Population Genetics of Soilborne Fungal Plant Pathogens

Population Genetics of *Pythium ultimum*

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This review summarizes our recent attempts to quantify the amount of genetic variation in populations of *Pythium ultimum*. We are interested in knowing how much variation exists, how the variation is distributed, and what role is played by sexual and asexual reproduction in generating and maintaining this variation. We began our studies to aid ongoing efforts to breed tomato cultivars with improved resistance to a ripe fruit rot, water mold, caused by *P. ultimum* (64). It is our hope that by studying genetic variation in the pathogen population we may learn something about the biology of the organism that will lead to more effective plant breeding and disease management strategies. In this paper, we will discuss the rationale for studying the population genetics of a soilborne plant pathogen, what we have learned about the genetics of *P. ultimum* populations, the potential pitfalls of our approach, and the implications of this work for controlling *Pythium* disease.

P. ultimum, like other species in the genus *Pythium*, is variable with respect to linear growth in culture, the ability to discharge zoospores, the production of sporangia, and the production of oospores (49,80). Variation in pathogenicity attributes has not been the subject of detailed quantitative studies with natural inoculum (49). However, a variety of studies suggest that variation in virulence and aggressiveness exists and that this variation affects the expression of host resistance (20,24,60; K. S. Aitken, D. M. Francis, and D. A. St. Clair, *unpublished data*). Efforts to develop resistant cultivars and chemical control strategies should take into account this variability and the mechanisms that generate and maintain the variation. The field of population genetics can offer theory, tools, and approaches that will help us understand more about the populations we are trying to control and give us insight into the ecology and biology of the organisms. In the following discussion, we consider "populations" to be isolates collected in the same location at the same time. The term "population structure" refers to the amount of genetic variation among individuals in a population, the spatial and temporal subdivision of variation, and the phenetic relationships between individuals (41).

The Rationale for a Population Genetics Approach

The knowledge that single gene changes in pathogen isolates can affect the durability of resistance in host plants has led to management strategies that require monitoring of the pathogen population. The survey of virulence in populations of airborne pathogens is routine for many diseases when a differential series of cultivars is available. The practical application of population genetics, therefore, is not new to plant pathology or breeding. In their

recent reviews, Leung et al. (41) and McDonald and McDermott (56) discussed gene deployment approaches to disease management advocated by Browning and Frey (12), Robinson (67), and Wolfe and Barrett (84). The approaches discussed use multilines and combinations of resistance genes to slow pathogen evolution and were initially based on an understanding of pathogen population structure derived from genetic studies with avirulence and host resistance genes. More recent work has incorporated molecular markers to bolster information on pathogen population genetics (10, 11,29,43). One notable feature of most of the studies discussed in the recent reviews is their emphasis on the development and deployment of major genes for resistance against pathogens displaying high levels of host specificity. A population genetics approach is also appropriate for pathogens displaying broad host ranges. Identification of the range of genetic variation in pathogen populations will improve the ability to screen and select for resistant germ plasm, facilitate the breeding of cultivars, and refine the development of cultivars for specific locations. Current interest in the population genetics of plant pathogens is driven by the desire to improve the durability of resistance (41), and several examples of how population genetics can facilitate this goal are provided in recent reviews (41,54,56).

One assumption behind the population genetics approach is that the forces that shape genetic variation also influence pathogenicity attributes. The converse of this assumption, that genetic differences as reflected by some measure of genetic distance will be reflected in differences in virulence and aggressiveness, is not necessarily true. When populations are clonal, such relationships may exist. However, for sexually reproducing populations we would not expect a correlation between genetic distance and pathogenicity attributes (41). It, therefore, is worth asking whether the focus of population studies should emphasize genetic markers or pathogenicity attributes. For organisms that exhibit strong host specificity, virulence phenotype itself is a powerful marker. In the absence of a suitable host differential, when the host-pathogen interaction is difficult to evaluate, and when the interaction is environmentally influenced, virulence data offers little insight into the structure of populations. The latter case exists for many soilborne plant pathogens and when the host-parasite interaction is mediated by quantitatively inherited resistance and virulence. Recent studies have indicated that some quantitative resistance may be race specific (39), and data on pathogen populations will be essential to using gene deployment strategies that seek to prolong the life of host resistance even for such quantitative interactions. In the case of *Pythium* species, the source of inoculum also may affect pathogenicity attributes such as sporulation (63) and, therefore, may affect the outcome of disease screens. Obtaining virulence data for large samples of *P. ultimum* isolates is therefore impractical yet it is precisely this sort of information that we would like to obtain.

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The use of genetic markers may offer insight about the distribution of alleles and genotypes (i.e., the structure of populations) that will assist in the development of new control strategies. In a practical sense, markers are often easier to score than pathogenicity attributes and are less subject to environmental variation. Markers also can complement virulence data, even in cases in which the host-pathogen interaction is easy to score and highly specific. Differences in genetic structure as measured by virulence data, fungicide adaptation, or neutral genetic markers can provide information about the forces that shape populations (85). Several other examples are cited in reviews (41,54,56). The challenge is to relate molecular marker data to the biology of the organism, using the genetic information to help formulate experimental approaches that elucidate the forces contributing to variation in pathogenicity attributes. The first step in applying genetic information to control strategies is to determine whether populations are similar in different growing regions and whether a population is sexual or clonal. The choice of isolates and selection of field test locations for breeding purposes depends on pathogen subdivision (56), and strategies for deploying resistant cultivars or applying pesticides differ for clonal organisms and sexually recombining organisms (41,56,78).

Features of ecology, life cycle, mating system, and genetics must be considered before applying the theory and methods of population genetics to any organism. In general, the basic mechanisms that generate variation are mutation, recombination, and migration. Variation is shaped by the forces of selection and genetic drift. Major selective forces may be imposed by the degree of specialization in host-pathogen interactions, control measures, or more general environmental constraints (17). The mating system of plant pathogens strongly influences how rapidly populations respond to selective forces (41). Population genetics theory was developed for obligately outcrossing diploid species, and much work has been done to transfer this theory to the study of haploids. Organisms that are soilborne, homothallic, diploid, and coenocytic, therefore, present a series of complications that affect genetic assumptions, and given these features, *P. ultimum* is not an ideal model for studies of population genetics. However, with proper sampling and selection of markers, the theoretical framework of population genetics may allow us to answer several questions concerning the biology, ecology, and genetics of *P. ultimum*.

Ecology

The soilborne habitat of *P. ultimum* is an important feature of the ecology that affects its population structure. It is unclear how much the soil restricts movement. Spores may be transported by insects (27,28). Water from rain and irrigation also facilitates movement, especially when zoospores are produced. Spread of spores also is possible in soil carried by wind or farm equipment. Despite these mechanisms, movement may be restricted relative to organisms that produce airborne spores. Studies on the population genetics of plants suggest that limited dispersal of seed or pollen can lead to population subdivision (44). In the case of the soilborne fungus *Fusarium oxysporum*, samples from undisturbed soil that were separated by a short distance contained genetically distinct populations, whereas samples from agricultural soil failed to show substructuring (30). It is probable that soilborne pathogens will display more subdivision than pathogens that produce airborne spores.

The mechanisms that allow *P. ultimum* to survive in soil also affect population structure. The sexual spores, oospores, can survive for months to years depending on soil type and environmental conditions (45). Oospore germination is not synchronous (31,45), and dormancy may extend the effective generation time by allowing an isolate's progeny to enter a population over a period of time in a way that is similar to seed dormancy in higher plants (44). The asexual spores of *P. ultimum* can survive for 11 months in soil (76), and these sporangia may also serve as a resting structure. Asexually reproduced clones, therefore, may be propagated year to year.

Taxonomy

Oomycetes such as *P. ultimum* commonly are considered fungi but are probably most closely related to the chrysophyte algae (4,13). Two taxonomic forms of *P. ultimum* are recognized, and a third is supported by morphological, immunological, molecular, and genetic criteria (71,80). *P. ultimum* var. *sporangiferum* isolates produce abundant motile spores at room temperature. In contrast *P. ultimum* var. *ultimum* does not produce zoospores. Most isolates are able to complete the sexual cycle in pure culture. The third taxonomic category consists of some self-sterile isolates belonging to *Pythium* groups. Isolates in several self-sterile *Pythium* groups (e.g., HS and G) are not given a species designation because they do not produce the sexual cycle in pure culture or in pairings with other heterothallic isolates (80). However, the growth rate, morphology in culture, and pathogenicity attributes of many self-sterile isolates belonging to *Pythium* group HS are similar to *P. ultimum* (48). This relationship is supported by a number of criteria reviewed by Saunders and Hancock (71). There is also some evidence that isolates belonging to *Pythium* group G are a morphological variant of *P. ultimum* (36). The morphological groups represent major classes in which more subtle intraspecific variation for traits such as growth rate in culture, sporulation, and pathogenicity attributes also exist (24,49). These classes represent variation that affects dispersal and sexual reproduction. Therefore, it is possible that populations of *P. ultimum* will be structured differently depending on the relative abundance of these morphological types.

Life Cycle

The life cycle of *P. ultimum* suggests several features that may lead to genetic variation in the absence of sexual reproduction. *Pythium* species typically are diploid throughout the vegetative portion of their life cycle (21,73), although higher ploidy levels may exist (21). The vegetative cells may reproduce asexually for extended periods of time. With sporangia serving as a resting structure (76), asexual reproduction could maintain a clone for years in nature. In the vegetative cells, the hyphae are coenocytic; no septae are present, and many nuclei coexist within the same cytoplasm. With coenocytic organisms, the definition of an individual for population level studies is complicated. Anastomosis of hyphae has not been demonstrated in any *Pythium* species, and hyphae generally are assumed to be homokaryotic. However, the potential for heterokaryosis to arise through mutation is great, because nuclei may be propagated for long periods of time without passing through the sexual cycle. Asexual spores are multinucleate and can germinate without producing the single-celled motile zoospore. Heterokaryosis arising from mutation, therefore, can be propagated indefinitely and is analogous to heterozygosity in a diploid monokaryon. In addition, it is believed that selection pressure for precise partitioning of the genome in some coenocytic organisms is low because the effect of mitotic errors is diluted. Inefficient partitioning in mitosis or "mitotic instability" can lead to polyploidy and aneuploidy (32). Although no detailed studies of mitosis have been performed for any *Pythium* species, the cell cycle in related organisms characteristically is short and prone to partitioning errors (32).

Mating System

In *P. ultimum*, sexual reproduction occurs through unequal gametangia. The oogonium provides most of the cytoplasm and nutrition for the thick-walled oospore and is considered the female gamete. The antheridium is the male gamete. Antheridia and oogonia often arise from the same thallus (i.e., monoclinal), a condition that favors self-fertilization. However, antheridia not directly connected to the oogonia occasionally participate in fertilization (Fig. 1; [80]). The occurrence of these declinal antheridia suggests there is no physical barrier to outcrossing in this homothallic spe-

cies. Based on morphological criteria, homothallism in *P. ultimum* does not appear to require obligate self-fertilization.

We used genetic markers to demonstrate that *P. ultimum* isolates representing the three morphological classes can intermate, providing genetic evidence that the different morphological types are members of the same species. Crosses in the lab yield viable hybrid progeny that can self-fertilize and produce viable progeny. Self-sterility segregates in hybrid progeny derived from crosses between homothallic and group HS parents. The genetic data favoring the placement of some *Pythium* group HS isolates with *P. ultimum* now include mitochondrial DNA polymorphisms (48), nuclear DNA polymorphisms, and the ability to intermate (24,25; D. M. Francis and D. A. St. Clair, *unpublished data*). It is likely that the self-sterile or HS isolates we examined are male forms of *P. ultimum* (70,71). The mating system of *P. ultimum*, therefore, is analogous to androecy in plants, both hermaphrodites and males exist, and both inbreeding and outcrossing are possible.

Genetics

Our understanding of the genetic implications of sexual reproduction in *P. ultimum* is based on a few cytological studies of a species of *Pythium* that is no longer recognized (*P. debaryanum* [80]) and the genetic studies summarized below. In *P. debaryanum* meiosis was observed in the oogonia and antheridia just prior to fertilization (68,69). Sansom's (68,69) work suggested that three of the four nuclei produced by meiosis degrade in the oogonium, leaving a single egg nucleus. In the antheridium, one postmeiotic nucleus enters the oogonium through the fertilization tube first. The remaining three nuclei enter the oogonium later but appear to degenerate. More recent cytological studies using electron microscopy imply that resting oospores of *P. ultimum* contain a single nucleus that proliferates as dormancy is broken (33). One nucleus from the antheridia and one from the oogonia appear to be involved in fertilization. There is also some cytological evidence that polyploidy may occur in *Pythium* species (21).

The inheritance of markers that we have studied in lab crosses is consistent with diploidy in some isolates of *P. ultimum* (24,25; Fig. 2). We have shown that *P. ultimum* can outcross by mixing isolates and plating them on media that allows the formation of oospores. Outcrossing rates from 1 to 10% could be detected in the single oospore progeny of such laboratory crosses (24,25; D. M. Francis and D. A. St. Clair, *unpublished data*). Selfing of hybrid progeny generated populations that behaved according to Mendelian expectations for F_2 segregation and independent assortment (24,25; Fig. 2). In addition to confirming diploid inheritance, the populations generated in our lab crosses have been useful in establishing the allelic relationship between restriction fragment length polymorphism (RFLP) bands.

Fig. 1. Oogonium of *Pythium ultimum* with A and B, both monoclinous antheridia and C, diclinous antheridia.

Our genetic studies also have identified some isolates that are mitotically unstable, heterokaryotic, and polyploid. In cultures derived from hyphal-tip transfers of the isolate Pu18, we identified subisolates that differed genetically from the original isolate (24) by loss of an RFLP band. Our recent work has clarified the genetics of the marker used, identified several other genetic variants, and incorporated karyotypic analysis using pulsed-field gel (PFG) electrophoresis (D. M. Francis, D. A. St. Clair, and F. N. Martin, *unpublished data*). We have found that genetic variants identified by loss of RFLP bands usually involve the loss of an allele. Karyotypic analysis showed that Pu18 and its asexual derivatives had many more chromosomes than other group HS isolates examined, which is consistent with polyploidy (D. M. Francis, D. A. St. Clair, and F. N. Martin, *unpublished data*). The chromosome banding pattern in mitotic derivatives often differed from the original isolate, with variation detected for both chromosome length and number. In addition to the allelic and karyotypic variation detected, we found single hyphal-tip derivatives that differed in growth rate, sensitivity to growth inhibitors, and pathogenicity attributes (D. M. Francis, D. A. St. Clair, and F. N. Martin, *unpublished data*). These data suggest that mitotic instability can cause genetic differences that create a heterokaryon. Not all isolates exhibit somatic instability, and the mechanisms involved may include either recombination or partitioning errors during nuclear division. The isolates that have exhibited mitotic instability appear to have more chromosomes or duplicate chromosomes relative to mitotically stable isolates. This observation suggests that polyploidy, aneuploidy, or heterokaryosis may play a role in mitotic instability, but more data are necessary to confirm this hypothesis. The genetic data suggesting the existence of heterokaryosis and polyploidy in some isolates is consistent with cytological data for *Pythium* species (21), morphological data for *P. ultimum* (14), PFG karyotypic analysis of *P. sylvaticum* (50), and genetic data for *Bremia lactucae* (37,72).

Population Genetics of *P. ultimum*

Genetic approaches have not been applied widely to natural populations of the genus *Pythium*, and descriptive questions about the amount and distribution of genetic variation must be answered be-

Fig. 2. Amplified fragment length polymorphisms were used to detect genetic differences between a *Pythium ultimum* group HS isolate, Pu23, and a homothallic *P. ultimum* isolate, Pu7. Self-fertile hybrid progeny derived from a mixed mating (24) can be used to generate segregating F_2 progeny. The resulting segregation ratios are consistent with diploid inheritance and suggest an independent assortment of the three polymorphic bands indicated by arrows (>) to the left of the columns.

fore mechanistic questions can be posed. For example, it is not possible to address questions of outcrossing rate and gene flow until we can answer questions concerning allele frequencies, the mating system of the organism, and the geographic scale over which variation is found. The number of locations and geographic distribution of sampling sites required will depend on population substructuring and allele frequencies that cannot be estimated without a prior survey; thus, iteration is often necessary (7). As a prerequisite to population genetics, polymorphic markers must be identified at each location, allele frequencies must be estimated (because these affect sample sizes), and Mendelian genetics of marker loci must be confirmed.

The first question we asked was “Is there genetic variation among isolates of *P. ultimum*?” Previous studies indicated we should find some variation, because protein electrophoresis (1), isozymes (17), and ethidium bromide banding patterns (42) had all been used to evaluate variation between isolates. The potential for differences in mating strategies between species in the genus *Pythium* and within populations of *P. ultimum* were not considered in previous studies, and marker data were not interpreted as if they were derived from a potentially outcrossing diploid species. Our study was designed to compare the ability of RFLP and polymerase chain reaction-based random amplified polymorphic DNA markers to detect genetic polymorphism. We were interested in obtaining rough estimates of the percentage of polymorphic loci and the average number of alleles per locus. In addition, we were interested in de-

tecting heterozygosity that might indicate outcrossing in the field. For these reasons, we chose to include codominant markers for which some allelic relationships had been determined. The ability to generate crosses in the lab (25) proved useful for establishing the Mendelian inheritance of markers and determining allelic relationships for the markers. Codominant markers indicated that the group HS isolates we examined have high levels of heterozygosity. These results suggest that the group HS isolates are outcrossing in nature. However, we chose to use a collection of isolates rather than isolates sampled from a population. This decision allowed us to work with well characterized representatives of the three morphological types but limited our ability to draw quantitative conclusions concerning the frequency of outcrossing in natural populations (25).

Recently, we have been collecting genetic data from a larger sample of isolates obtained from two fields and a greenhouse. Because this work is still in progress, we will limit our discussion to the more qualitative conclusions. Discussion of the quantitative analysis in progress will be limited to a review of the approaches we are using. The goal of this study was to begin to collect allele-frequency data that would allow us to ask more quantitative questions concerning the distribution of genetic variation, the rate of outcrossing, and the frequency of heterokaryosis. The first lesson we learned was that markers polymorphic in a collection representing a large geographic area may not be useful when the scale of sampling is changed. Despite detecting an average of two alleles per locus in the collection, only one of the low-copy RFLP markers used in our first study detected polymorphism between isolates from the two fields and greenhouse. We have detected more polymorphisms with midreplicative RFLPs that hybridize to 25 to 35 bands (Fig. 3). The midreplicative RFLPs were scattered throughout the genome, based on both segregation analysis and hybridization to blots of PFG karyotypes (D. M. Francis, D. A. St. Clair, and F. N. Martin, *unpublished data*). We have not yet been able to elucidate allelic relationships in the complex banding patterns characteristic of the midreplicative markers and, therefore, have not been able to accurately quantify levels of heterozygosity in the field samples. However, in the rare cases in which we can interpret bands as alleles, the two banded phenotypes associated with heterozygosity also are associated with self-sterility. This observation is consistent with the results of our first study, in which we concluded that group HS isolates tend to be heterozygous but that heterozygosity is rare in homothallic isolates.

Our sampling was based on a hierarchical design (3), with multiple isolates collected from the same infected tissue, the same grid location in a field, and multiple grids per field. Sixty-seven isolates were collected from a field planted with processing tomatoes, twenty-one from a field planted with beans, and fifteen from infected tomato roots in a greenhouse. Several qualitative statements can be made based on the sampling and the genetic data. First, there is a patchy distribution of inoculum in the field, because adjacent grids had different concentrations of *P. ultimum*. Both seasonal and spatial fluctuations in inoculum distribution have been reported previously for *Pythium* species (2,22). Second, genetically distinct isolates are found in the same soil sample and infect the same tissue (Fig. 3). Third, some clonal structure is ap-

Fig. 3. Random amplified polymorphic DNA (RFLP) banding patterns indicate that isolates from the same field are genetically diverse and that distinct isolates infect the same plants. Banding patterns detected with RFLP probe PG303 are shown for 11 isolates from two locations (grids 69 and 72) in the same field. Isolates indicated by an asterisk were collected from the same infected tomato fruit after a late-season rain during October 1994.

TABLE 1. Number^a of individuals^b from two samples required to detect differences in the frequency (*f*) of alleles *A* and *a*

Allele frequency (<i>f_A</i>)					Difference (<i>f_A</i> - <i>f_a</i>)
0.55	0.70	0.80	0.90	0.95	
2,081	1,766	1,345	756	400	0.05
520	444	337	189	102	0.10
132	110	85	50	50	0.20
22	20	14	25	25	0.50

^a Number based on a chi-square test for homogeneity with a significance level of 5% and a power of 90% (7).

^b Numbers are based on diploid individuals.

parent, because 23 genotypes were found in the tomato field, 8 in the bean field, and only 1 in the greenhouse. One genotype accounted for 32% of all isolates in both fields. All 15 isolates from the greenhouse had an identical genotype that was not present in either field. The qualitative data we collected suggests several questions worth investigating. A few of these questions and possible approaches are discussed below.

Future Population Genetic Studies

Over what scale are differences between “populations” detected? The question of how genetic variation is distributed must be answered before questions that seek to clarify the mechanisms that generate and maintain variation can be answered. Approaches and statistical considerations for measuring genetic variation are described by Brown and Weir (9). Understanding the geographic range over which patterns of genetic differentiation among samples exists is important to plant breeders. The answer to this question allows us to determine whether we are breeding for different populations in different regions. A practical consequence is the determination of the geographic range over which field trials should be performed (56) or the specific isolates that should be used in artificial screens. The sizes of the samples taken from the two fields were small, and our ability to detect differences between the two fields was limited to large differences in allele frequencies. As a point of comparison, population level studies for pathogens with airborne spores can involve thousands of samples (85). Based on our data we cannot conclude that the two fields are different, suggesting that the patchy distribution of inoculum is not extended to a patchy distribution of genotypes. Because our ability to detect differences in allele frequency is limited by the size of the samples (Table 1; 7) we would like to use other methods to analyze substructuring within a field.

A second approach to defining geographic substructuring is to perform a spatial analysis of genotypes by matrix correlation analysis (46) as described by Milgroom et al. (58,59) and Kohli et al. (40). Nonrandom clustering of genotypes may be interpreted as evidence of restricted dispersal, and the absence of clustering may indicate that gene flow is not restricted locally (58). Spatial analysis of genotypes is probably a more appropriate approach for the smaller samples that generally are available to those studying soil-borne pathogens.

Is there gene flow between populations? As we accumulate genotypic data from a number of locations, the data can be compared using methods that facilitate the estimation of more complex functions of gene frequencies, such as hierarchical F statistics (86) and gene diversity (61,62). Data from the field sites and subdivisions from each field can be analyzed and compared using these approaches. Calculation of hierarchical F statistics and Nei's diversities allows us to measure how the populations have differentiated by describing how variation is partitioned between and within individuals. The theory and methods used to estimate gene flow are discussed by Slatkin (75) and in previous reviews (54). The sample size required for these studies generally is large.

What is the contribution of outcrossing to genetic variation? A sexual cycle that permits both inbreeding and outcrossing could provide a powerful mechanism for generating new combinations of alleles. The rate of outcrossing within a population greatly affects the distribution of genetic variation within a population and could affect the durability of host resistance. In addition, some isolates are mitotically unstable, and variation also may be generated in the absence of sex (24). Understanding the relative importance of sexual and asexual reproduction and self-fertilization and outcrossing has important implications for estimating how fast populations will respond to selection. For example, in pathogens that reproduce by asexual means only, the destiny of a new mutation is directly related to the fitness of the clone. Favorable selection depends on both the novel mutation and the genetic background in which it arises. In contrast, new mutations that arise in sexually

reproducing and outcrossing populations can be recombined into new genetic backgrounds, some of which may be more favorable to the propagation of the mutation. The approaches used to study population genetics can help us understand which mechanisms play a role in nature and at what frequency they occur. Several approaches are available for estimating the rate of outcrossing, but inferences are limited by the scale of sampling. In addition, the features of the ecology and life cycle discussed above also affect the interpretation of results.

The data we collected suggests that sexual outcrossing may play a role in generating and maintaining variation in *P. ultimum* populations. Isolates are capable of outcrossing in the laboratory; heterozygosity was detected in isolates recently obtained from the field, and genetically distinct isolates infected the same tissue. The analytical approaches discussed above may allow us to estimate the frequency of outcrossing in nature once we have collected genotypic data using a larger number of codominant markers. Our estimates of outcrossing based on the observed frequency in lab crosses range from 1 to 10%. Field rates are likely lower due to the requirement of genotypes coinfecting the same tissue.

The number of markers and sample sizes required for estimating outcrossing may differ from those required to estimate whether two samples are collected from the same population. The actual level of outcrossing is the most important factor in calculating the number of markers required (74). For inbreeding populations, large samples analyzed with a few polymorphic markers would minimize the variance of the outcrossing estimate. For outcrossing populations, more statistical efficiency would be gained by genotyping a smaller sample with more markers (74). Multilocus approaches are favored as the outcrossing rate approaches 0.5 (74). For studies aimed at estimating the amount of outcrossing, the most useful markers are those with gene frequencies between 0.2 and 0.5 (8). However, this is the region of highest sampling variance, when F is between 0 and 0.3 as is found in outcrossing species (8). Tests for departure from the Hardy-Weinberg equilibrium (HWE), therefore, are insensitive for outcrossing species, and the sampling variance associated with inbreeding ($F > 0.9$) is lower by one-fourth (8). In this case, the sampling properties of the F statistic favor studies on inbreeding species.

Estimations of outcrossing also may be obtained by examining the tendency of unlinked markers to be inherited together. Tests of linkage disequilibrium are a logical next step to studies based on HWE and may be tested for in nonrandom mating populations (81). We do not expect *P. ultimum* populations to be under HWE, because of self-fertilization and restricted movement caused by its location in the soil. The expected violations of HWE due to the life cycle and natural habitat of *P. ultimum* would bias the population toward inbreeding, and estimations of outcrossing, therefore, would be conservative. The use of measures of linkage disequilibrium, therefore, are an attractive approach to the problem of estimating outcrossing.

In addition to different approaches to analysis of genetic data, different sampling schemes may increase the ability to estimate outcrossing rates in natural populations. For example, Wright's F statistics can be calculated from “clone-corrected” data sets (55). An alternative would be to resample the fields and collect isolates from oospores using methods described by Stasz and Martin (77), because these represent only the sexually reproducing portion of the population. This approach is similar to that used by Brown and Wolfe (11) who timed their sampling to correspond to the release of sexual spores.

What is the role of asexual variation and heterokaryosis? Our lab studies have suggested that some isolates are somatically unstable (24) and these isolates tend to be heterokaryotic, polyploid, or heterozygous (D. M. Francis and F. N. Martin, *unpublished data*). The occurrence of somatic instability and heterokaryosis must be accounted for in sampling schemes and marker choice. Markers that detect more than two alleles at a single locus are

useful for detecting heterokaryosis or polyploidy in diploids (37). In the absence of such markers, estimates of heterokaryosis or polyploidy may be obtained by including karyotypic analysis of samples collected from populations. The importance of heterokaryosis, mitotic instability, polyploidy, and aneuploidy to genetic variation in *P. ultimum* populations remains unclear. For example, mitotic recombination in a heterozygous diploid leads to both heterokaryosis (increased diversity of nuclei within a common cytoplasm) and reduced heterozygosity in some nuclei (less diversity within an individual nucleus). In the absence of sexual outcrossing, asexual mechanisms may be capable of generating new combinations of genes or alleles. A better understanding of the frequency in nature and the mechanisms involved may help to clarify the importance of these non-Mendelian phenomenon.

Conclusions

Does the genetic data tell us something about the organism that will allow us to control disease more effectively? This question highlights the greatest future challenge, the attempt to relate genetic data to important phenotypic traits. Although our studies are still largely descriptive, several observations suggest areas of future work that may help relate the genetic data to important questions concerning the biology of *P. ultimum*.

First, for breeding purposes it is clear that plant material should be screened with more than one isolate. These isolates should be abundant in fields where disease is a problem, and the isolates should be aggressive. Unmodified field soil is not a good source of material for screening plant germ plasm or breeding lines due to the patchy distribution of isolates and undefined genetic make up of the isolate population. Without further studies on the interaction between host genes with specific isolates, it is difficult to make recommendations of the exact number of isolates to use for such screening. For now, the number of isolates used will likely be decided by practical constraints imposed by disease screening. The ability to detect interactions between isolates and host genes will be facilitated by breeding approaches to generate populations of genetically uniform lines (e.g., inbred backcross, advanced backcross, and recombinant inbred designs) that provide enough plant material to test against a variety of isolates. In addition, the application of methods that allow dissection of Mendelian factors underlying quantitative resistance may allow breeding progress that would have been difficult until recently (57).

Second, an interesting observation from our data is that group HS isolates tend to be heterozygous, whereas homothallic isolates tend to be homozygous. Our observations from lab crosses suggest that the HS phenotype segregates in F1 progeny (24). A cross in nature, therefore, should result in heterozygous HS isolates and heterozygous homothallic isolates. Theoretical studies have suggested that androdicey would be unstable unless males have a higher fitness relative to hermaphrodites (15). Reevaluation of androdicey based on these theoretical considerations revealed that functional androdicey was rare in plants (16), and in cases where androdicey was not ruled out, studies have confirmed a higher fitness for males (26). The low abundance of heterozygous homothallic isolates found in our studies may suggest that the HS isolates largely are maintained through asexual reproduction, whereas homothallic isolates continue to reproduce sexually by self-fertilization or that HS isolates have a higher fitness than their homothallic siblings. Although these observations should be considered preliminary, they provide an example of how genetic data may allow inferences to be made about the biology of the organism studied. Our genetic observations suggest that studies should be conducted to evaluate the relative fitness of group HS isolates and homothallic isolates. If future studies reveal a difference in fitness or reproductive strategy between group HS isolates and homothallic isolates, it may be possible to modify cultural practices to differentially target populations.

The utility of a population genetics approach for clarifying features of oomycete biology, with subsequent implications for con-

trol of disease, has been demonstrated by work on *Phytophthora sojae* and *B. lactucae*. Marker data has suggested the existence of sexual outcrossing in field isolates of the homothallic oomycete *Phytophthora sojae* (23). Work based on phenotypic markers (5,6) suggested that outcrossing could occur under lab conditions, and evidence based on neutral genetic markers has provided data supporting the occurrence of outcrossing in the field (23). Markers have allowed controlled crosses to be selected and pathogen genes controlling virulence to be mapped in *P. sojae* (79,82,83). The existence of sexual outcrossing suggests that the race concept may prove inadequate to describe variation in virulence and suggests that strategies of resistance breeding in soybean that rely on tolerance may provide more durability (51,52). In the case of *B. lactucae*, data on fungicide resistance, virulence gene distribution, and markers has been used to facilitate control decisions that differ for the clonal population in California and sexually reproducing populations in Europe (18,19,37,38,72).

In addition to the implications of population level genetic analysis for resistance breeding and disease management strategies, comparative studies of the genus *Pythium* represent a good opportunity to elucidate the role of pathogen specialization and sexual reproduction in determining the structure of pathogen populations. Considerable variation in pathogenic specialization exists within the genus *Pythium* (35,53). For example, *P. ultimum* has a wide host range encompassing monocots and dicots, whereas *P. arrhenomanes* is restricted to monocots (17,35). Species in the genus *Pythium* also express diverse patterns of sexual reproduction. Most species in the genus are homothallic (self-fertile), but heterothallic (outcrossing) species have been identified (34,35,80). In one heterothallic species, *P. sylvaticum*, the mating system is sexually dimorphic, with antheridia and oogonia separated on different individuals (47,67). There also are reports of *P. sylvaticum* isolates that are not strictly male or female (65,66), although conclusions were based on morphological comparisons, not on the inheritance of markers. Mating systems may be equally complex for homothallic species in the genus (24,25,48,71). The genus *Pythium*, therefore, contains pathogens with a range of host-pathogen interactions involving different levels of specialization and diverse mating systems involving varying degrees of outcrossing and inbreeding. Data for species representing various levels of host-pathogen specialization, diverse niches, and different mating systems could be used to elucidate the forces contributing to population structure.

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