Phytophthora sojae Races Have Arisen by Clonal Evolution and by Rare Outcrosses

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Received 26 October 1993. Accepted 15 August 1994.

An extensive set of nuclear and mitochondrial restriction fragment length polymorphisms (RFLPs) was used to examine the genetic relationships among 48 isolates of *Phytophthora sojae*, an oomycete pathogen of soybean. This organism is diploid and homothallic. The isolates examined encompassed 25 physiological races of the pathogen, including races 36 and 37 which we describe here for the first time. The results reveal a moderate degree of diversity within the species; about 18% of all detected nuclear restriction fragments were polymorphic in at least one isolate. One group of isolates, representing seven physiological races, had RFLPs nearly identical to isolates of the first described race, race 1, and probably arose from race 1 isolates clonally by mutation. There was much more genetic variation among the remaining isolates. The distribution of RFLP alleles among these isolates suggests most of the genetic variation in the species is found in four genotypes (progenitor lines) represented by isolates P1658 (race 1), P7064 (race 7), P7074 (race 17), and P7076 (race 19), respectively. All the other isolates appear to have been produced by rare outcrosses between representatives of these four genotypes. The distribution of avirulence phenotypes against *Rps* genes (soybean resistance genes against *P. sojae*) is consistent with the reassortment of single avirulence genes as a result of the same outcrosses. Therefore it is proposed that occasional outcrosses have been a major contributor to the origin of new physiological races of *P. sojae*, in addition to clonal evolution. In concordance with these mechanisms, no correlation was observed between particular RFLPs and race types. Thus it will be very difficult to use RFLP or RAPD markers to directly identify race types of new field isolates unless the markers are derived directly from avirulence genes.

Additional keywords: avirulence genes, evolution, Phytophthora root rot.

Phytophthora sojae Kaufmann & Gerdemann (syn. *Phytophthora megasperma* Drecsh. f. sp. *glycinea* Kuan & Erwin) causes a root and stem rot of soybean (Schmitthenner 1989). *P. sojae* is an oomycete, a group of organisms which have classically been included among the fungi, but which are more closely related to chromophyte algae such as diatoms (Förster *et al.* 1990). *P. sojae* is an important problem in all of the major soybean-growing areas of Canada and the United States, notably the Midwest and lower Mississippi valley (Schmitthenner 1989). Little is known about the origin of *P. sojae*. It is believed to be native to the United States, perhaps as a pathogen of native legumes, as the disease was identified for the first time shortly after soybeans were introduced on a large scale into the Midwest. *P. sojae* is also a pathogen of lupines (Jones and Johnson 1969), which may be the natural host. However, the origin of *P. sojae* has not been investigated systematically. Twelve single dominant resistance genes (*Rps* genes) against *P. sojae* have been characterized in soybean (Anderson and Buzzell 1992b; Buzzell and Anderson 1992; Buzzell *et al.* 1987). Five genes (or alleles) are clustered at one locus (*Rps1a, Rps1b, Rps1c, Rps1d*, and *Rps1k*) and three are clustered at a second locus (*Rps3a, Rps3b,* and *Rps3c*). *Rps7* is closely linked to, but separable from, the *Rps1* locus (Anderson and Buzzell 1992b), while the remaining genes, *Rps4, Rps5,* and *Rps6* appear to be unlinked. Several *Rps* genes have been incorporated into commercial cultivars. However, the effectiveness of these genes has been progressively lost as new races of the pathogen have appeared (Keeling 1982; Schmitthenner 1991). To date, 37 physiological races of the pathogen have been isolated from diseased soybean plants (Layton *et al.* 1986; Schmitthenner *et al.* 1994; Wagner and Wilkinson 1992; S. Abney, personal communication; this paper). In addition, Hobe and Schmitthenner (1981) isolated over 40 previously uncharacterized pathotypes of *P. sojae* directly from soil, suggesting that there exists a large reservoir of genetic diversity in the *P. sojae* population. However, it is not known how this extensive diversity might be created.

Förster *et al.* (1989) and Whisson *et al.* (1992) examined the genetic relationships of a small sample of isolates using mitochondrial (Förster et al 1989) and nuclear (Whisson *et al.* 1992) restriction fragment length polymorphisms (RFLPs). They observed a poor correlation between race types and RFLP pattern types, suggesting that particular races (e.g., race 4) arose several times independently, or that genetic recombination was occurring. *P. sojae* is homothallic. Therefore it has generally been assumed that genetic outcrossing is not an important source of diversity (Layton and Kuhn 1988; Long and Keen 1977). However, Layton and Kuhn (1990) showed that *P. sojae* could form heterokaryons when different isolates were co-inoculated onto a single plant, and Long and Keen (1977) showed that selfing of heterokaryons could produce hybrid progeny. Outcrossing can also occur between isolates grown together in mixed culture (Bhat and Schmit-

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MPMI Vol. 7, No. 6, 1994, pp. 780-791

thenner 1993; this paper). Therefore the potential exists for outcrossing in the field if different strains infect the same plants. In fact, oospores are readily found in plants infected with *P. sojae*, and the pathogen overwinters in the Midwest of the United States in the form of oospores (Schmitthenner 1989). To examine the mechanisms by which diversity is generated in this species, and to look for evidence of genetic recombination, we used 62 nuclear RFLPs to survey the distribution of genetic variation among a large collection of isolates of *P. sojae* from different geographical regions, including representatives of most of the described physiological races. The distribution of RFLP alleles among the isolates suggests that new races of *P. sojae* have arisen both by progressive mutation and by infrequent outcrosses.

RESULTS AND DISCUSSION

There is substantial nuclear RFLP diversity among *P. sojae* isolates.

Random cloned nuclear DNA fragments from an isolate of *P. sojae* (P3114) were used as probes to investigate DNA polymorphisms by hybridization analysis of Southern transfers among 48 isolates of *P. sojae* representing 25 different races. Twenty of the DNA probes appeared to correspond to single-copy sequences based on the visualization of a single band on the autoradiograms; only four of them detected RFLPs among the initial subset of twenty isolates. Sixteen of the DNA probes detected multiple bands, presumably corresponding to non-tandem repeats; 11 of these probes revealed polymorphisms among at least some isolates (examples are shown in Fig. 1). Several DNA probes, such as p87 (Fig. 1B), p13, and p123 (not shown), appeared to detect differences in the copy numbers of conserved repeat sequences; particular bands were present on the autoradiograms at either low (low copy) or high intensity (high copy) (Fig. 1B) or were completely absent in some isolates (probe p123, not shown).

Although the majority of DNA fragments detected on the autoradiograms (approx. 350 altogether) appeared to be identical among all isolates, 62 fragments were polymorphic with at least one of three enzymes. Fragments were considered probable alleles if they were detected by a probe with similar intensity and appeared in a mutually exclusive fashion among the isolates (e.g., 121P1 in Fig. 1A). Two fragments were ruled out as possible alleles if they were missing simultaneously from any isolate. Two alleles were commonly observed for each polymorphic locus, but some displayed up to four alleles (Fig. 1). With greater numbers of bands (some probes detected over 20) it was often difficult to unambig-

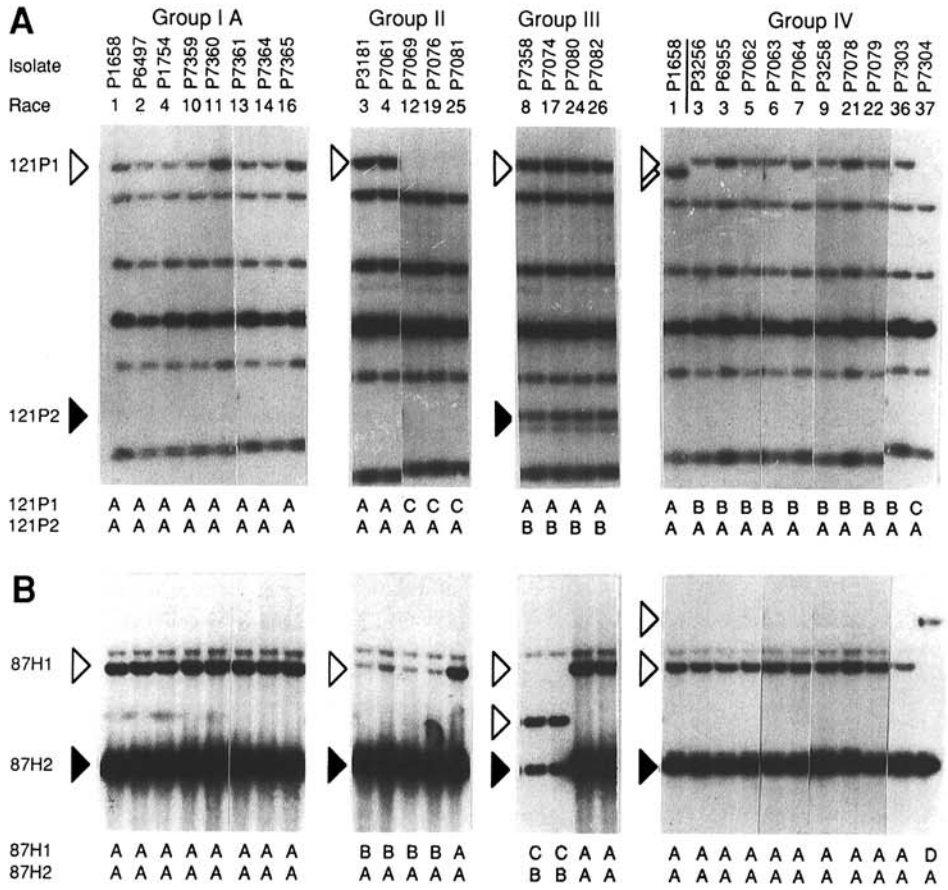

Fig. 1. Nuclear RFLPs in *Phytophthora sojae* detected by blot hybridization with random cloned probes. **A,** RFLPs detected by probe p121 after digestion of DNA with *Pst*I. **B,** RFLPs detected by probe p87 after digestion of DNA with *Hin*dIII. Groups are as described in the text and in Figures 3 and 7. Open triangles indicate presumptive alleles of the RFLPs 121P1 (A) and 87H1 (B), while the closed triangles indicate presumptive alleles of 121P2 (A) and 87H2 (B). The allele states of each isolate for each RFLP, as inferred from the hybridization pattern, are given below each panel. The B alleles of 87H1 and 87H2 appear to represent copy number polymorphisms. The faint bands halfway down panel 1 of B are gel artefacts.

uously identify allelic fragments. In those cases, alleles were scored as presence or absence of the band. No attempt was made to determine whether individual bands on the autoradiograms corresponded to more than one hybridizing restriction fragment.

Use of genetic crosses to confirm alleles.

To validate this method of identifying allelic fragments, we genetically crossed two isolates (P6497 and P7064) which show a substantial number of RFLP differences. Since *P. sojae* is homothallic, F_1 hybrids were identified from mixed cultures using RAPD markers specific for each parent, a strategy developed for *Pythium ultimum* (Francis and St. Clair 1993). A single zoospore line from one F_1 hybrid was then selfed (details of the cross procedure will be published elsewhere). Of the resultant F_2 progeny, 47 were scored for segregation of 26 of the 36 RFLPs differing between the two parents. Figure 2A shows segregation of RFLP 121P1 (i.e., the same RFLP shown in Fig. 1A). Figure 2B shows a complex pattern of fragments detected by probe p82. Five RFLPs detected by p82 were assigned to three loci (82P1 and 82P3 with two codominant alleles, 82P2 with one observable allele) on the basis of mutual exclusivity; all five of these assignments were supported by the segregation data in Figure 2B. In fact, in all 26 cases, the segregation of the RFLPs confirmed the initial allele assignments. All of the 10 untested RFLPs were unambiguous, being comprised of a single polymorphic band. Therefore, the use of mutual exclusivity to identify alleles appears largely reliable in this species, probably because the level of heterozygosity is low (see below), and the genetic diversity of the species is not high. However, we cannot rule out the possibility that a small number of the untested allelic relationships might be incorrect, due to chance or to the presence of multiple alleles. Interestingly, no isolates were heterozygous at any of the 15 loci at which codominant alleles were confirmed by the genetic cross, nor at a further 6 loci at which codominant alleles were defined by mutual exclusivity. The genetic cross also showed that isolates P6497 and P7064 were homozygous at a further 11 loci having dominant alleles. A high degree of homozygosity is expected in a species like *P. sojae* that undergoes a high frequency of selfing relative to the rates of outcrossing.

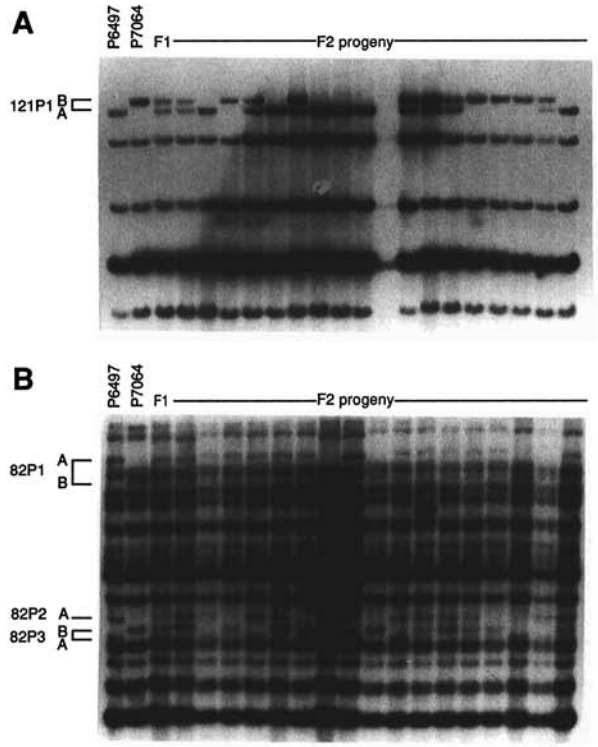

Fig. 2. Confirmation of allelic relationships of RFLPs by genetic crossing. Hybridization of two RFLP probes to Southern blots of DNAs from P6497 and P7064, from an F_1 hybrid of the two isolates, and from 17 F_2 progeny produced by selfing the F_1 hybrid. Hybridizations to DNAs from an additional 30 F_2 progeny are not shown. **A,** Probe p121 (same as for Fig. 1A). **B,** Probe p82. In both A and B, DNAs were digested with *PstI*. The A and B alleles of 121P1, 82P1, and 82P3 are indicated by the linked lines. 82P2 is dominant and the B allele is scored as a missing band. Similar hybridizations were carried out with 13 other probes detecting 22 other RFLPs, as indicated in Figure 7.

Genetic relatedness of isolates.

To begin the analysis of the RFLP data, we used unweighted pair group mathematical average (UPGMA) clustering analysis of the nuclear RFLP data to identify groups of genetically similar isolates. The resulting dendrogram (Fig. 3), based on pairwise comparisons of RFLP patterns, summarizes the relative genetic similarity of the isolates to one another (but not necessarily the pathways by which the isolates actually evolved). The dendrogram identifies four overall groups of isolates, indicated by the vertical bars. The first group contains a large group of isolates (group IA) which have identical or near-identical RFLP alleles. This group includes all isolates of races 1, 2, 10, 11, 13, 14, 15, and 16 and one isolate of race 4. The dendrogram also shows that isolates of race 12 and 19 (in group II) have identical or near-identical RFLPs. Similarly, isolates of race 6 or race 7 (in group IV) are nearly identical, including an isolate (P7469) reported by Whisson *et al.* (1992) to be highly divergent. In our specific virulence assays, isolates designated races 12 and 19 are identical, as are those designated races 6 and 7. In contrast, the dendrogram shows that isolates of race 4 are located in different groups, one in group IA (P1754) and four isolates identical to one another in group II (P3180, P7061, P7375, P7468). Similarly, although the three isolates of race 3 (P3181, P3256, P6955) occur together in group IB, they are substantially divergent from one another. There was no clear correlation evident between geographic origin and RFLP groups. Three race 1 isolates (P7225, P7226, P7470) and a race 15 isolate (P7227) from Australia were nearly identical to race 1 isolates from the United States, consistent with the Australian population being derived from recent ancestors common to the United States (Whisson *et al.* 1992). Interestingly, all four Australian isolates, including those of races 1 and 15, shared a single RFLP allele, 74P2B, not found in any U.S. isolates (not shown).

As indicated in Table 1 and in the Materials and Methods, there is some uncertainty as to whether certain accessions represent duplicates or not. Two of the four potential pairs of duplicates had identical RFLP patterns, though this does not prove they are the same strain. One exception was a race 12 isolate, P7369, which was obtained from T. Anderson (Harrow, Ontario) via J. Paxton (Urbana, Illinois) and B. Tyler

(Davis, California). This isolate displays two polymorphic differences to a race 12 isolate, P7069, obtained from T. Anderson several years later (not shown), and to the genetically identical race 19 isolate P7076. Similarly, the race 7 isolate P1656, which was obtained directly from J. Paxton, also shows two polymorphic differences with the race 7 isolate P7469 obtained from J. Paxton via J. Manners (Queensland, Australia). Remarkably, the two polymorphisms that distinguish P7369 and P7069 are identical to those that distinguish P1656 and P7469, namely the presence of two novel *Eco*RI fragments detected by the probe p38, suggesting that this is a hotspot for mutation. Both pairs of isolates had identical mtDNAs (see below).

MtDNA RFLPs.

Restriction fragment polymorphisms of the mtDNAs of a subset of 20 *P. sojae* isolates were scored following digestion with four restriction endonucleases. One fragment in each case showed extensive minor length variations (e.g., Fig. 4). These length variations appeared to be mainly localized in the ribosomal RNA coding region when fragments were compared with a published mtDNA restriction map of *P. sojae* (Förster *et al.* 1989). The length variation appeared to occur at an extremely high frequency, as three pairs of isolates with a very recent common origin (P1139 and P3114, P6951 and P6956, and P1658 and P7374) showed length differences in this region (Fig. 4). For example, P6956 was isolated as a metalaxyl resistant mutant of P6951, while P7374 was separated from P1658 by only 4 yr of passaging in B. Tyler's laboratory. P1139 and P3114 both originated from C. Grau's isolate 1-16. As shown in Figure 5, the UPGMA dendrogram of this analysis had a very similar branching pattern compared to the one generated from nuclear DNA RFLP data. Exceptions were isolates P3256 and P7081 that clustered in different places within group I, and isolate P3258 that clustered in group IV rather than group I. Genetic distances of the two dendrograms in Figures 3 and 4 cannot be compared because in the nuclear DNA RFLP analysis only informative DNA fragments (those that differed in at least one isolate) were included, while the mtDNA analysis was based on the complete database of 78 restriction fragments, including the hypervariable fragments, and including 24 fragments which were conserved among all isolates. However, the branching patterns and the relative branch lengths within each analysis are comparable between the two studies.

Polymorphisms in mtDNA have been investigated in a previous study (Förster *et al.* 1989) and only three different mtDNA types generated by six restriction enzymes were found among

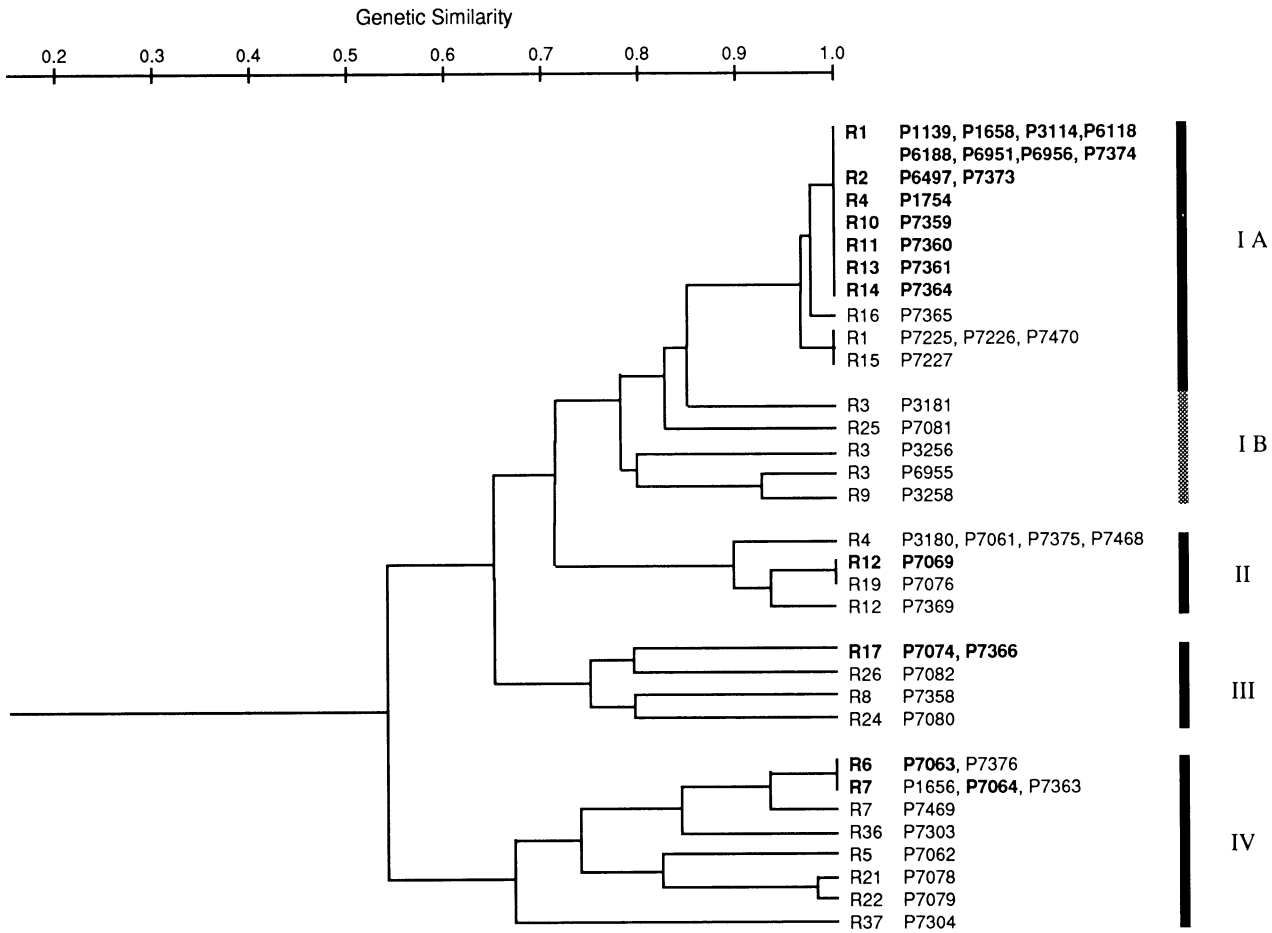

Fig. 3. Genetic similarity among isolates of *Phytophthora sojae* based on nuclear RFLPs. Four-digit isolate numbers indicate accession number in the U.C. Riverside cryogenic *Phytophthora* species collection. R numbers indicate race types. Vertical bars indicate groups of similar isolates. Strains marked in bold are the most similar (I A) or most divergent (II–IV) from P1658 (R1) in each group (see text).

Table 1. Lists of isolates used

Strain[a]	Origin[b]	Obtained through[c]	Race	Notes
P1139	Wiscosin (C.G., 1–16)	D.E. (324)	1	Different mtDNA than P3114[d]
P1658	Ohio (F.S.)	N.T.K. ($P900_5$)	1	IMI 333381; ATCC 34000
P3114	Wisconsin (C.G., 1–16)	D.M. (115)	1	Different mtDNA than P1139[d]
P6118	Arkansas (J. J.)	IMI-ATCC	1	IMI 131555; ATCC 18010
P6188	Virginia (R.W., 66)		1	
P6951	Indiana (K.A., F. L.)	D. K. (5–58)	1	Different mtDNA than P6956[d]
P6956	Indiana	D. K. (5–58 FTP)	1	Different mtDNA than P6951[d]
P7225	New South Wales (J. I.)	J. M. (CIFR 11)	1	
P7226	Queensland (J. I.)	J. M. (UQ 60)	1	
P7227	Queensland (J. I.)	J. M. (9933)	1	
P7374	Ontario (A. H.)	N. T. K. ($P174_5$)/B.T.	1[e]	
P7470	New South Wales (J. I.)	J. M. (CIFR)	1	
P6497	Mississippi (B. K.)	T. A.	2[e]	
P7373	Mississippi (F. L.M.)	N. T. K. ($P406_5$)/B. T.	2[e]	ATCC 32988
P3181	Wisconsin (S.N.)	D. M. (285)	3	
P3256	Ohio (F. S. #573)	N. T. K. ($P892_5$)	3[e]	
P6955	Indiana (K. A., F. L.)	D. K. (76–4.4)	3	Metalaxyl mutant
P1754	Ohio (F. S.)		4	
P3180	Wisconsin (S. N.)	D. M. (276)	4	
P7061	Ontario (E. W.)	T. A.	4	
P7375	Ontario (T. A.)	J. P./B. T.	4[e]	
P7468	Wisconsin (C. G.)	J. M. (W18750N)	4	
P7062	Ontario (E. W.)	T. A.	5	
P7063	Ontario (E. W.)	T. A.	6	
P7376	Ontario (T. A.)	J. P./B. T.	6[e]	
P1656	Illinois	J. P.	7	May be same as P7363[f]
P7064	Canada (C. M.)	T. A.	7	
P7363	Ontario (T. A.)	J. P./B.T.	7[e]	May be same as P1656[f]
P7469	Illinois (J. P.)	J. M. (US7)	7[e]	Different nuclear RFLPs than P1656
P7358	Indiana (K. A., F. L.)	T. A./J. P./B. T.	8[e]	
P3258	Illinois	N. T. K.	9	
P7359	Mississippi (B. K.)	T. A./ J. P./B T.	10[e]	
P7360	Ontario (C. M.)	T. A./J. P./B. T.	11[e]	
P7069	Mississippi (B. K.)	T. A.	12	Different nuclear RFLPs than P7369
P7369	Mississippi (B. K.)	T. A./J. P./B. T.	12[e]	Different nuclear RFLPs than P7069
P7361	Mississippi (B. K.)	T. A./J. P./B T.	13[e]	
P7364	Mississippi (B. K.)	T. A./J. P./B. T.	14[e]	
P7365	Mississippi (B. K.)	T. A./J. P./B. T.	16[e]	
P7074	Arkansas (B. K.)	T. A.	17	May be same as P7366[f]
P7366	Arkansas (B. K.)	T. A./J. P./B. T.	17[e]	May be same as P7074[f]
P7076	Mississippi (B. K.)	T. A.	19[e]	
P7078	Indiana (F. L.)	T. A.	21	
P7079	Indiana (F. L.)	T. A.	22	
P7080	Indiana (F. L.)	T. A.	24	
P7081	Indiana (F. L.)	T. A.	25	
P7082	Mississippi (B. K.)	T. A.	26[e]	
P7073	Japan (M. N.)		36[e]	
P7304	Japan (M. N.)		37[e]	

[a] Accession number in the U.C. Riverside *Phytophthora* species collection.

[b] Original site of isolation and investigator who conducted the isolation. Where known, the investigator's strain designation is given. C.G. = C. Grau (U. Wisconsin); F. S. = A. F. Schmitthenner (Ohio State); J. J. = J. P. Jones; R. W. = R. L. Wick (Virginia Polytech, Blacksburg); K. A. = K. Athow (formerly Purdue U., IN); F. L. = Francis Laviolette (formerly Purdue U., IN); J. I. John Irwin (U. Queensland, Australia); A. H. = A. A. Hildebrand (formerly Agriculture Canada, Harrow, ON); B. K. = B. L. Keeling (USDA, Stoneville, MS); F. L. M. = F. L. Morgan (formerly USDA, Stoneville, MS); S. N. = S. L. Nygard (formerly U. Wisconsin); E. W. = Ed Ward (formerly Agriculture Canada, London, ON) T. A. = Terry Anderson (Agriculture Canada, Harrow, ON); C. M. = C. Meharg; J. P. = Jack Paxton (U. Illinois); M. N. = Megumi Nagahama (Kamikawa A. E. S., Japan).

[c] Investigators who maintained and passed on the isolate prior to its deposit at U.C. Riverside. Several isolates were passed through several laboratories before reaching U.C. Riverside, e.g., T.A./J.P./B.T. indicates isolates acquired from the original investigator by T. Anderson, who then sent the isolate to J. Paxton, who sent the isolate to B. Tyler, who desposited the isolate at U.C. Riverside. Where known, the investigator's strain designation is given. Blanks indicate that the isolate was deposited directly by the original investigator. D. E. = D. Erwin (U.C. Riverside); N. T. K. = N. Keen (U.C. Riverside); D. M. = Doug Maxwell (U. Wisconsin); IMI = International Mycological Institute, ATCC = American Type Culture Collection; D. K. = D. Kuhn (Miami International U.); B. T. = B. Tyler (U.C. Davis); J. M. = J. Manners (U. Queensland, Australia).

[d] These strains had a recent common origin, and should be identical, but have minor mtDNA differences.

[e] The pathotypes of these isolates were confirmed as part of this study.

[f] These isolates are possibly (P1656 and P7363) or probably (P7074 and P7366) of common origin, but this cannot be confirmed due to lack of records. These pairs of isolates have identical nuclear and mitochondrial RFLPs.

24 isolates of *P. sojae* of North American origin. In contrast, the present study revealed 16 different mtDNA types among 20 isolates when DNAs were digested by four restriction enzymes. In additional isolates, even more polymorphisms were evident, although the overall similarity among isolates was high (minimum similarity coefficient between isolates = 0.68). No correlation was evident between mtDNA pattern group and geographic origin.

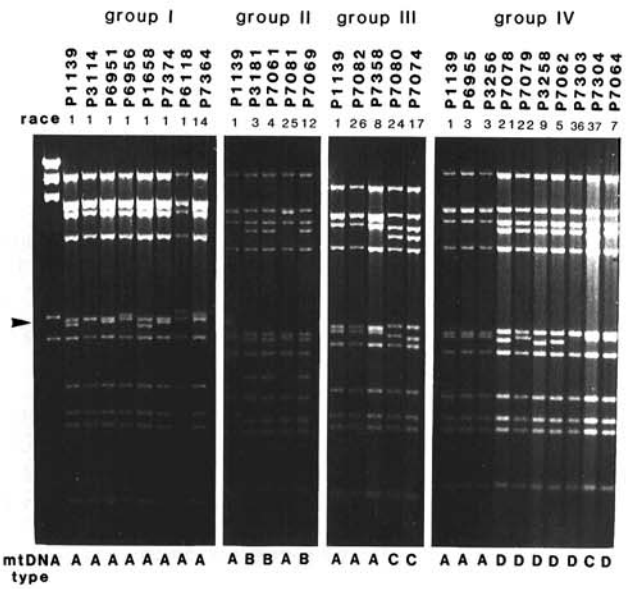

Fig. 4. Variation in mtDNA restriction digestion patterns. Restriction fragment patterns of *Hin*dIII digested mtDNAs from selected isolates. The arrowhead indicates a hypervariable fragment. A–D indicate four distinct restriction patterns identified after excluding the hypervariable fragments. Groups I–IV are as described in the text and in Figures 5–7. Isolate P1139 is included with groups II to IV for comparison. The following pairs of isolates had a recent common origin: P1139 and P3114, P6951 and P6956, P1658 and P7374.

Some race types may have arisen by clonal evolution.

The isolates placed in group IA have identical or near identical nuclear RFLPs. Figure 6 shows the pathotypes of the eight race types occurring among these isolates. Isolates of race 1 are avirulent against every *Rps* gene except *Rps7*. In contrast, the isolates of the other races are virulent against various *Rps* genes. The first isolates of *P. sojae* obtained from soybean crops were race 1. Additional races were identified as cultivars were planted containing *Rps* genes. A likely explanation for the fact that these isolates have near-identical RFLPs (compared to the other more diverse isolates), but differ in their virulence against *Rps* genes, is that these isolates arose clonally from a small number of strains of race 1 by mutations in specific avirulence genes, conferring a selective advantage in fields planted to soybeans containing *Rps* genes. The evidence seems particularly strong for the evolution of a new race type by mutation in the case of race 15 isolates in Australia. The three isolates of race 1 from Australia analyzed here contained a unique RFLP allele (74P2B) not found among any U.S. isolates. Subsequent to the extensive planting of the cultivar Davis in Australia, isolates of race 15 were commonly recovered from diseased plants (Ryley and Obst 1992). The one isolate of race 15 from Australia examined here (P7227) had identical nuclear RFLPs to the Australian race 1 isolates, including the presence of the allele 74P2B, consistent with it having derived directly from an Australian race 1 strain. The genetic near-identity of these

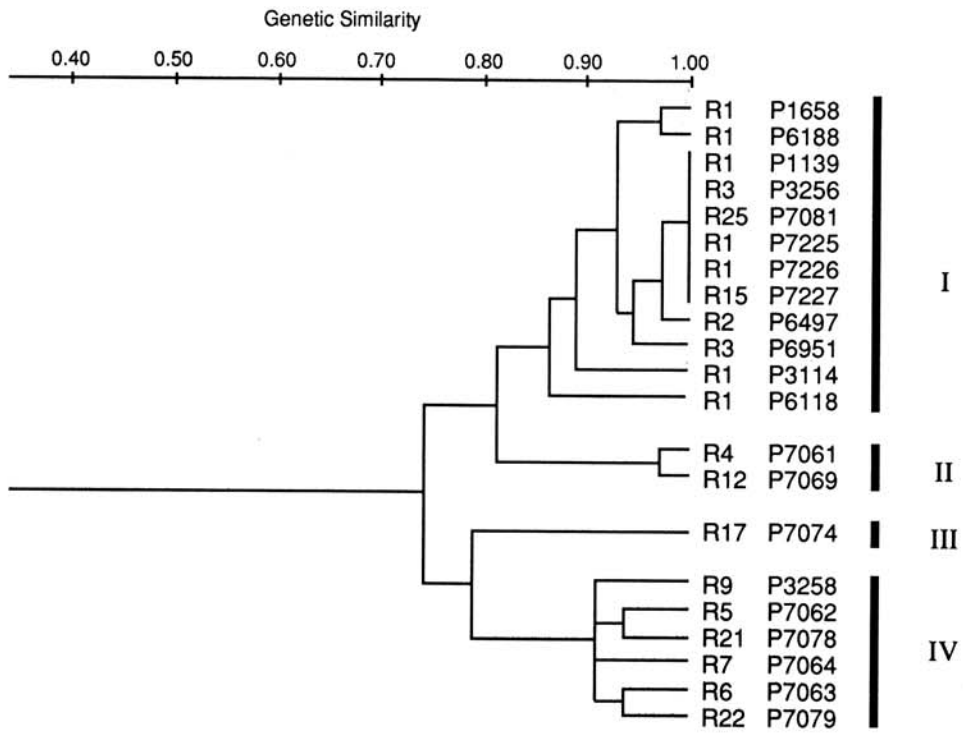

Fig. 5. Genetic similarity of selected isolates based on mtDNA RFLPs. Four-digit isolate numbers and R numbers indicate race types are as in Figure 3. Vertical bars indicate the same groups of similar isolates as in Figure 3. Length variation in the hypervariable rRNA region was included in the analysis.

isolates means also that it will be very difficult to use RFLPs or random amplified polymorphic DNAs (RAPDs) to identify the race type of isolates in this group, unless the probes are derived directly from the relevant avirulence genes.

A few highly divergent isolates encompass most of the variation in the species.

Based on the similarity comparisons, several isolates highly divergent from the isolates of group IA were identified: P7069 (race 12) in group II, P7074 (race 17) in group III, and P7063 and P7064 (races 6 and 7, respectively) in group IV. These are shown in bold in Figure 3. In group IV, P7376, P1656, and P7363 (races 6 or 7) were identical to P7063 and P7064, while P7469 (race 7) was more divergent (two additional RFLPs). However, these four isolates were only scored with the smaller subset of 20 probes. Similarly, in group II, P7076 (race 19) was identical to P7069, while P7369 (race 12) was slightly more divergent (two additional RFLPs), but these two isolates also were scored only with 20 probes. Figure 7A shows that altogether, the three sets of divergent isolates, together with the isolates of group IA, contain virtually all of the RFLP alleles found in the complete set of 48 isolates. Many of the alleles are unique to one of the four divergent sets of isolates. The only alleles not present in any of the most divergent isolates (heavy-outlined in Fig. 7) are restricted to just one or two isolates, suggesting that those alleles arose by recent mutations.

A "CLONAL" ISOLATES

		Reactions against Rps Resistance Genes										
Rps gene		*1a*	*1b*	*1c*	*1d*	*1k*	*3a*	*3b*	*3c*	*4*	*5*	*6*	*7*
Isolate	Race												
P1658	1	A	A	A	A	A	A	A	A	A	A	A	V
P6497	2	A	V	A	A	A	A	A	A	A	A	A	V
P1754	4	V	A	V	A	A	A	A	A	A	A	A	V
P7359	10	A	V	A	A	A	V	A	A	A	V	A	V
P7360	11	A	V	A	A	A	A	A	A	A	A	V	V
P7361	13	A	A	A	A	A	A	A	A	A	A	V	V
P7364	14	V	A	V	A	A	A	A	A	A	A	A	V
P7227	15	A	A	A	A	A	V	A	A	A	V	A	V
P7365	16	A	V	V	A	V	V	A	?	A	A	A	A

B "PROGENITOR' ISOLATES

P1658	1	A	A	A	A	A	A	A	A	A	A	A	V
P7064	7	V	A	V	A	A	V	A	V	V	V	V	V
P7069	12	V	V	V	V	V	A	V	A	A	A	A	A
P7074	17	A	V	A	V	A	V	V	V	V	V	V	V

C GROUP II ("PROGENY" OF CROSS LIKE P1658 X P7069)

P1658	1	A	A	A	A	A	A	A	A	A	A	A	V
P3181	3	V	A	A	A	A	A	A	A	A	A	A	V
P7061	4	V	A	V	A	A	A	A	A	A	A	A	V
P7081	25	V	V	V	A	V	A	A	A	A	A	A	V
P7069	12	V	V	V	V	V	A	V	A	A	A	A	A

D GROUP III ("PROGENY" OF CROSS LIKE P1658 X P7074)

P1658	1	A	A	A	A	A	A	A	A	A	A	A	V
P7358	8	V	A	A	V	A	A	A	V	V	V	A	V
P7080	24	A	V	A	A	A	V	A	A	V	V	V	V
P7082	26	A	V	A	V	A	V	V	V	V	V	V	V
P7074	17	A	V	A	V	A	V	V	V	V	V	V	V

E GROUP IV ("PROGENY" OF CROSS LIKE P1658 X P7064)

P1658	1	A	A	A	A	A	A	A	A	A	A	A	V
P6955	3	V	A	A	A	A	A	A	A	A	A	A	V
P3256	3	V	A	A	A	A	A	A	A	A	A	A	V
P7062	5	V	A	V	A	A	A	A	V	V	A	V	V
P3258	9	V	A	A	A	A	A	A	V	V	V	A	V
P7078	21	V	A	A	A	A	V	A	A	A	V	A	V
P7079	22	V	A	V	A	A	V	A	A	V	V	V	V
P7303	36	A	A	A	A	A	V	A	A	V	V	V	A
P7304	37	V	A	V	A	A	V	V	V	V	V	V	V
P7064	7	V	A	V	A	A	V	A	V	V	V	V	V

Fig. 6. Specific virulence of clonal, progenitor, and progeny isolates. **A,** Isolates proposed to have arisen by clonal evolution. Genetically identical isolates of the same race are not shown. **B,** Isolates representative of proposed progenitor strains. **C–E,** Isolates proposed to have originated by outcrossing among progenitor strains. In C–E the relevant progenitor-type isolates are shown for comparison. "A" indicates specific avirulence of pathogen, i.e., an incompatible reaction in the presence of the particular Rps gene. "V" indicates specific virulence of pathogen, i. e., a compatible reaction in the presence of the particular Rps gene. "?" indicates unknown virulence. Shaded boxes indicate virulence phenotypes which differ between the two proposed progenitors for the group (C–E). Heavy-outlined boxes indicate virulence phenotypes not present in either of the progenitor isolates shown; these phenotypes may have arisen by recent mutation or (in C–E) by crossing to a different progenitor.

Four non-overlapping groups of isolates are defined by RFLP alleles specific to the groups.

A second important feature of the data is that each of the divergent races contains all or nearly all of the B alleles (i.e., those different than in group IA) present within the isolates of the same similarity group. For example, in group II the B alleles found in P3181 (race 3), P7061 (race 4), P7081 (race 25), and P7076 (race 19) are all found in P7069. Likewise in group III, the alleles present in P7358 (race 8), P7080 (race 24), and P7082 (race 26) are all present in P7074 (race 17), with a single exception (the B allele of 8P2 found in P7080). Furthermore, in group IV the B alleles present in races P7062 (race 5), P3258 (race 9), P7078 (race 21), P7079 (race 22), P7303 (race 36), and P7304 (race 37) in group IV are nearly all present in P7063 and P7064 (races 6 and 7). Most of the exceptional alleles not present in P7063 or P7064 are unique to P7304. Figure 7B–D shows the distribution of alleles within the three groups. These figures show that the isolates of groups II, III, and IV are related not only quantitatively, but also because they share RFLP alleles unique to the group.

The distribution of mtDNA restriction pattern types further supports the definition of these four groups. As shown in Figures 4 and 7, four basic mtDNA patterns were observed if the hypervariable fragments are disregarded. Three of these (patterns B, C, and D) are specific to groups II, III, and IV, respectively.

Examination of the nuclear RFLP alleles present in the isolates placed in group IB in Figure 3 shows that although they are quantitatively more similar to the isolates of group IA, they in fact contain alleles unique to either group II or group IV. For example, isolates P3181 (race 3) and P7081 (race 25) carry the B alleles of RFLPs 87H1 (Fig. 1B), 8P2, and/or 68H4, and/or the C allele of 121P1 (Fig. 1A), which are specific to group II (Fig. 7B). Similarly isolates P3256 and P6955 (both race 3) and P3258 (race 9) contain the B alleles of RFLPs 121P1 (Fig. 1A), 74P8, 128P3, and 285R2 which are unique to and nearly ubiquitous in group IV (Fig. 7D). On the basis of these and other distinctive alleles, P3181 and P7081 were assigned to group II, while P3256, P6955, and P3258 were assigned to group IV, as shown in Figure 7B and D. The reassignment of P3258 to group IV is also supported by the analysis of mtDNA RFLPs (Fig. 4). Therefore, all of the 48 isolates we examined can be placed into one of four distinct groups (IA, II, III, and IV) defined by alleles specific to the group, and all of the specific alleles of a

group are found in the most divergent member of the group. The only partial exception is isolate P7304 (race 37); although this isolate contains predominantly alleles specific to group IV, it carries one allele specific to group II (121P1C) and one allele specific to group III (38H4B), as well as three RFLPs unique to itself. P7304 also has the mtDNA restriction digestion patterns specific for group III.

The distribution of alleles among isolates within each group indicate genetic outcrossing has occurred.

Two different evolutionary hypotheses could explain why there are three distinct groups (II, III, and IV) of *P. sojae* with genotypes divergent from that of group IA, and why each group includes an isolate containing all of the alleles specific to the group. In the first hypothesis, the isolates of any group are clonally derived from a group IA isolate by successive mutations which create RFLPs, culminating in the most divergent isolate. Alternatively, the isolates of intermediate divergence could result from genetic crosses between a group IA isolate and the most divergent isolate of a group (or one similar to it). As discussed in detail in the following paragraphs, the data strongly support the latter hypothesis. Consequently, we have designated isolates having RFLP genotypes identical to group IA isolates, to P7069 (race 12), to P7074 (race 17), or to P7064 (race 7) as progenitor isolates. All isolates with genotypes of intermediate divergence are termed progeny isolates.

We evaluated the two hypotheses described above against the RFLP data using an analysis based on transmission genetics and on parsimony. The RFLP data are not suitable for analysis by the methods of population genetics because the isolates analyzed were collected over a long period (20–30 yr), from a wide variety of locations, and often on the basis that they represented novel race type.

In the following discussion, for convenience, we refer to the genotype of an isolate at a particular locus to be A, B, or C etc., rather than AA, BB, or CC despite the fact that *P. sojae* is diploid. This does not affect the basic arguments, especially since heterozygosity appears to be rare in *P. sojae*. To distinguish between successive mutation and outcrossing, we examined the RFLP data within each group for reassortment of alleles at pairs of RFLP loci. Reassortment was inferred from the occurrence of sets of isolates with the following configuration of alleles.

	Locus 1	Locus 2
progenitor isolate 1	A_1	A_2
intermediate isolate 1	A_1	B_2
intermediate isolate 2	B_1	A_2
progenitor isolate 2	B_1	B_2

The presence of all four pairwise combinations of alleles (A_1A_2, A_1B_2, B_1A_2 and B_1B_2) as shown above could easily arise by a genetic cross between the two progenitor isolates, if the loci were unlinked, yielding the two intermediate isolates as progeny. However, it could not occur by successive mutation, unless the exact mutation creating one RFLP allele occurred twice, e.g., $A_1\underline{B_2} \leftarrow A_1\underline{A_2} \rightarrow B_1\underline{A_2} \rightarrow B_1\underline{\underline{B_2}}$, or a reversion occurred, e.g., $A_1\underline{A_2} \rightarrow A_1\underline{B_2} \rightarrow B_1\underline{\underline{B_2}} \rightarrow B_1\underline{A_2}$. Such double mutations or reversions might occur occasionally, for

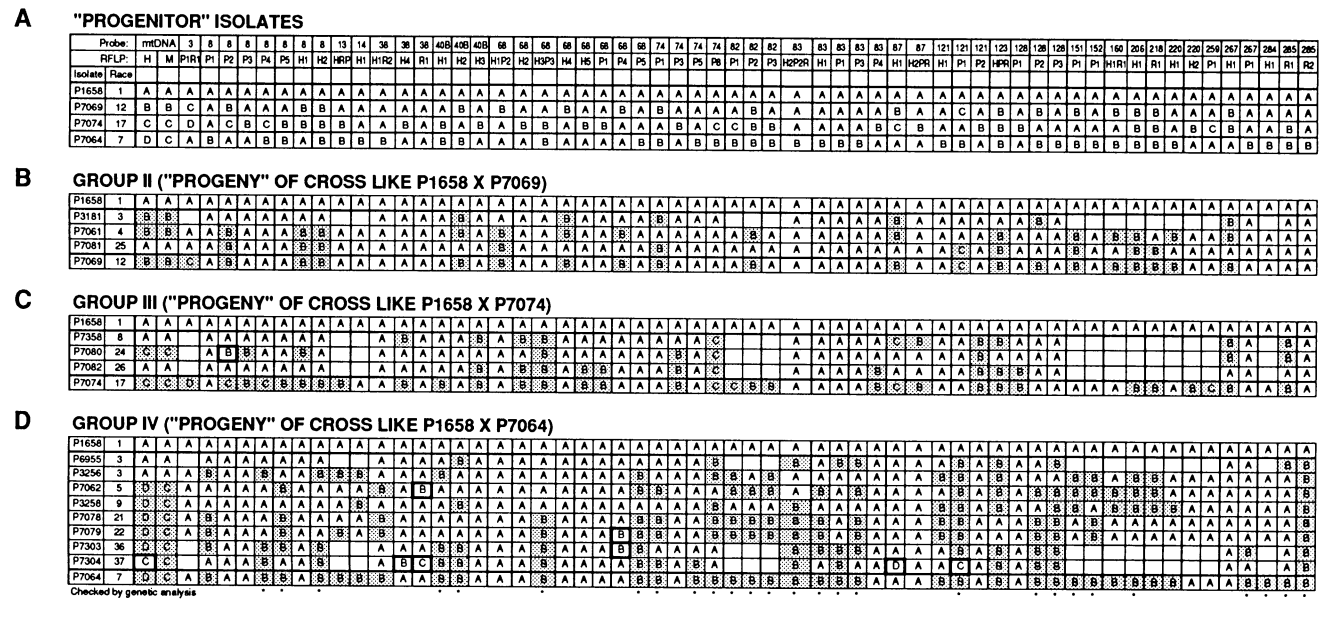

Fig. 7. Distribution of RFLP alleles among proposed progenitor and progeny isolates. **A,** Isolates representative of proposed progenitor strains. All isolates in group IA are identical, or nearly identical to P1658. **B–D,** Isolates proposed to have originated by outcrossing among progenitor strains similar to P1658 and P7069 (B), P1658 and P7074 (C), or P1658 and P7064 (D). In B–D the relevant progenitor-type isolates are shown for comparison. RFLP alleles are arbitrarily designated A, B, C etc. on the basis that the allele state of P1658 (race 1) is always A. mtDNA patterns were scored on the basis of the entire digestion pattern, excluding hypervariable bands, created by *Hin*dIII (H) and *Msp*I (M). Blanks indicate RFLPs not scored. Four additional RFLPs not shown here appear only in one or two of the isolates not included in this diagram. Shaded boxes indicate alleles which differ between the two proposed progenitors for the group. Heavy-outlined boxes indicate alleles present in a progeny isolate but not in a progenitor; such alleles may have arisen by recent mutation or by crossing to a different progenitor. Asterisks (*) indicate loci at which the alleles were confirmed by analysis of the cross between P6497 and P7064.

example at a transposon insertion hotspot. However, the likelihood that such double mutations would occur for many pairs of loci is low.

In group II, proposed as resulting from crosses involving strains similar to P1658 (race 1) and P7069 (race 12) there were 13 polymorphic loci scored for all the isolates of the group, and thus 78 pairwise comparisons of polymorphic loci (13 × 12/2). Of these, 42 (54%) show reassortment; i.e., all four combinations of alleles (A_1A_2, A_1B_2, B_1A_2, and B_1B_2) occur within the group, including the proposed progenitor isolates. In group III, proposed as resulting from crosses involving isolates like P1658 and P7074 (race 17), there were 23 polymorphic loci fully scored and so 253 pairwise comparisons. Of these, 54 (21%) showed reassortment. In group IV, proposed as resulting from crosses involving isolates like P1658 and P7064 (race 7), there were 24 polymorphic loci fully scored and so 276 pairwise comparisons. Of these, 150 (54%) showed reassortment.

If the observed configurations of alleles had arisen by successive mutation, then reversions or double mutations must have occurred and become fixed within group II at seven of the 13 polymorphic loci, within group III at eight of the 22 polymorphic loci and within group IV at 18 of the 24 polymorphic loci, even following the most parsimonious paths (determined using PAUP [Swofford 1990]). The probabilities that particular numbers of double mutations and reversions could have occurred and become fixed can be estimated using the frequency of polymorphic loci within a group (e.g., in group II, [23 polymorphic fragments detected by all probes]/[350 fragments detected by all probes] = 6.6%) as an estimate of the average frequency with which any mutations at a given locus can become fixed within the group. On this basis, the probabilities that double mutations and reversions could have occurred and become fixed at the above frequencies, or greater, are 6.4×10^{-6}, 4.8×10^{-4}, and 2.6×10^{-12}, respectively for groups II, III, and IV, using an exact binomial calculation. In other words, it is extremely unlikely that the observed configurations of alleles could have arisen by successive mutations.

A different measure of the likelihood that the observed RFLP distribution could have arisen by successive mutation is provided by the skewness of the length distribution of all possible phylogenetic trees produced from the data during parsimony analysis (Hillis and Huelsenbeck 1992). If the data are mostly random, as would be expected among genetic progeny, then the distribution should be symmetrical. If the data contain as little as 10% phylogenetic information, produced by successive mutation, the distribution will be significantly skewed to the left (Hillis and Huelsenbeck 1992). The g_1 statistic measures the skewness of a distribution; for a symmetrical distribution, $g_1 = 0$ (Hillis and Huelsenbeck 1992). As calculated by PAUP, g1 for groups II, III, and IV was –0.33, –0.98, and –0.39, respectively, compared to 5% significance levels (Hillis and Huelsenbeck 1992) of –1.09, –1.04, and –1.02,

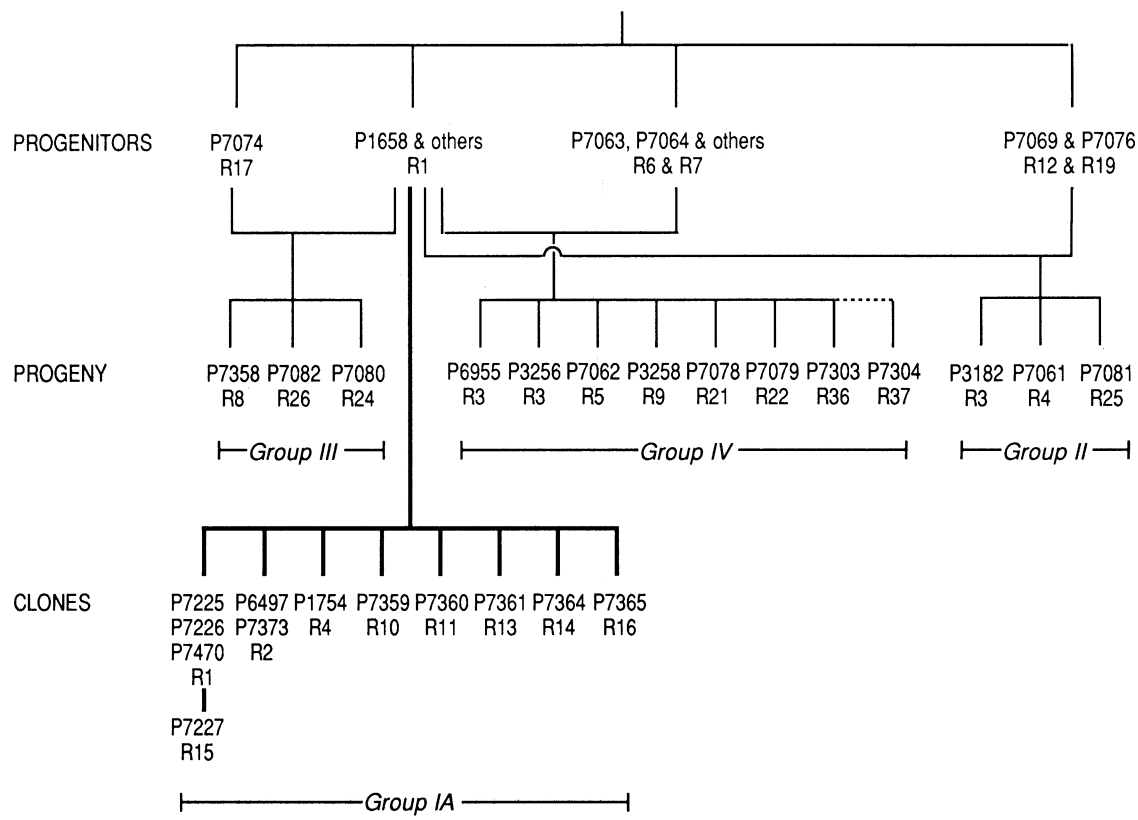

Fig. 8. Isolates of *Phytophthora sojae* arose clonally and by outcrossing of progenitor strains. Four digit P numbers are isolates. R numbers are race types. Bold lines indicate clonal descent by successive mutation. Progenitors, progeny, and clones are as described in the text. Progenitor isolates are representative; the actual progenitors may have been slightly different. Although a single cross is shown for each of groups II–IV, repeated crosses could have occurred within groups. The origin of P7304 is shown by a dashed line as it probably included crosses with strains from other groups (see text).

respectively. Therefore, this analysis shows that the distribution of RFLP alleles in all three groups is not significantly different from random, i.e., consistent with the distributions being produced by outcrossing.

The distribution of mtDNA restriction patterns also is consistent with genetic outcrossing. *P. sojae*, mtDNAs are maternally inherited (as in other oomycetes) and all fertile strains are hermaphrodites (since they can self). Thus the genetic progeny of outcrosses should acquire the mtDNA of one parent or the other, depending on which parent contributed the oogonium and which contributed the antheridium. Within each RFLP group, each progeny isolate displayed either the pattern of group IA, or the pattern specific for the group, i.e., the pattern of one of the two putative progenitors. A third explanation for the observed combinations of alleles, which is a formal possibility, is that the observed distribution arose as a result of self-crosses by progenitor strains which were extensively heterozygous as a result of repeated mutations, rather than the result of outcrosses. This explanation is very unlikely for several reasons. Firstly, for this to have occurred, the mutation rate must have been very high in comparison to the rate of selfing, which is unlikely given the high frequency of selfing by *P. sojae* in the field. Also, if this process were continuing in the present, *P. sojae* would show extensive heterozygosity, which it does not. Finally, selfing of a single heterozygous isolate would be extremely unlikely to produce any pair of isolates which differ at nearly every locus at which there is variation within the group as a whole. In the case of group IV, for example, there are 24 polymorphic loci and nine progeny (including, for this argument, two progenitor isolates such as P1658 and P7064). Therefore, the probability of finding a pair of isolates like P1658 and P7064, which differ at every locus for which there is variation, would be $(8 \times 9 \times 0.5) \times 2^{-24} = 2.1 \times 10^{-6}$. For groups II and III the probabilities would be 1.2×10^{-3} and 2.4×10^{-6}, respectively.

Taken together, the above arguments strongly support the hypothesis that the three divergent groups of isolates (II, III, and IV) arose by outcrossing, resulting in genetic reassortment, as summarized in Figure 8. Therefore, in *P. sojae*, outcrossing appears to be an important mechanism by which new genotypes (e.g., new races) arise. Although it is most likely that the mechanism of outcrossing is sexual mating, it cannot be ruled out that some outcrossing may occur by parasexual mechanisms (Layton and Kuhn 1988, 1990).

Outcrossing is probably rare.

It is possible, though unlikely, that only a single outcross occurred between the progenitor isolates of each group. At the other extreme, the data do not rule out the occurrence of repeated outcrosses within groups, perhaps interspersed with additional mutations. There also could have been some limited outcrossing between groups. For example P7304 (group IV) carries the C allele at 121P1, whereas this allele is otherwise only found in group II (Fig. 1A), and it carries C type mtDNA characteristic of group III. However, several features of the data suggest that outcrossing between genetically different isolates is rare relative to the frequency of selfing: 1) the lack of observed heterozygosity, 2) the presence in the field (Anderson and Buzzell 1992a; Keeling 1982, 1984; Schmitthenner 1991) of pairs of progenitor isolates differing at all polymorphic loci, for a similar reason as described in the previous paragraph, and 3) the lack of genetic mixing between isolates of different groups. Genetic drift, resulting in loss of rare hybrids produced by outcrossing, could further suppress the apparent rate of outcrossing. The present data do not indicate whether the apparent rate of outcrossing is low because genetically different isolates are usually geographically separated, or because outcrossing is infrequent relative to selfing even when two isolates are present in the same field or are infecting the same plant, or both. When two *P. sojae* isolates are mixed in culture, 4–10% (our unpublished data) or up to 76% (Bhat and Schmitthenner 1993) of the oospores produced were genetic hybrids. Detailed sampling and analysis of *P. sojae* from single fields and plants will be required to resolve this question.

Outcrossing may be an important mechanism for generating new race types.

Outcrossing appears to have increased the genetic diversity of *P. sojae* isolates, as measured using RFLPs. Outcrosses also could potentially have increased the number of different race types by reassortment of genes conferring specificity against *Rps* genes (avirulence genes). Alternatively, the different races observed within groups II, III, and IV could have arisen by changes in specific virulence (i.e., by new mutations) subsequent to establishment of the different genotypes. If the first hypothesis were correct, the distribution of virulence alleles among the isolates should fit the progenitor-progeny relationships inferred from the RFLP alleles. If the second hypothesis were true, then there should be little relationship between the distribution of virulence alleles and the distribution of RFLP alleles. As shown in Figure 6C–E, the progenitor isolates of groups II, III, and IV are virulent against the greatest number of *Rps* genes, while the respective progeny isolates are virulent against subsets of the relevant *Rps* genes. Moreover, the specific virulence of isolates, when considering pairs of *Rps* genes, is consistent with genetic reassortment of avirulence loci, based on the same analysis as described above for the RFLPs. These observations are consistent with the hypothesis that most of the virulence alleles found in the progeny isolates derive from progenitor isolates. It also is consistent with the presence of single avirulence genes which determine the reactions against individual *Rps* genes. The hypothesis that there are individual avirulence genes that segregate in a Mendelian fashion is supported by the results of laboratory crosses we have conducted between several races (unpublished). Thus genetic outcrossing may be an important mechanism by which new races are generated and virulence against particular *Rps* resistance genes is spread through the pathogen population. Together with successive mutation, which appears to be responsible for generating new race types within group IA, outcrossing can explain the large number of races described so far for *P. sojae*.

In *Phytophthora infestans*, which is a heterothallic outbreeding species, sexual reproduction appears to be rare outside of central Mexico, even when strains of both mating types coexist (Fry *et al.* 1992). In central Mexico, where sexual reproduction is common, all possible combinations of specific virulence phenotypes (i.e., races) occur. Outside of central Mexico, the range of virulence phenotypes is more restricted, but new races appear rapidly when potato cultivars with resis-

tance genes are planted, presumably as a result or mutation and/or mitotic crossing over (Fry *et al.* 1992). In contrast to *P. sojae*, most *P. infestans* isolates show substantial heterozygosity, probably because selfing is rare (Fry *et al.* 1992).

Both the independent reassortment of genetic variation among *P. sojae* isolates of different race types, and the clonal appearance of new races with identical RFLPs (e.g., group IA) means that it will be very difficult to use of molecular markers such as RFLPs or random amplified polymorphic DNAs (RAPDs) to try to identify virulence phenotypes, unless the markers are tightly linked to, or coincident with, the relevant avirulence genes. In contrast to *P. sojae*, nonsexually reproducing pathogens such as the rice blast fungus, *Magnaporthe grisea* (Levy *et al.* 1991) and certain but not all *Fusarium* species (Gordon and Okamoto 1992; Whitehead *et al.* 1992), the parallel accumulation of mutations which alter specific virulence or create sequence polymorphisms has resulted in a strong correlation between RFLP types and race types. In outbreeding pathogens, such as *Venturia inaequalis* (Day 1974), *Bremia lactucae* (Gustafsson *et al.* 1985), several rusts (Day 1974), and *Phytophthora infestans* (in central Mexico) (Fry *et al.* 1992), outcrossing is an important mechanism by which new race types are generated. In these pathogens, the concept of races as genetically distinct groupings has little meaning, as a very large number of virulence phenotypes can be generated by sexual crosses. Since sexual outcrosses appear to be responsible for many race types in *P. sojae*, it can be predicted that a very large number of races eventually could be identified in the field (already there are 37). In support of this prediction, Hobe and Schmitthenner (1981) identified 40 new race types among isolates obtained directly from soil using leaf baiting. Therefore, in the near future it may be necessary to adopt a different system for describing the virulence phenotypes of different isolates of *P. sojae*. The appearance of novel races of *P. sojae* due to outcrossing will also impact strategies for the breeding of resistant soybean lines using combinations of *Rps* genes.

MATERIALS AND METHODS

P. sojae isolates.

The isolates included in this study are from the *Phytophthora* species collection at the University of California, Riverside, and are listed in Table 1. Isolates of several race types were obtained from the same source (e.g., T. Anderson, Agriculture Canada, Harrow) on several different occasions by different researchers. These researchers (e.g., M. D. Coffey, J. Paxton, J. Manners, B. Tyler) later deposited their versions of the strains at Riverside. Therefore it is likely that several of the Riverside accessions, obtained from the same original source by different routes, may be identical. These are indicated in Table 1. However, it was the practice of several source laboratories to occasionally replace isolates showing incorrect reactions against *Rps* genes with fresh field isolates of the correct race type (T. Anderson and J. Paxton, personal communication). Therefore it is not certain that all of the Riverside accessions in question are in fact identical. Consequently, separate numbering has been retained for these accessions. Working stocks of *P. sojae* were maintained on clarified V8 agar (Ribeiro 1978).

Specific virulence tests.

The specific virulence of *P. sojae* strains, including the new races 36 (isolate P7303) and 37 (isolate P7304), was determined by inoculating seedlings of a standard set of soybean cultivars carrying particular *Rps* resistance genes, namely: Williams (no *Rps* genes), L75-6141 (*Rps1a*), Harosoy 63 (*Rps1a, Rps7*), L77-1863 and HARO13 (*Rps1b*), L75-3735 (*Rps1c*), HARO1472 (*Rps1c, Rps7*), PI103091, and HARO16 (*Rps1d*), Williams 82 (*Rps1k*), HARO1572 (*Rps1k, Rps7*), L83-570 (*Rps3a*), HARO3272 (*Rps3a, Rps7*), PRX146-47 (*Rps3b*), PRX147-48 (*Rps3c*), L85-2352 (*Rps4*), L85-3059 (*Rps5*), HARO5272 (*Rps5, Rps7*), HARO6272 (*Rps6, Rps7*) and Harosoy (*Rps7*). Races 36 and 37 were also screened on HARO(1-7)1 (no *Rps* genes), Asgrow 1937 (*Rps1a*), Altona (*Rps6*), and L89-1581 (*Rps6*). 7- to 12-day-old seedlings were raised in the greenhouse, then placed at 25° C in the light for 24 hr in a growth chamber prior to inoculation. For each *P. sojae* isolate/soybean cultivar combination, five to six seedlings were inoculated by inserting small pieces of infested V8 agar into small slits in the hypocotyl. Individual seedlings were scored after 5 days at 25° C in the light as susceptible (total collapse), resistant (continued strong growth), or intermediate (slowly spreading black lesion, but plant continues growing). Pathogen/cultivar interactions were scored as compatible if no more than one of six seedlings was resistant or two were intermediate, and incompatible if no more than one seedling was susceptible, or two were intermediate. Otherwise the interaction was scored as intermediate. Tests with unknown races or tests showing intermediate or unexpected results were repeated 3 to 4 times using *Rps* genes in several different soybean genetic backgrounds. In our virulence assays, isolates designated races 4 and 14 are avirulent against identical sets of *Rps* genes. Similarly we found no consistent differences between the avirulence reactions of isolates designated races 6 and 7, between the reactions of isolates designated races 12 and 19, nor between the reactions of isolates designated races 17 and 26. Two isolates recently acquired from Japan (P7303 and P7304) did not match any of the published races in their virulence responses against soybean resistance genes. Therefore, we have assigned them to new races 36 and 37, respectively. The reactions of these new races to the different *Rps* genes is shown in Figure 6E.

DNA manipulations and origin of nuclear DNA probes.

DNA manipulation procedures were carried out as described previously (Förster *et al.* 1990). DNA of all isolates was purified by cesium chloride gradient centrifugation. Nuclear DNAs were digested with *Hin*dIII, *Pst*I, or *Eco*RI. MtDNAs were digested with *Hin*dIII, *Hae*III, *Msp*I, or *Eco*RV. Nuclear DNA probes were obtained by cloning random *Hin*dIII fragments of nuclear DNA from *P. sojae* isolate P3114 into pUC19 using standard protocols (Maniatis *et al.* 1982).

Analysis of nuclear DNA and mtDNA RFLP patterns.

Twenty isolates (Table 1) were screened with 39 nuclear DNA probes, detecting 62 polymorphic bands among a total of about 350 bands. The 13 most informative probes were selected for RFLP analysis of an additional 28 isolates. These probes detected 126 restriction fragments including 46 of the

62 RFLPs. RFLPs were named according to the probe used, the restriction enzyme detecting the polymorphism, and when more than one polymorphic band was detected, by a number beginning with the largest polymorphic fragment, e.g., 121P1 indicates an RFLP detected in the largest polymorphic *Pst*I fragment hybridizing to probe p121. Polymorphisms detected with a single probe, and by more than one enzyme, were assumed to correspond to the same locus and therefore were only entered once, e.g., 83H2P2R is a polymorphism detected by digestion with *Hin*dIII, *Pst*I, or *Eco*RI and hybridization with probe p83. The alleles of the RFLPs were designated as A, B, C, etc. with the allele of strain P3114 (race 1) arbitrarily designated as A in every case. Only those nuclear DNA fragments that displayed a polymorphism in at least one isolate were included in the database for similarity analysis. The similarity analysis was performed using the NTSYS 1.6 computer program (Rohlf 1990) using Dice similarity coefficients (Dice 1976). Parsimony analysis was carried out using the PAUP 3.0s computer program (Swofford 1990). Mitochondrial DNA restriction patterns were scored for fragments in common between isolates and for new fragments. All mitochondrial restriction fragments generated by four endonucleases were entered into the database and the analysis by the NTSYS program was done as described previously (Förster *et al.* 1990).

ACKNOWLEDGMENTS

We would like to acknowledge John Reed, Bella Chun, and Areelak Kashemsanta for virulence assays, Terry Anderson and Richard Buzzell (Agriculture Canada, Harrow), Jack Paxton (University of Illinois, Champagne-Urbana), Noel Keen (University of California, Riverside), David Kuhn (Miami International University), John Irwin and John Manners (University of Queensland, Australia), and Megumi Nagahama (Kamikawa Agricultural Experiment Station, Japan) for *P. sojae* isolates, Terry Anderson and Richard Buzzell (Agriculture Canada, Harrow) and Richard Bernard (University of Illinois, Champagne-Urbana) for soybean seed, and Jeff Hall for photography. This work was supported by the University of California Genetic Resources Conservation Program, by the University of California Agricultural Experiment Station and by USDA-NRICGP grant 91-37303-6694 to B. M. T.

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