Genetic Variation in Homothallic and Hyphal Swelling Isolates of *Pythium ultimum* var. *ultimum* and *P. utlimum* var. *sporangiferum*

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Genetic variation in a collection of 22 *Pythium ultimum* isolates was analyzed using restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), and sequence characterized amplified regions (SCARs) as genetic markers. Qualitative evidence for the occurrence of sexual outcrossing in the field, asexual mechanisms affecting variation, and differences in aggressiveness between isolates was found. Codominant SCAR and RFLP markers detected multiple alleles in several isolates. Genetic analysis of F_1 progeny from a cross indicates that heterozygosity is one cause of multiple alleles and contributes to genetic variation. Segregation analysis of F_2 progeny fit diploid expectations and supported the use of the molecular markers for phenetic analysis. One isolate contained three alleles at one locus suggesting that polyploidy, aneuploidy or heterokaryosis may also contribute to genetic variation. Phenetic analysis using UPGMA clustering of Nei's distance calculated from RFLP data, UPGMA clustering of similarity matrixes calculated from RAPD data, and principle component analysis of RAPD data revealed no clustering of the three morphological types of *Pythium ultimum* (var. *ultimum*, var. *sporangiferum*, and group HS). Our results suggest that the three morphological variants of this homothallic oomycete are not genetically distinct.

The oomycetes express diverse and complex patterns of sexual reproduction. They are typically diploid (Shaw 1983), although higher ploidy levels may exist (Dick 1972). The genus *Pythium* contains mostly homothallic (i.e., self-fertile) species (Van der Plaats-Niterink 1981), but heterothallic (i.e., outcrossing) species have been identified (e.g., Campbell and Hendrix 1967; Hendrix and Campbell 1968). In one heterothallic species, *P. sylvaticum* Campbell & Hendrix, the mating system is sexually dimorphic for specific pairings (Pratt and Green 1971; Martin 1989). However, many isolates are not strictly male or female, and some isolates are homothallic (Pratt and Green 1971, 1972; Gavino and Martin 1993). This complex pattern of sexuality is common for other heterothallic oomycetes in the genera *Achlya* (Raper 1940, 1960) and *Phytophthora* (Gallindo and Gallegly 1960). We previously observed that the homothallic oomycete, *P. ultimum* Trow var. *ultimum*, is capable of outcrossing and that *Pythium* group HS isolates (Van der Plaats-Niterink 1981) can produce viable hybrid progeny in crosses to homothallic *P. ultimum* isolates (Francis and St.Clair 1993). *Pythium* group HS isolates are unable to complete the sexual stage in pure culture, and the terminal hyphal swellings are thought to be antheridia (Saunders 1986; Martin 1990). The existence of the HS trait in some *P. ultimum* isolates may force outcrossing and suggests that mating systems may be equally complex for homothallic species in the genus *Pythium*.

Although *P. ultimum* has few morphological characters suitable for population studies, there is variation in traits that affect both the mode of dispersion and sexual reproduction. For the purposes of this paper, we consider three morphological groups: homothallic (HO), hyphal swelling (HS), and zoospore-producers (var. *sporangiferum*). The most common isolates of *P. ultimum* are homothallic and fail to produce zoospores (Van der Plaats-Niterink 1981). In contrast, *P. utlimum* var. *sporangiferum* Crechsler produces abundant zoospores at room temperature (Van der Plaats-Niterink 1981). Group HS isolates of the genus *Pythium* (Van der Plaats-Niterink 1981) belong with *P. ultimum* based on mitochondrial polymorphisms (Martin 1990) and on the ability to cross to homothallic *P. ultimum* isolates (Francis and St.Clair 1993).

Little is known about the relationship among the three morphological groups in *P. ultimum*. Studies to date have used protein electrophoretic patterns (Adaskaveg *et al.* 1988), isozymes (Chen *et al.* 1992), and banding patterns in ethidium bromide-stained gels of digested DNA (Levesque *et al.* 1993). In the study of Adaskaveg *et al.* (1988), an isolate of *P. utlimum* var. *sporangiferum* could not be distinguished from isolates which fail to produce zoospores based on protein banding patterns (Adaskaveg *et al.* 1988). The data of Chen *et al.* (1992) suggest that isolates which do not produce oospores in pure culture, presumably HS isolates, are closely related to homothallic isolates. These data suggest that the morphological types are not genetically distinct.

As a prerequisite for studying genetic variation in field populations of *P. ultimum*, this study was conducted to examine genetic polymorphisms among three morphological variants, to determine levels of heterozygosity in isolates, to examine the potential for asexual mechanisms to affect variation, and to evaluate differences in pathogen aggressiveness between genetically distinct isolates. The isolates examined in this study do not represent a population

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isolated at the same time from the same geographic location. Rather, we chose to examine well-characterized morphological variants isolated from a variety of hosts as a first step toward future studies of *P. ultimum* populations. We used phenetic approaches to estimate the genetic similarity among isolates but did not attempt to deduce evolutionary relationships because the validity of using distance measures to infer phylogeny is disputed (Hillis and Huelsenbeck 1992; Crawford 1990). Our study provided qualitative evidence for the occurrence of sexual outcrossing in the field, asexual mechanisms affecting variation, and differences in aggressiveness among isolates. Such information establishes a foundation for future studies to assess the rate of outcrossing and the relative contribution of sexual and asexual mechanisms to generate and maintain variation in field populations.

RESULTS

Morphological characterization of isolates.

All isolates were examined for the ability to produce oospores in pure culture and for the ability to produce zoospores. Isolates that failed to produce oospores were classified as hyphal swelling (HS) types (Table 1). Both isolates of *P. utlimum* var. *sporangiferum* (Pu22 and Pu33) were observed to produce zoospores. In contrast, none of the other 20 isolates in our collection produced motile spores under the same laboratory conditions (Table 1).

Genetic characterization of isolates with RFLPs.

Of the 21 probes tested, four (pPG111, pPG134, pPG303, and pPG305) hybridized to more than 10 fragments. Banding patterns of Southern blots probed with pPR06 and pPG132 were less complex (five to 10 bands), but alleles could not be unambiguously assigned for all 22 isolates. The remaining 15 probes were used to characterize all 22 isolates using only the 26 probe-enzyme combinations for which alleles could be unambiguously assigned (Table 2). Among all 22 isolates, 77% of the probe-enzyme combinations detected polymorphisms. Restriction fragment length polymorphism (RFLP) markers detected between one and five alleles per locus, with an average of 2.23 in both *Eco*RI and *Bam*HI digests. For the 19 isolates from California, 62.4% of the probe-enzyme combinations were polymorphic, with an average number of 2.07 alleles/locus. Several isolates had RFLP banding patterns that suggested the isolates were heterozygous (Fig. 1, Table 3). One isolate, Pu18, had all three alleles detected by probe pPG123 in *Bam*HI digests. This three-allele banding pattern was confirmed in hyphal tip derivatives of Pu18 (Fig. 2, and data not shown) and thus was not the result of a mixed culture. One hyphal tip derivative out of 12 examined was missing a band detected by pPG123 (Fig. 2, and data not shown). Further studies are being conducted to determine the frequency of this somatic event. One probe, pPR06, hybridized to approximately eight fragments in *Eco*RI and *Bam*HI digests in the isolates from the USA but to greater than 40 in Pu22 (data not shown).

Phenetic characterization of isolates with RFLPs.

The unweighted pair-group method with arithmetic average (UPGMA) clustering using a matrix of Nei's genetic distance among 22 isolates resulted in a phenogram in which all three morphological types were interspersed (data not shown), indicating that the HO, HS, and var. *sporangiferum* isolates

Table 1. *Pythium ultimum* isolate collection used in this study

Isolate number	Morphological variety[a]	Ability to produce		Geographical origin/ host[b]	Culture source[c]
		Oospores	Zoospores		
Pu1	*ultimum* (HO)	+	–	CA, Yolo Co./Tomato fruit	DMF
Pu5	*ultimum* (HO)	+	–	CA, Merced Co./Almond	EEB 1579
Pu6	*ultimum* (HO)	+	–	CA	EEB 1696
Pu7	*ultimum* (HO)	+	–	CA	EEB 1563
Pu8	*ultimum* (HO)	+	–	CA/Cotton	ATCC 32939
Pu11	*ultimum* (HS)	–	–	CA/Cotton	JH 78-11
Pu12	*ultimum* (HO)	+	–	CA, Yolo Co./Tomato fruit	DMF
Pu13	*ultimum* (HO)	+	–	CA, San Joaquin Co./Tomato fruit	DMF
Pu14	*ultimum* (HO)	+	–	CA, San Joaquin Co./Eggplant	DMF
Pu15	*ultimum* (HO)	+	–	CA, Yolo Co./Tomato fruit	DMF
Pu17	*ultimum* (HO)	+	–	CA, Fresno Co./Cotton	JH 67-1
Pu18	*ultimum* (HS)	–	–	CA	JH 86-2
Pu19	*ultimum* (HS)	–	–	MD	JH 86-3
Pu20	*ultimum* (HO)	+	–	CA, Fresno Co./Tomato root	JH 90-2
Pu21	*ultimum* (HO)	+	–	CA, Fresno Co./Tomato root	JH 90-8
Pu22	*sporangiferum* (HO)	+	+	Europe/*C. album*	ATCC 13647
Pu23	*ultimum* (HS)	–	–	CA, Kern Co./Soil	MD
Pu24	*ultimum* (HO)	+	–	CA, Kern Co./Soil	MD
Pu25	*ultimum* (HS)	–	–	CA, Kern Co./Cotton	MD 249
Pu31	*ultimum* (HO)	+	–	CA, San Joaquin Co./Cotton	JD F-367
Pu32	*ultimum* (HS)	–	–	CA, Kern Co./Soil	DMF
Pu33	*sporangiferum* (HO)	+	+	WA/Wheat	ATCC 5811

[a] Isolates were tested for both the ability to produce oospores in pure culture and zoospores as described in Materials and Methods. For sexual type, isolates capable of producing oospores are referred to as HO for homothallic. Isolates that failed to produce oospores are referred to as HS after Van der Plaats-Niterink (1981).

[b] CA, MD, WA refer to California, Maryland, and Washington. The county of origin in California is given and the host, if known.

[c] Isolates were obtained from Ed Butler (EEB), Mike Davis (MD), Jim DeVay (JD), and David Francis (DMF) at the University of California, Davis, and Joe Hancock (JH) at the University of California, Berkeley.

were not genetically distinct groups. The European isolate Pu22 was only distantly related to the isolates from the United States, showing a 0.43 probability of allelic identity at any randomly chosen locus (data not shown).

Phenetic characterization of isolates with RAPDs.

The 20 primers used detected 101 bands. Among all 22 isolates, 87% of the random amplified polymorphic DNA (RAPD) bands were polymorphic. Fewer (61%) were polymorphic among the 19 isolates from California. Two methods for calculating similarity coefficients based on RAPD data, simple matching (SM) and Jaccard's (Rohlf 1992), were compared. The Jaccard coefficient ($a/[n - d]$) and the SM coefficient ($a + d/n$) differ in how missing bands (class d) are treated. The parameters a, d, and n are defined from a two-way contingency table such that for any two isolates, four possible classes of comparisons are possible based on the presence (designated as 1) or absence (designated as 0) of a band: $a = (1,1)$, $b = (0,1)$, $c = (1,0)$, and $d = (0,0)$, and the total number of comparisons is $n = a + b + c + d$ (Rohlf 1992). When both isolates showed the presence of a RAPD band of the same molecular weight (class a), the assumption of a high level of sequence similarity was made. This assumption was tested by hybridization with gel-isolated RAPD fragments A06(0.8) and A11(0.4) to Southern blots of gels containing DNA amplified with A06 and A11. These RAPD fragments were selected as probes because they appeared to be monomorphic across all 22 isolates. Autoradiography revealed hybridization to the expected fragment across all lanes (data not shown), validating the assumption for these markers. When one isolate had a band that was missing in another isolate (class b or c), sequence

Table 2. Restriction fragment length polymorphism (RFLP) genotypes[a] for 26 RFLP probe-enzyme combinations in 22 *Pythium ultimum* isolates

Locus[b]	Pu1	Pu5	Pu6	Pu7	Pu8	Pu11	Pu12	Pu13	Pu14	Pu15	Pu17	Pu18	Pu19	Pu20	Pu21	Pu22	Pu23	Pu24	Pu25	Pu31	Pu32	Pu33
PG104B	1	1	2	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1
PG105B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PG105E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PG106B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1,2	1	1	1	1,2	1
PG106E	1	1	1	1	1	1	1	1	1	1	1	1,2	1	1	1	1	1	1	1,2	3	1,2	1
PG109B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1,2	1	1	1,3	1,2	1
PG110B	1	1	1	1	1	2	1	1	1	1	1	2	1,2	1	1	2	1,2	1	1	1	1,2	2
PG110E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1,3	1	1	1	1,3	1
PG112B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
PG112E	1	1	1	1	1	1,2	1	1	1	1	1	1	1	1	1	3	2	1	1	1	1,2	1
PG115B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PG115E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PG121E	1	1	1	1	1	1	1	1	1	1	1	1	1,2	1	1	2	1	1	1	1	1	1
PG121B	1	1	1	1	1	1	1	1	1	1	1	1	1,2	1	1	1	1	1	1	1	1	1
PG123B	1	1	2	1	1	1	1	1	1	1	1	1,2,3	1	1	1	3	1,3	1	1	1	1,3	1
PG123E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PG124B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PG124E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	1	1	1	1	1	1
PG133E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PG312E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PG312B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
PG313B	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
PG313E	1	1	1	1	1	1	1	1	1	1	1	1,3	1	1	1	2	1	1	1	1	1,3	1
PG314B	1	1	1	2	1	3	2	2	2	2	5	1,5	2,4	2	1	1	2	2	1	1	2	2
PG314E	1	1	2	3	1	4	3	3	3	3	5	2,3	3	3	1	3	1,3	3	1	1	2	3
PG315E	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1,2	1

[a] Numbers denote alleles.
[b] Loci are denoted *PG* for *Pythium* genomic clone followed by the clone number. B = *Bam*HI and E = *Eco*RI digested DNA.

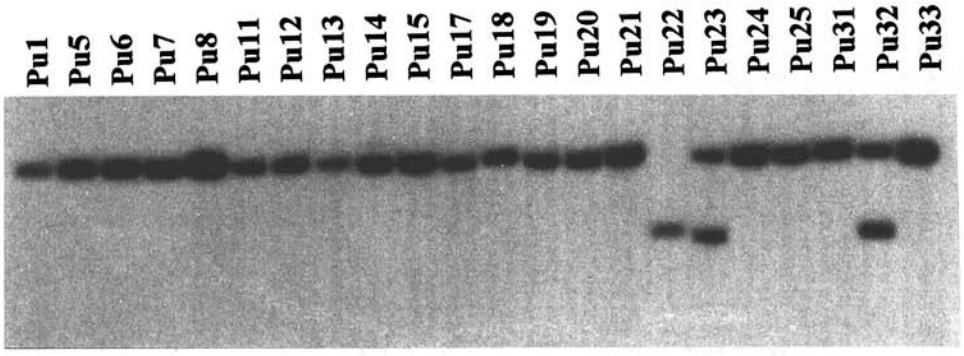

Fig. 1. Putatively heterozygous isolates were detected with RFLPs. Hybridization of pPG106 to *Bam*HI-digested DNA from 22 *Pythium ultimum* isolates revealed three genotypic classes. Isolates with two-banded genotype such as Pu23 and Pu32 were initially hypothesized to be heterozygous. Genetic analysis confirmed heterozygosity for Pu23.

divergence due to base substitution, deletions, or insertions was assumed. The absence of a band in both isolates (class *d*) was the least informative class since any number of distinct mutations could have caused the lack of amplification.

Phenograms based on UPGMA clustering of similarity matrixes derived from SM and Jaccard's coefficients both placed Pu22 as the most distinct isolate. The three morphological types were interspersed in both phenetic trees, and the two trees were nearly identical in topology (Fig. 3, and data not shown). The phenograms generated by RFLP and RAPD data revealed similar clustering of isolates (Fig. 3, and data not shown), although RFLP markers could not uniquely distinguish all 22 isolates.

Principal component analysis (PCA) was performed to provide an alternative method to UPGMA clustering using similarity coefficients to evaluate genetic variation among the isolates. PCA also allowed visualization of the data in three dimensions to evaluate isolate groupings not apparent in the two-dimensional phenetic trees. The first three principal components explained 28.4, 17.5, and 9.5% of the total variation, respectively. These three components do not appear to be directly related to characteristics such as morphological type and therefore do not have an obvious biological interpretation. The three-dimensional PCA plot (Fig. 4) summarizes 55.5% of the variation in the collection. Each additional dimension summarizes less than 9% of the variation. All three morphological types were found within the main cluster and are also represented in the more distant isolates (Fig. 4). The clustering observed in the phenetic trees (Fig. 3, and data not shown) and in the PCA (Fig. 4 and data not shown) was very similar, with the three morphological types interspersed.

Segregation analysis.

In order to confirm the existence of heterozygosity in *P. ultimum* isolates, progeny were selected from a cross between Pu7 (HO) and Pu23 (HS). Five individuals with Pu23 alleles were identified out of nearly 300 single oospore progeny screened using the sequence characterized amplified region (SCAR) markers SC20 and SC24 (Fig. 5, Table 4). All five individuals were confirmed to be F_1 hybrids in RFLP analysis using pPR06 to detect dominant Pu7 polymorphisms in *Eco*RI digests (Table 4). No Pu23 selfed progeny were identified. Markers SC24 and SC20 each identified hybrids not detected by the other marker (Table 4), suggesting that the primers amplified heterozygous loci in Pu23. Hybridization of low-copy RFLP markers to the five F_1 progeny also showed segregation and independent assortment, presumably due to heterozygosity of Pu23. Three of the progeny, F_1 (Pu7 × Pu23) 33, F_1 (Pu7 × Pu23) 43, and F_1 (Pu7 × Pu23) 217, were able to produce oospores in pure culture. These results support the classification of isolates as heterozygous based on RFLP banding patterns (Fig. 1).

Mendelian diploid inheritance and independent assortment of 10 RAPD bands, two SCARs, six codominant RFLPs, and five dominant RFLPs was confirmed in the F_2 populations from Pu6 × Pu8 (Francis and St.Clair 1993, and data not shown) and Pu7 × Pu23 (Fig. 5, and data not shown). No significant linkages beyond the possible linkage between *B08(1.5)* and *PG104* reported previously (Francis and St.Clair 1993) were detected. Segregation of SC20 in the F_2 from F_1 (Pu6 × Pu8) 22 fit the expected 1:2:1 segregation ratio ($\chi^2 = 3.45$, $0.10 < P < 0.25$), as did SC24 in the F_2 from F_1 (Pu7 × Pu23) 43 ($\chi^2 = 3.6$, $0.10 < P < 0.25$). These results confirm both the independence of markers used for the phenetic analysis and the allelism of several codominant markers.

Disease evaluation to detect variation in rate and severity of infection.

Based on the analysis of RFLP and RAPD data, Pu11, Pu13, Pu22, and Pu31 were selected to represent genetically distinct isolates. To assess the ability of the four isolates to cause water mold, a ripe fruit disease of tomato (Pearson and Hall 1973), both a soil assay and an agar disk assay were performed on ripe fruit. *L. esculentum* cultivars VF145B-7879 and Hunt100 were chosen as susceptible lines and

Table 3. *Pythium ultimum* isolates in which multiple alleles were detected and proportion of heterozygosity

Isolate	Sexual type[a]	Heterozygosity[b] (het. loci/ 26 total loci)
Pu11	HS	0.04
Pu18	HS	0.19
Pu19	HS	0.15
Pu23	HS	0.23
Pu25	HS	0.04
Pu31	HO	0.04
Pu32	HS	0.35

[a] HO = homothallic, HS = hyphal swelling.

[b] Multiple alleles are assumed to be due to heterozygosity. This assumption has been tested by genetic analysis of F_1 hybrids and F_2 progeny from Pu7 × Pu23. Heterokaryosis is a possible explanation for multiple alleles in Pu18.

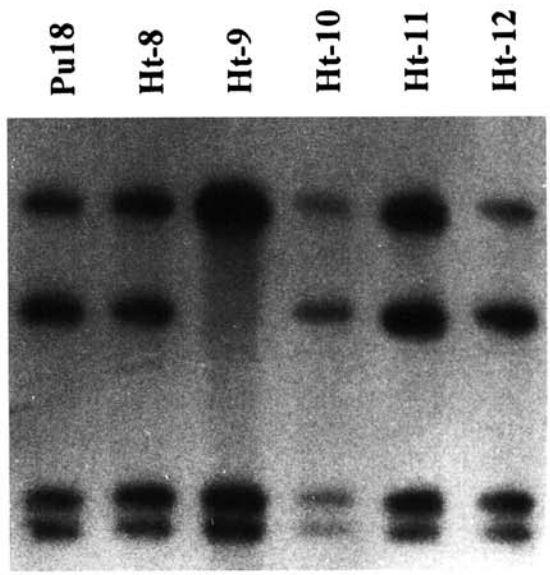

Fig. 2. A sample of hyphal tip (HT) derivatives of isolate Pu18 probed with pPG123. RFLP bands 1, 3, and 4 (from the top of the figure) represent alleles (designated 1, 2, and 3 in Table 2) as determined by segregation analysis, whereas band 2 was invariant in all 22 isolates in the survey. The HT derivatives usually exhibited the same banding pattern as Pu18, indicating the pattern was not the result of a mixed culture. In a sample of 12 HT derivatives, one (HT-9) was missing band 2, suggesting that somatic events may alter the genotype.

Lycopersicon cheesmanii LA422 and *L. esculentum* cultivar Arkansas 82-86 as resistant lines. In the soil assay, the inoculum density affects the severity of symptoms, with more dilute mixtures producing less disease (expressed as a percentage of infected fruit; data not shown). The 5:1 mixture used to evaluate and compare isolates fell well within the range of inoculum densities where the disease severity versus inoculum density graph has reached a plateau (slope = 0) for all four isolates tested (data not shown). This mixture corresponded to 750–1,500 infection units per gram of soil and is within the range for *Pythium* species found in agricultural soils (Dick and Ali-Shtayeh 1986). All isolates produced water mold symptoms on the susceptible cultivars Hunt100 and VF145B-7879.

When an analysis of variance was performed on the data from the soil assay for the isolates and tomato genotypes, significant isolate ($P = 0.0006$), genotype ($P = 0.0001$) and isolate-by-genotype ($P = 0.0028$) effects were detected for percentage infected fruit at 3 days after inoculation, but only the genotype effect was significant ($P = 0.0001$) at four days (data not shown). Subsequent analysis revealed that Pu11 and *L. cheesmanii* LA422 were the primary causes of the significant effects. LA422 was the most resistant to infection ($P \leq 0.05$) at both 3 and 4 days, relative to the cultivars. Pu11 was significantly ($P \leq 0.05$) the least infective of the isolates at 3 days on all genotypes, producing less infected fruit, but this difference was not significant at 4 days, suggesting that Pu11 infected fruit at a slower rate than Pu13, Pu22, and Pu31.

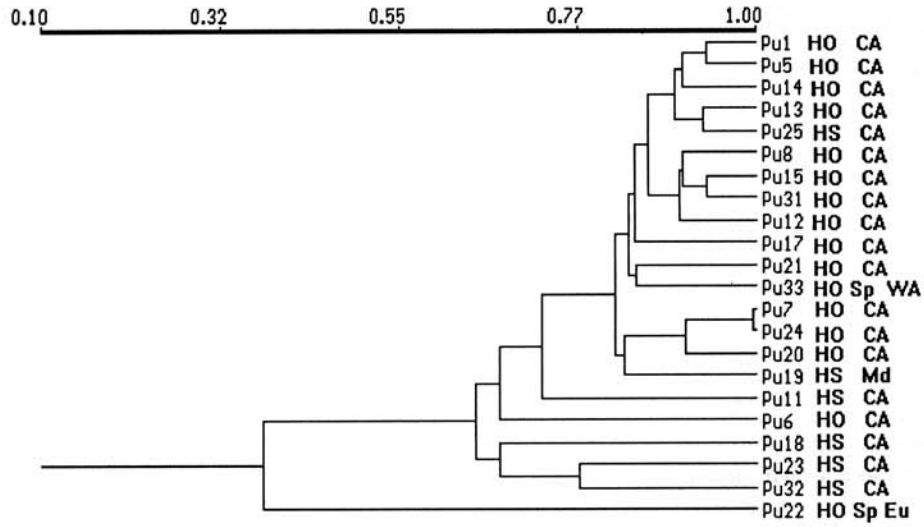

Fig. 3. Phenetic tree of 22 *Pythium ultimum* isolates. The tree was produced using UPGMA clustering of a similarity matrix of Jaccard's coefficient calculated from 101 RAPD bands. Cophenetic correlation was $r = 0.903$. A phenetic tree with similar topology was produced using the simple matching coefficient calculated from the same RAPD data set, but is not shown.

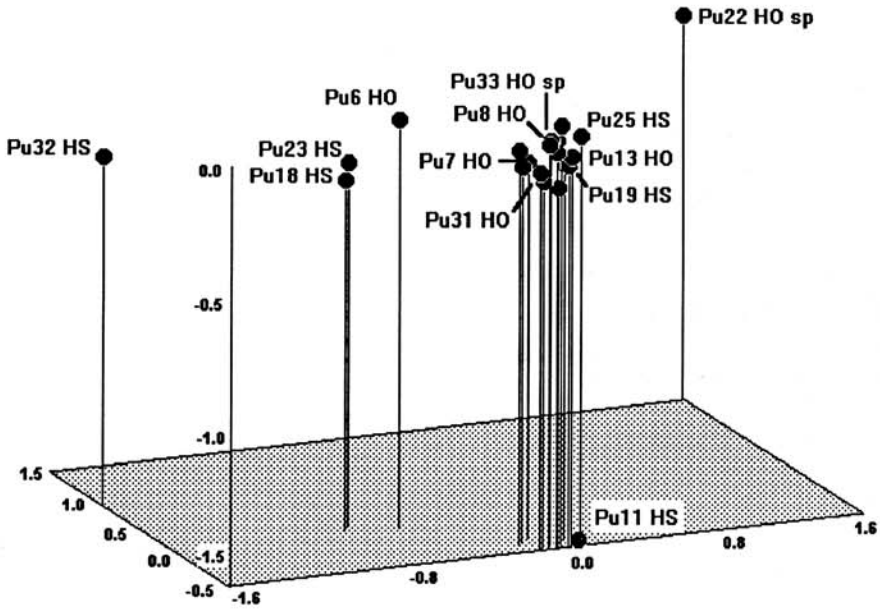

Fig. 4. Principle component analysis of 22 *Pythium ultimum* isolates based on 101 RAPD bands. The first three principle components are represented in this three-dimensional plot and account for 55.5% of the variation in the collection.

The agar disk assay allowed us to evaluate infection by these four isolates at an earlier time point than the soil assay and to evaluate both the percentage infection and lesion diameter. The assay was used on the three large-fruited cultivars, Hunt100, VF145B-7879, and Arkansas 82-86, on which lesion diameters could be measured. For lesion diameter, the analysis of variance revealed highly significant ($P < 0.001$) isolate, genotype, and isolate-by-genotype effects, whereas for percentage infection only isolate ($P = 0.02$) and genotype ($P = 0.003$) effects were significant (data not shown). Further analysis revealed that isolate Pu11 infected a significantly lower percentage on all genotypes ($P \leq 0.05$), and Arkansas 82-86 had significantly lower percentage infection and lesion diameter than the other two cultivars ($P \leq 0.05$). In addition, when Pu11 was successful at infecting fruit, the lesions were significantly smaller than lesions produced by Pu13, Pu22, and Pu31 ($P \leq 0.05$).

DISCUSSION

Our results suggest that the three morphological types, HO, HS, and var. *sporangiferum*, are not genetically distinct. However, these results do not imply phylogenetic relationships. In three methods of phenetic analysis (i.e., UPGMA clustering of Nei's distance from RFLP data, UPGMA clustering of similarity matrixes from RAPD data, and PCA of RAPD data) the HO, HS, and var. *sporangiferum* isolates did not show clustering according to morphological type (Figs. 3 and 4, and data not shown). These results are supported by data from isozymes (Chen *et al.* 1992) and protein banding patterns (Adaskaveg *et al.* 1988). Lack of clustering suggests a close genetic relationship between all three types, multiple independent origins of HS types and var. *sporangiferum*, or intermating between all three types. The European isolate, Pu22, was distinct, displaying less than 50% similarity to the other 21 isolates based on RAPD markers using the SM coefficient (which interprets a missing band in two isolates, i.e. class *d*, as a similarity) and less than 40% similarity based on Jaccard's coefficient (which omits class *d* from consideration). However, the RFLP probes generated from a California isolate hybridized well to DNA from all isolates, including Pu22, under high stringency conditions. Allelic differences detected with the RFLP markers still placed this isolate distant to the isolates from the United States. Further sampling will be necessary to determine whether these differences represent population divergence or reflect the small number of isolates sampled.

The existence of a sexual cycle that permits outcrossing in California is suggested by the occurrence of heterozygosity in field isolates (Table 3, Figs. 1 and 5). We cannot estimate the frequency of outcrossing from our data because isolates from the survey have not been derived from populations collected at the same time and location. Heterozygosity was most closely associated with the HS isolates, although the homothallic isolate Pu31 was also heterozygous at one locus out of 26 probe-enzyme combinations (Table 3). Failure to identify selfed progeny from Pu23 in nearly 300 single oospore individuals from the Pu7 × Pu23 cross suggests that this HS isolate may be predominantly outcrossing. Although we have not confirmed the segregation of alleles in all putative heterozygotes, the data from Pu7 × Pu23 F_1 progeny provide convincing evidence of heterozygosity in Pu23 (Table 4, Fig. 5). Low levels of heterozygosity could be maintained due to heterozygote advantage in the absence of outcrossing (for discussion, see Brown 1979). However, the high level of heterozygosity in isolates Pu23 and Pu32 makes heterozygote advantage an unlikely explanation for our observations (Table 3). While we cannot rule out heterokaryosis, aneuploidy or polyploidy as an explanation for the occurrence of more than one allele in many isolates, our data for Pu23 support heterozygosity in a diploid. Self-fertilization of F_1 (Pu7 × Pu23) 43 yielded an F_2 population that demonstrated segregation and independent assortment of RAPD, SCAR, and RFLP markers consistent with the expectations of Mendelian inheritance in a diploid (Fig. 5, and data not shown). Our previous analysis (Francis and St.Clair 1993) of F_2 from the HO × HO cross, Pu6 × Pu8, also supports the occurrence of diploidy in *P. ultimum*.

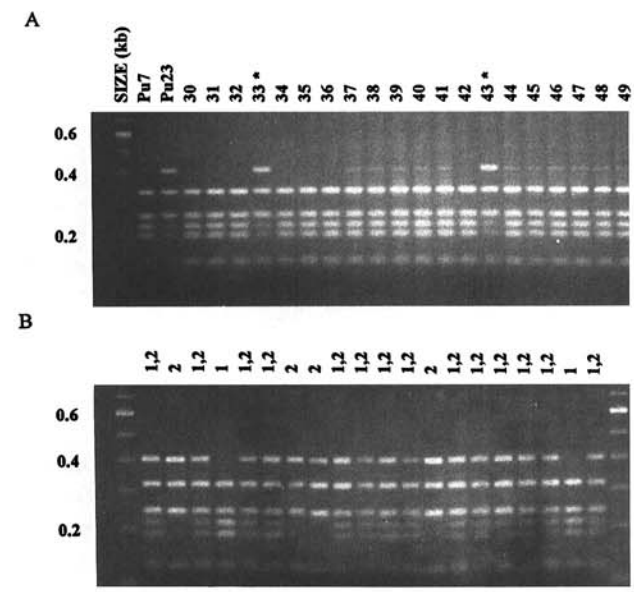

Fig. 5. Identification of putative F_1 hybrids (**A**) and segregation of alleles in F_2 progeny (**B**) with SCAR marker SC24. **A,** SC24 was used to identify single oospore progeny with Pu23 alleles (designated *) from the cross Pu7 × Pu23. Using additional markers, all individuals with Pu23 alleles were confirmed as F_1 hybrids (see Table 4). **B,** Single oospore progeny from self-fertilization of the hybrid F_1(Pu7 × Pu23)43 show segregation for alleles "1" and "2" of SC24.

Table 4. Segregation and independent assortment of SCAR and RFLP alleles[a] in five F_1 progeny of Pu7 × Pu23

Locus[b]	Pu7	Pu23	$F_1$33	$F_1$43	$F_1$102	$F_1$217	$F_1$323
SC20	1	1,2	1,2	1	1,2	1,2	1
SC24	1	1,2	1,2	1,2	1	1,2	1,2
PG106B	1	1,2	1	1,2	1	1,2	1
PG109B	1	1,2	1	1,2	1,2	1,2	1,2
PG123E	1	1,2	1,2	1,2	1	1	1
PG314E	3	1,3	3	1,3	1,3	1,3	3
PR06E	1	null	1	1	1	1	1

[a] Numbers denote alleles. pPR06 hybridized to 8 bands in an *Eco*RI digest and it was not possible to distinguish alleles, thus it was scored as a dominant marker.

[b] Loci are denoted *PG* for *Pythium* genomic clone followed by the clone number. B = *Bam*HI and E = *Eco*RI digested DNA.

Sexual outcrossing and diploidy do not explain all of the genetic variation observed in the *P. ultimum* isolates we examined. The duplication of sequences hybridizing to pPR06 in isolate Pu22 suggests that genome rearrangement may contribute to variation at the molecular level. We have also identified an isolate, Pu18, which appears to have three RFLP alleles detected by a single marker (Table 2), suggesting the existence of heterokaryosis, aneuploidy, or polyploidy. The existence of more than the expected diploid number of alleles in the oomycete *B. lactucae* has been termed "hyperploidy" as it has not yet been possible to distinguish between polyploidy, aneuploidy, or heterokaryosis (Hulbert and Michelmore 1988; Schettini *et al.* 1991). Our observation of three alleles in Pu18 is consistent with the proposed occurrence of heterokaryosis in *P. ultimum* (Campbell and Sleeth 1946). They observed that hyphal tip transfers of one isolate yielded subcultures that differed from the original isolate with respect to the ability to produce oospores in pure culture. The loss of an RFLP band in a hyphal tip transfer from Pu18 (Fig. 2, and data not shown) suggests that the phenotypic changes observed by Campbell and Sleeth (1946) may have a genetic basis. Further studies will be necessary to determine whether the three alleles detected in isolate Pu18 are the result of heterokaryosis, polyploidy, or aneuploidy.

Four isolates that could be distinguished on the basis of genetic markers (Fig. 3, Table 2) were tested to compare their ability to infect ripe tomato fruit using a soil inoculation technique and an agar disk assay. The agar disk assay does not reproduce all of the variables of a natural infection, but the consistency of results obtained with this method and those obtained using artificially infected soil are encouraging for studies on pathogen virulence and aggressiveness. Results from both assays were consistent with other studies that suggest a wide range of aggressiveness in *P. ultimum* (DeVay *et al.* 1982; Moulin *et al.* 1994). The agar disk assay will permit quantitative data to be obtained and efficient testing of a larger number of isolates than is practical with the soil assay. Studies designed to detect a relationship between the genotype and pathogenicity attributes of isolates are not appropriate for the collection we examined as any association would depend on either linkage of genetic markers to pathogenicity factors, linkage disequilibrium in *P. ultimum* populations, or the existence of *P. ultimum* races. To adequately address the possible association of markers with the virulence and aggressiveness of isolates would require the examination of a large natural population (Wolfe and Knott 1982).

We have found qualitative evidence for the occurrence of an active sexual cycle involving outcrossing, evidence for the occurrence of asexual mechanisms generating variation, and evidence for variation in virulence and aggressiveness in *P. ultimum* isolates. While some data on pathogen variation is available for *Pythium* species (Adaskaveg *et al.* 1988; Chen *et al.* 1992; Levesque *et al.* 1993), little information is available on gene frequencies and genetic diversity in natural populations. This is in contrast to the data available for the heterothallic oomycetes *Bremia lactucae* (Ilott *et al.* 1987; Hulbert and Michelmore 1988; Schettini *et al.* 1991) and *Phytophthora infestans* (for review, see Fry *et al.* 1992). Quantitative statements about the relative importance of the sexual cycle and asexual mechanisms in generating and maintaining genetic variation in *P. ultimum* will require approaches based on population genetics. Our codominant markers detect sufficient genetic polymorphism in *P. ultimum* to estimate the rate of outcrossing, the frequency of hyperploidy, and allele frequencies in both HS and HO isolates collected from the same location at the same time. Appropriate population sampling will permit assessment of deviation from Hardy-Weinberg equilibrium (Wright 1968; Brown 1979) and linkage disequilibrium (Brown *et al.* 1980) in order to estimate rates of outcrossing in natural populations. Such approaches may also permit the study of the distribution of genetic diversity, migration, and gene flow in order to help determine what defines a population of *P. ultimum* (Slatkin 1985). Our results also suggest that the differences in virulence and aggressiveness observed between isolates, combined with the abundance of genetic polymorphism and the ability to make crosses, will allow quantitative genetic approaches to dissect the genetic factors underlying pathogenicity attributes in *P. ultimum*.

MATERIALS AND METHODS

Fungal isolates and culture conditions.

A collection of *Pythium ultimum* isolates was assembled and consisted of 22 isolates obtained from E. Butler (University of California, Davis), J. Hancock (U.C. Berkeley), M. Davis (U.C. Davis), J. DeVay (U.C. Davis), and isolates from our own collection (Table 1). The isolates represent three morphological types: homothallic (*P. utlimum* var. *ultimum*; referred to as HO), hyphal swelling (*P. utlimum* var. *ultimum*; referred to as HS), and zoospore producers (*P. utlimum* var. *sporangiferum*). Nineteen of the 22 isolates were selected from a variety of locations in California; isolates from Europe (Pu22), Maryland (Pu19), and Washington (Pu33) were also included because they represented well-characterized morphological variants of *Pythium ultimum*.

Culture conditions.

Media and growth conditions for the maintenance of cultures, isolate crosses, identification of progeny, and hyphae for DNA preparations were as described previously (Francis and St.Clair 1993). All 22 isolates were tested for the ability to produce oospores in pure culture by replicated testing on half-strength clarified V8 media (Ribeiro 1978) supplemented with 5 μg/ml of β-sitosterol. Isolates were also tested for the ability to produce zoospores. Inoculated agar disks 1 cm in diameter were placed in 10-cm diameter petri dishes containing quarter-strength liquid V8 medium. After two days, mycelium from the liquid medium was transferred to sterile distilled water. Sporangia were then examined microscopically for vesicle formation and zoospore release at 2, 4, and 6 hr after transfer.

Genetic markers.

Construction of a size-selected genomic library and selection of RFLP probes were described previously (Francis and St.Clair 1993). Probes for RFLP analysis were designated by pPG followed by a number, e.g., pPG106. Segregating RFLP loci were identified according to the probe designation (e.g., *PG106*). One RFLP marker was derived from a

subcloned RAPD fragment and was designated pPR06. Twenty-one probes were hybridized to *Eco*RI and *Bam*HI digested DNA from all 22 isolates. Bands were assigned alleles based on their unambiguous differences in molecular weight and on segregation in F_2 populations (see segregation analysis below) when possible. For probes that detected more than one or two bands per isolate, it was sometimes not possible to make allelic designations; therefore data from these probes was not used. RFLP markers were scored as codominant loci.

Ten base pair oligonucleotide primers used to generate RAPD markers (Williams *et al.* 1990). RAPD primers from kits A, B, C, and D (Operon, Inc., Alameda, CA) were screened, and 20 primers were chosen for their ability to amplify clear bands in *P. ultimum*. All bands, polymorphic and monomorphic, were scored based on the following criteria: 1) bands were less than 2 kb, 2) amplification was reproducible in at least two separate amplifications, and 3) the amplified fragment was a clear and distinct band. Segregating loci detected by RAPD markers were designated by the Operon primer number followed by the size in kb of the segregating band, e.g., *A05(1.5)*. RAPD markers were scored as dominant markers (i.e., presence or absence of the band).

Selected RAPD bands were subcloned and sequenced to develop codominant PCR-based markers using 24 base primers unique to the end of the original fragment, as described by Paran and Michelmore (1993). Two such sequence characterized amplified regions (SCARs) were used for this study, and their primer sequences 5′ to 3′ were SC20, consisting of the primers CAATCGCCGTCACAACAACT-GATC and CAATCGCCGTTGCATTTTGCTACC, and SC24, consisting of the primers GACCGCTTGTGTAGCTT-TTGTTTC and GACCGCTTGTAGGTGGAGCAGCAG. Unambiguous visualization of the polymorphisms between Pu7 and Pu23 required digestion of the amplified product with *Rsa*I for SC24 and digestion with *Hae*III for SC20. These primer sequences are appropriate for detecting polymorphisms between a number of isolates when used in conjunction with other restriction endonucleases.

Extraction of DNA.

DNA for RAPD, RFLP, and SCAR analysis was extracted from frozen mycelia according to a modification of the CTAB procedure (Bernatzky and Tanksley 1986) as described previously (Francis and St.Clair 1993). For the identification of progeny using SCARs, the DNA preparation was scaled down in volume to allow all steps to be accomplished in 1.5-ml microfuge tubes. A 16-gauge needle was used to scrape a small amount of mycelium from cultures into the 1.5-ml tubes. The tubes were frozen in liquid N_2 or overnight at –80° C. The mycelium was then vortexed in 300 µl of extraction buffer. Volumes of lysis buffer and chloroform extractions were correspondingly scaled down. This small-scale DNA preparation allowed 48 samples to be easily processed in 4 hr.

PCR conditions.

Amplification reactions for RAPD markers were performed as described previously (Francis and St.Clair 1993). Reactions for SCARs were in a total volume of 50 µl. Each reaction contained 5–10 ng of *P. ultimum* DNA, 200 nM each of the 24-bp oligonucleotide primers, 1 unit of *Taq* Polymerase (Perkin Elmer Cetus, Norwalk, CT), 100 µM dNTPs, 10 mM Tris (pH 8.2), 50 mM KCl, 1.9 mM $MgCl_2$, and 0.001 % w/v gelatin (cell culture tested, Sigma, St. Louis, MO). Thermal cycling parameters for amplification with the SCAR primers were 94° C for 30 sec, followed by 30 cycles at 94° C, 1 min; 62° C, 1 min; 72° C, 2 min. After 30 cycles, the reaction was completed by a single cycle of 72° C for 5 min. All amplifications were in a DNA thermal cycler model N801-0150 (Perkin Elmer Cetus).

Electrophoresis and Southern analysis.

Horizontal electrophoresis gels for RFLP analysis, Southern transfer, and hybridization conditions were as described (Francis and St.Clair 1993). PCR amplification products for both RAPD and SCAR reactions were analyzed on 2% agarose gels (1% NuSieve GTG, FMC Corp., Rockland, ME; 1% Ultrapure, GIBCO BRL Life Technologies, Gaithersburg, MD) in 1 × TBE buffer under the same conditions described previously (Francis and St.Clair 1993).

Analysis of segregation.

Five isolates (Pu6, Pu7, Pu8, Pu11, and Pu23; Table 1) were used to generate progeny for use in identifying alleles and in segregation analysis. We have previously described the use of RAPD markers and RFLP markers to demonstrate that outcrossing can occur between homothallic (HO) isolates and between homothallic and hyphal swelling (HS) isolates of *P. ultimum* (Francis and St.Clair 1993). A similar approach using SCAR markers was used to identify hybrids between the HO isolate Pu7 and HS isolate Pu23. Hybrids from this cross were then analyzed for the ability to complete the sexual stage in pure culture and for the segregation of RFLP markers. Hybrid progeny were designated by the prefix F_1, the two isolates used in the mixed mating, and a number, e.g., F_1(Pu6 × Pu8)22. The prefix F_2 was added to progeny derived from selfed F_1. Chi-square goodness-of-fit values for expected segregation ratios in F_2 populations from F_1(Pu6 × Pu8)22 and F_1(Pu7 × Pu23)43 were calculated. The chi-square values for independent assortment and the maximum likelihood estimate of recombination frequency between selected markers were calculated with the LINKAGE-1 program (Suiter *et al.* 1983).

Phenetic data analysis.

Genetic distances (Nei 1973) based on RFLP data were calculated with a statistical package provided by K. Ritland, University of Toronto. Genetic distance was calculated according to Nei (1975, pages 176-177). The calculation of genetic distance was based on the number of shared alleles, not on nucleotide substitutions as in Nei (1987). The matrix of genetic distances was used to construct a phenogram using UPGMA clustering (Sneath and Sokal 1973). The computer program NTSYS-pc (Rohlf 1992) was used to calculate two similarity coefficients, SM and Jaccard's, based on RAPD data as a measure of genetic similarity for each pair of isolates. Phenograms for matrixes based on UPGMA clustering were constructed for the two similarity matrixes, and the cophenetic correlation for each tree was calculated as described by Rohlf (1992).

As an alternative method to analyze variation among isolates, Q-type principal component analysis (PCA) was performed using NTSYS-pc according to the approach outlined by Sneath and Sokal (1973). Briefly, the data matrix for isolates by RAPD bands was standardized (0 mean, unit variance), product-moment correlation coefficients were calculated among variables, and eigenvectors were extracted from the resulting matrix. The standardized data were then projected onto the eigenvectors to obtain a plot of the first three principal components.

Disease evaluation.

For the soil assay, ripe fruit was placed on inoculated soil. Ten days before the soil inoculum was desired, sterile 100-mm diameter petri dishes containing 30 ml of half-strength V8 were inoculated with 0.5-cm diameter blocks from a corn meal agar (CMA) plate containing *P. ultimum*. Liquid cultures were grown for 3 days prior to inoculation of a vermiculite/V8 mixture. Mason jars (approximately 1 L) were prepared for inoculum by drilling a 1 cm diameter hole in each lid. The hole was plugged with cotton to maintain sterility while allowing the jar to vent. The jars were filled with vermiculite and 600 ml of clarified, half-strength V8, and autoclaved for 45 min. Seven days before the inoculation of soil, 3-day-old liquid cultures were added to the sterilized vermiculite/V8 (1 dish/liter jar). In 5 to 7 days, mycelium was visible from top to bottom in the mason jars. The soil inoculum was prepared with an autoclaved soil-sand mixture consisting of four parts shredded clay loam and one part coarse sand. The infected vermiculite was mixed in a 5:1 v/v ratio of soil to vermiculite just prior to use for disease evaluation. The disease screen was carried out in plastic chambers (40.5 cm × 28 cm × 15 cm) containing enough soil-inoculum mixture to cover the bottom with approximately 2–3 cm of soil (approximate volume 1.5 L). Initially, 700 ml of sterile water was added; the soil and water were mixed and distributed evenly in the chamber, and additional water was added to obtain a saturated mixture, with standing water present in depressions only.

Ripe fruit was harvested no more than 24 hr prior to the screen. Fruit not immediately used was stored at 13 °C. The pedicel was left on all fruit as the stem scar provides an avenue for infection. Fruit were surface-sterilized in 1.2% sodium hypochlorite for 15 min, rinsed three times in deionized water, placed in the infected soil blossom-end down, and the plastic chambers were covered to maintain high humidity. The percentage of fruit showing the presence of a water-soaked lesion or localized softening was determined at 3 and 4 days after inoculation. Ten to 20 fruit per tomato genotype were used for each observation, with four to six replicates for each isolate-by-genotype combination.

The agar disk assay involved placing agar disks from *P. ultimum* cultures on ripe fruit (harvested and sterilized as above). Preliminary experiments were conducted using cultures grown on CMA, V8 agar, and potato dextrose agar, and no significant differences attributed to media were observed (data not shown). Therefore, all subsequent isolate comparisons were performed with cultures grown on CMA. Disks 10 mm in diameter were placed on the fruit with the mycelium down. The fruit were placed in plastic chambers (40.5 cm × 28 cm × 15 cm), misted with water, and polyvinylidene chloride wrap was placed over the top of the box. Ten to 18 fruit per genotype were inoculated with each isolate, and the experiment was replicated three times. After 36 hr, percent infection was scored as in the soil assay, and diameters of lesions on successfully infected fruit were measured (in millimeters) using calipers.

Data from the soil assay and the agar disk assay were analyzed using the SAS statistical package (SAS Institute, Cary, NC). The general linear model (GLM) procedure was used for the analyses of variance, and Tukey's test and Fisher's protected LSD were used for mean separations. Data for percentage infection from both the soil and agar disk assays were transformed using arcsin square root to stabilize the variance (Steel and Torrie 1980).

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