes on the left. Along with other genetic markers, the loci presented in this paper (EGHV2, EGH15, and EGH18) have been shown to segregate in the 1:1 ratio expected from a haploid organism and are currently being used to generate a linkage map of *E. g. hordei* (U. Haemmerli, K. Müller, J. M. McDermott, and M. S. Wolfe, *in preparation*). The IGS-*Msp*I digest products have not been screened on the progeny of cross K5; however, the products we are detecting are most likely the result of amplification of the major length variant in the tandem array rDNA locus (14). The SCARs presented in this paper were not linked with >35% recombination among the markers in a progeny set of 146, so they are well distributed throughout the genome (U. Haemmerli, K. Müller, J. M. McDermott, and M. S. Wolfe, *in preparation*). The rDNA locus has not been mapped.

Most population-genetic analyses are based on knowing the inheritance and nature of the genetic variation being used. Whether allelic variation is due to insertions or deletions or base substitutions affects how one uses and interprets the data gener-

ated. For example, forward or reverse mutation rates would be very different for the above cases. Also, knowing the linkage of markers is essential in determining where associations are expected among variation detected at different loci. When sampling 10 loci, it is best to know if these are distributed throughout the genome or are tightly linked and, thus, represent a more limited sampling of genomic variation. In the mildew pathosystem, without the linkage data, any associations found between virulence and molecular variation are not easily interpreted.

**Local population.** When 10 primers were used to detect RAPD variation in a local sample of 48 individuals collected in a stationary spore trap, less variation was detected as compared to the polymorphism at the European level. Only 19 polymorphic bands were detected in the local sample as compared to 30 for the smaller test set of 16 European isolates. This is a decrease of approximately one-third of the number of polymorphisms per primer and is an indication of the geographic substructuring of populations.

With these 19 RAPD polymorphisms, 2<sup>19</sup> or 524,288 genotypes are theoretically possible. Obviously, the number detected is limited by the sample size, but in practice, the expected number of genotypes for a given population sample size will be reduced considerably due to variation in allele frequencies and associations among loci. Even with these constraints there is more than sufficient molecular genetic variation maintained at the field level to study local populations in detail, and sufficient variation is maintained over European samples to make precise comparisons of the spatial distribution of variation among populations.

VNTR SEG7A detected seven alleles in the European-wide test set, and only five of these alleles were detected in the Zürich population of 48 individuals. This is in accord with the results of the RAPDs, in which the local population sample had a more limited amount of variability than in the small European test set.

Our work indicates that PCR-based markers coupled with genetic analysis promises to contribute significantly to the study of the population biology of *E. g. hordei*, addressing questions concerning the population genetic structure, gene flow, selection, and reproductive system (38). Importantly, these new DNA markers will be used to complement a considerable data bank on virulence and fungicide sensitivity from the same isolates collected throughout Europe. In addition, we hope that our efforts will eventually result in the use of "standard" DNA marker sets for the analysis of barley mildew populations to facilitate cooperation and comparison of results in this model pathosystem.

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