Genetics

Genetic Relationships Among Australian and North American Isolates of *Phytophthora megasperma* f. sp. *glycinea* Assessed by Multicopy DNA Probes

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ABSTRACT

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Genetic relationships among five Australian and five North American isolates of *Phytophthora megasperma* f. sp. *glycinea* were explored using 10 random multicopy clones from a genomic DNA library of *P. m. glycinea*. Total DNA was cut with eight restriction endonucleases, giving 80 probe × enzyme combinations for each *P. m. glycinea* isolate. Analysis of restriction fragment length polymorphisms (RFLPs) generated by each probe revealed two size classes of RFLP difference between isolates. A large class was observed only with two probes, each distinguishing a single isolate of apparent genetic distance, d (expected frequency of nucleotide substitution), of about 0.05 from the other nine isolates. These two probes provide highly diagnostic DNA fingerprints for particular isolates and should be useful to track further closely related isolates in

population studies. A small class, detected by the remaining eight probes, was characterized by values of d of about 0.01 or less between isolates. Cluster analysis using polymorphisms pooled from these eight probes generated a phenogram that clearly separated a U.S. isolate of race 7 from the other nine isolates (average d of 0.0066). These other isolates formed a discrete cluster of four U.S. isolates (average d within 0.0026) and a discrete cluster of five Australian isolates (average d within 0.0022), with these two clusters being separated by an average d of 0.0031. The close relationships between these geographically separate groups suggest that Australian *P. m. glycinea* isolates are derived from recent ancestors common to the U.S. isolates.

Additional keywords: fungal genetics, genetic variation, soybean.

Members of the genus *Phytophthora* cause a large number of economically important plant diseases on a wide range of field and horticultural crops (1,8,21). Control of *Phytophthora*-incited diseases of field crops has been largely achieved through the breeding of disease-resistant cultivars (8). A major problem with this strategy is that resistance is often overcome by the development of new physiological races (pathotypes) of the pathogen (26). In particular, this situation has arisen for the soybean-specific pathogen *P. megasperma* Drechs. f. sp. *glycinea* T. Kuan & D. C. Erwin, which is widespread in North America, Australia, and elsewhere (29). Soybean (*Glycine max* (L.) Merr.) has been cultivated in North America more extensively and for a longer time than in Australia. Before 1970, soybean was a minor crop with a total area on the order of a hundred hectares grown annually in Australia, mostly in southeast Queensland. Subsequent rapid expansion saw this crop reach about 50,000 ha by the mid-1970s (11). Phytophthora stem rot was first recorded in Australia in 1979 (24), and plant mortalities of up to 90% were recorded in commercial crops of susceptible cultivars in 1981 (27). The disease is now widespread throughout all of the major soybean-growing areas of Australia. Until 1988, only two races (1 and 15) had been identified in Australia, but recently race 4 and isolates with both race 4 and race 15 specificities have been recovered (28,31). Rose et al (27) also reported the occurrence of a high proportion of atypical race 1 isolates in the Australian population. These isolates killed 100% of plants of the cultivar Harosoy upon inoculation and varying proportions of the cultivars Altona and Sanga, whereas typical race 1 isolates only killed Harosoy plants.

Although homothallic, *P. m. glycinea* has demonstrated considerable variation in virulence. Over 20 races have been reported in North America (10,12), and at least three races have been reported in Australia (27,28,31). The small number of races identified in Australia is presumably a consequence of the few

soybean resistance genes so far deployed. Because genetic markers have been restricted in the past to virulence traits (13), proteins (9), or isozyme polymorphisms (22), the data base from which to compare homologies among *P. m. glycinea* isolates has been limited. For example, 224 isolates of *P. m. glycinea*, representing 19 races, had identical patterns when tested with 11 isozymes (22). Because *P. m. glycinea* is homothallic, it might be expected that continuous selfing of field populations would result in segregation towards a series of genetically uniform, homozygous individuals, with the most virulent and aggressive individuals being positively selected. Nonetheless, mixing of germ plasm by recombination could occur if anastomosis (30) and heterokaryosis (14,16) also occur in the field. To investigate the amount of heterozygosity in natural populations, alternative genetic markers to those listed above are necessary.

The advent of molecular techniques for the detection of nucleotide sequence differences in DNA (17) offers an alternative solution to assess differences in the genetic structure of field populations of *P. m. glycinea*. Förster et al (5–7) sized the mitochondrial genome of *P. m. glycinea* at 45.3 kb and constructed maps of restriction sites. They found close homology among North American isolates of *P. m. glycinea*, yet they found considerable differences between isolates of *P. m. glycinea* and isolates of *P. megasperma* obtained from hosts other than soybean. However, the mitochondrial genome codes only for tRNA, rRNA, and a few respiratory enzymes. An analysis of nuclear DNA would provide a wider genetic base for the comparison of different races of *P. m. glycinea* and isolates originating from different geographical locations. At present, there is no information available on the genetic relationships between *P. m. glycinea* isolates, based on nuclear DNA comparisons.

This investigation represents an initial survey of genetic diversity among a geographically diverse group of isolates of *P. m. glycinea*. The primary objectives were to use DNA markers to explore genetic relationships among Australian and North American isolates of this organism. We used random genomic DNA clones

as probes to generate restriction fragment length polymorphisms (RFLPs) in order to identify DNA fingerprints, which uniquely specify individuals, and also to scan the genomes of isolates to estimate genetic distance between the individuals surveyed.

MATERIALS AND METHODS

Fungal isolates. The 10 isolates of *P. m. glycinea* used in this investigation are listed in Table 1, using the race designations of Laviolette and Athow (10). Five isolates belonged to race 1, four of which were from different localities in Australia and one of which was from the United States. One Australian isolate belonged to race 15. The remaining four isolates were from the United States and belonged to races 4, 5, 7, and 9. Cultures of each isolate were initially grown on clarified V8 juice agar (25). Approximately five agar plugs (5 mm³) with fungal mycelium were transferred to 50 ml of sterile clarified V8 liquid (25) in 250-ml flasks and incubated 2–3 days on an orbital shaker (150 rpm, 28 C). This liquid culture was blended aseptically, and 10 ml was used to inoculate 200 ml of sterile clarified V8 liquid in 500-ml flasks. Cultures were grown for an additional 5–7 days, and then the fungal mycelium was harvested by vacuum filtration through Miracloth (Calbiochem Australia, Sydney). Mycelium was then stored at −70 C until used for DNA extraction.

DNA extraction, enzyme digestion, and electrophoresis. DNA was extracted from the frozen fungal mycelium, using a method modified from Panabières et al (23), by RNase A (0.4 µg/ml pretreated at 100 C; Sigma, St. Louis, MO) treatment at 37 C for 2 h to dissolve and digest the DNA pellet after the first ethanol precipitation. The concentration of these DNA preparations was determined using UV spectrometry. The DNA yield was 30–300 µg/g fresh weight for different samples of mycelia. All DNA samples were stored at −70 C. The DNA samples (10 µg) were digested to completion with 30 units of a restriction enzyme (Boehringer-Mannheim, Sydney; New England Biolabs, Beverly, MA) for 4–5 h at 37 C in 10 µl of the buffer recommended by the manufacturer. The following restriction enzymes (each with a six-base recognition sequence) were used: *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Eco*RV, *Kpn*I, *Xho*I, and *Pvu*II. Restricted DNA (5 µg) was separated on 0.8% agarose gel and transferred onto Zeta-probe membrane (Biorad Laboratories) using a vacuum blotting apparatus (Pharmacia-LKB, Uppsala, Sweden).

Probe preparation and hybridization. A genomic DNA library of *P. m. glycinea* isolate, UQ 60, race 1, was prepared as described by Braithwaite et al (3), using *Sau*3A as a restriction enzyme and pUC19 as the vector. DNA was extracted from recombinant plasmids using a rapid alkaline procedure and screened for insert size (2). Medium- to high-copy clones (Table 2) were identified by intense autoradiogram stains when slot blots of DNA from recombinant plasmids were probed with ³²P-labeled total genomic DNA of UQ 60 (3). Ten such medium- to high-copy clones (Table 2), which gave clear banding patterns when used to probe Southern blots of genomic DNA of *P. m. glycinea*, were selected for further work. Lambda DNA (Bresatec, Adelaide, Australia) digested with *Hin*dIII was used as a molecular weight reference. Whole plasmids were used as probes. All probing was conducted at high stringency: probe labeling, hybridizations, filter washing, and exposure to X-ray film were performed as described by Braithwaite et al (3) except that the hybridization temperature was 65 C, and forma-mide was omitted from hybridization mixtures; the high-stringency filter washes involved washing twice at room temperature with 5× SSPE (1× SSPE = 10 mM NaH₂PO₄, 180 mM NaCl, and 0.1 mM ethylenediaminetetraacetic acid, pH 7.4), once at 65 C with 1× SSPE + 0.1% sodium dodecyl sulfate, and then twice at 65 C with 0.1× SSPE + 0.1% sodium dodecyl sulfate. Labeled probes were removed from Southern blots and the blots reprobed as described by Braithwaite et al (3).

Screening of *P. m. glycinea* isolates for RFLPs. In preliminary work, genomic DNA of isolate A2, cut separately with the restriction endonucleases listed above, was used to screen medium- to high-copy clones for those giving the clearest banding patterns, which resulted in the selection of 10 clones as probes. In the complete screening experiment, DNA from the 10 isolates of *P. m. glycinea* each cut by the same restriction endonuclease were

TABLE 1. Physiological race and source of isolates of *Phytophthora megasperma* f. sp. *glycinea* used for genetic analysis[a]

Code	Isolate	Race	Source
A1	UQ60[b]	1	Queensland
A2	9933R15	15	Queensland
A3	CIFR6	1	New South Wales
A4	CIFR10	1	New South Wales
A5	CIFR11	1	New South Wales
U1	US1[c]	1	Illinois
U4	W18750N[d]	4	Wisconsin
U5	US5[c]	5	Illinois
U7	US7[c]	7	Illinois
U9	US9[c]	9	Illinois

[a] All isolates were from the culture collections of J. A. G. Irwin, University of Queensland, and M. J. Ryley, Queensland Department of Primary Industries.

[b] Isolate used to prepare genomic DNA library.

[c] Originally obtained from J. Paxton, Department of Plant Pathology, University of Illinois.

[d] Originally obtained from C. R. Grau, Department of Plant Pathology, University of Wisconsin.

TABLE 2. Hybridization characteristics of probes used during this investigation

Code[a]	Probe[a]	Insert size (kb)[b]	Copy number (slot blot)[c]	Average number of bands[d]	Example of F[e]
P2	pPmgS2	2.5	M	5.3	78/87
P3	pPmgS3	0.3	H	17.4	246/285
P4	pPmgS4	0.8	H	7.8	112/127
P5	pPmgS5	0.1	L/M	5.3	76/88
P6	pPmgS6	1.9	M	4.3	64/78
P7	pPmgS7	1.2	H	20.1	280/334
P8	pPmgS8	2.5	M	17.5	240/262
P16	pPmgS16	0.6	H	20.5	272/320
P19	pPmgS19	0.35	M	10.3	142/154
P22	pPmgS22	0.4	M	9.4	136/146

[a] Probe abbreviations and name. All probes were pUC19 clones of *Sau*IIIA-digested total DNA isolated from *Phytophthora megasperma* f. sp. *glycinea* (isolate UQ60).

[b] Insert size of cloned *P. m. glycinea* DNA fragment.

[c] Relative copy number, determined by hybridization of slot-blotted probe with ³²P-labeled total DNA of *P. m. glycinea* isolate A1, indicates relative size of homologous regions in the *P. m. glycinea* genome. H = high copy number, M = medium copy number, L = low copy number.

[d] Average number of bands per lane observed after probing DNA from isolate A2. Genomic DNA of isolates was cut separately with each of the following restriction endonucleases: *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Eco*RV, *Kpn*I, *Xho*I, and *Pvu*II.

[e] Comparison between isolates A1 and U7: bands generated by all eight restriction endonucleases were summed and expressed as similarity, F, according to Nei (18).

compared side by side on the same Southern blot; a series of such blots were prepared for each restriction endonuclease. All blots were hybridized to each of the 10 probes in turn; examples of autoradiograms from single blots probed by one clone are presented in Figure 1. The use of eight restriction endonucleases thus generated a total of 80 probe × enzyme DNA fingerprints for each isolate. To enable clear scoring of all intense and faint bands for each isolate, autoradiograms were exposed for a series of times after preparation. Control restriction digests showed complete cutting of plasmid DNA (pUC19) added to genomic
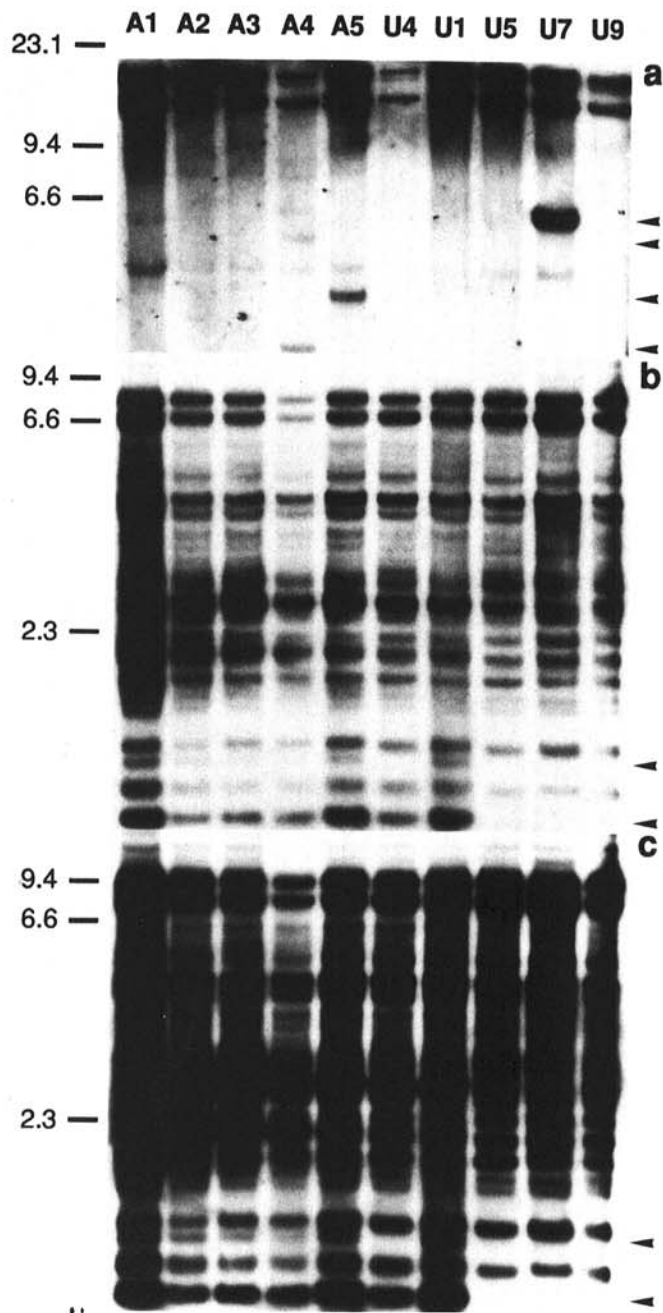
Fig. 1. Autoradiograms of Southern blots showing examples of polymorphic bands detected in total DNA from isolates of *Phytophthora megasperma* f. sp. *glycinea* after hybridization with selected probes. Size markers (λ phage/*Hin*dIII) are indicated as base pairs × 10⁻³; arrows indicate polymorphisms; **A**, DNA samples cut with *Bam*HI, probed with P6 (medium-copy) showing polymorphic bands of varying hybridization intensity; intense bands represent putative tandem repeats; **B**, DNA samples cut with *Pvu*II, probed with P16 (high-copy, putative dispersed repeat) showing polymorphisms in the lower portion of the autoradiogram; **C**, the same as **B**, but with longer exposure time to verify polymorphisms in the lower portion of the autoradiogram.

DNA of *P. m. glycinea*, indicating that the conditions of digestion were sufficient to cut the genomic DNA fully. The reproducibility of our methodologies was demonstrated in an independent second experiment, which gave F values similar to the full survey, for two representative isolates (A4 and U7) using probes P2 and P6.

RFLP data analysis. The computation procedures adopted by Wang and Tanksley (33), based on the theory of Nei (18) and Nei and coworkers (19,20), were used to compare polymorphisms between pairs of isolates. This involved estimating the fraction of fragments, F, in common between the two isolates, using the formula $F = 2m_{xy}/(m_x + m_y)$, where m_{xy} was the number of restriction fragments shared by the two isolates, and m_x and m_y were the numbers of restriction fragments in each isolate. Unless otherwise stated, scores of m_x, m_y, and m_{xy} from restriction fragments generated by each of the eight restriction endonucleases in turn were summed to obtain a pooled F value for each probe. Pooled F values were used to calculate the genetic distance, d, between the two isolates, where d was the expected number of nucleotide substitutions per site (i.e., base pair) using the formulas of Nei (18, p. 106): this involved first using an iterative spreadsheet procedure to calculate a quantity G, which was related to F by the relationship $F = G^4/(3 - 2G)$. Genetic distance, d, was then calculated from the formula $d = -(2/r)\ln G$, where r is the number of nucleotides in the recognition sequence of the restriction enzyme (i.e., $r = 6$ for all the enzymes used in this investigation). The SAS computer program (SAS Institute Inc., Cary, NC) was used for cluster analysis of the d values (McQuitty procedure of SAS, based on the Unweighted Pair Group Method with Arithmetic Mean, UPGMA), and the Proc Tree procedure of SAS was used to generate a dendrogram.

RESULTS

Assessment of probes and banding patterns. Table 2 summarizes the characteristics of the 10 clones selected as probes to detect restriction fragments in genomic DNA of the 10 isolates of *P. m. glycinea*. The size of DNA inserts in clones varied from 0.1 to 2.5 kb. When slot blots of the cloned DNA were probed with ³²P-labeled whole genomic DNA of *P. glycinea*, intense autoradiogram spots indicated that each clone hybridized to medium-high copies of homologous regions of the genome. When used to probe restriction endonuclease-digested whole genomic DNA on Southern blots, each of the 10 clones generated a medium to high number of bands; e.g., when DNA from *P. m. glycinea* isolate A2 was probed, the average number of bands per restriction enzyme varied from 4.3 bands for probe P6 to 20.5 bands for probe P16 (Table 2).

The probes appeared to be homologous either to tandem repeats, as evidenced by intense bands after autoradiography of hybridized Southern blots; to dispersed repeats, as evidenced by a series of bands of weak to moderate intensity; or to both. Some bands were relatively faint, perhaps because they were of relatively low homology to the probe. Such sequences could represent parts of the genome related to a common ancestral sequence or mutations within a tandem repeat giving single-copy fragments of different size. Figure 1 illustrates some of the characteristic patterns obtained with selected probes. However, all bands were given equal weight in calculating F and d values for each probe. Bands of apparently identical size but different intensity were treated as common to each isolate, as such bands were likely to have arisen by the restriction endonuclease cutting within the sequence of tandem repeats, which differed in copy number between isolates.

Assessment of polymorphic DNA fingerprints generated by each probe. Examples of polymorphic bands that distinguished the isolates of *P. m. glycinea* from each other are indicated by arrows in Figure 1. Polymorphisms between any two isolates cut by the same restriction endonuclease were recorded by scoring the number of bands in common and the total number of bands; scores were summed from all eight restriction endonucleases to calculate F values for each probe (e.g., as shown in Table 2 for

the comparison between isolates A1 and U7).

To evaluate the potential for each probe to provide unique DNA fingerprints of individual isolates, individual F values were determined for each probe. This analysis revealed two general size classes of F values, as exemplified in Table 3, which compares isolate A1 with each of the other nine isolates of *P. m. glycinea* in turn. Most F values were within the range 0.9–1.0 (U7 gave somewhat lower values, ranging down to 0.82); substantially lower F values were observed only with the specific probe/isolate combinations P2/A4 ($F = 0.523$) and P5/U4 ($F = 0.407$) (Table 3).

Further pairwise comparisons with the isolates other than A1 (data not presented) confirmed the two general size classes of F values described above: (i) generally high F values between most isolates (about 0.9–1.0), with somewhat lower values when U7 was compared to the other isolates (range 0.82–0.98), and (ii) very low F values when any isolate was compared with A4 using probe P2 (range 0.43–0.54) or when any isolate was com-

pared with U4 using probe P5 (range 0.38–0.48). Class (i) will also be referred to as the small class of RFLP differences and class (ii) as the large class of RFLP differences. Of the 100 isolate × probe combinations evaluated, only two (A4/P2, U4/P5) generated the large class of RFLP differences.

Because small RFLP differences were observed between most pairs of isolates, the two examples of probe/isolate combinations that gave large RFLP differences with other isolates were considered most likely to provide unique DNA fingerprints with a single restriction endonuclease. This was demonstrated by the pairwise comparison of isolates A4 and U7 in a further experiment

Fig. 3. Computer-generated phenograms of 10 isolates of *Phytophthora megasperma* f. sp. *glycinea*, showing genetic distance, d, for individual probes using pooled restriction fragment length polymorphism data generated by all eight restriction endonucleases; **A**, probe P2; **B**, probe P5; **C**, probe P16. Symbols for isolates and probes are explained in Tables 1 and 2, respectively; for determination of d (18), F values were obtained as described in Table 2.
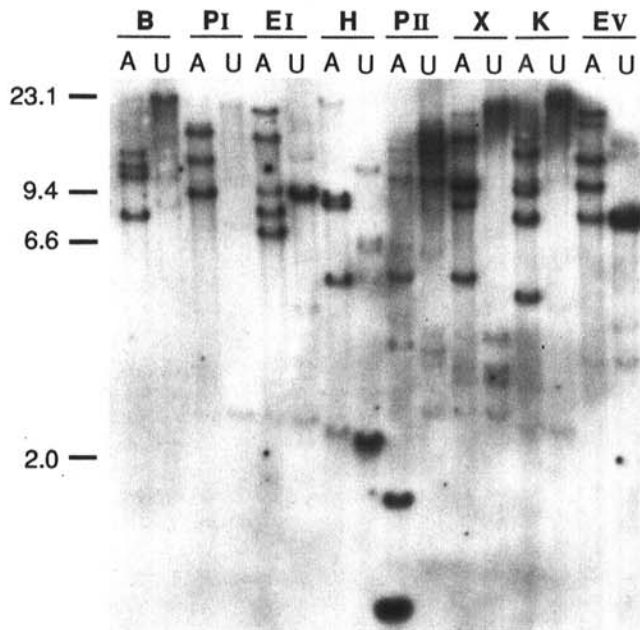
Fig. 2. Autoradiogram of Southern blot, showing DNA fingerprints of *Phytophthora megasperma* f. sp. *glycinea* obtained with the highly diagnostic probe P2 with genomic DNA of isolates A4 (A) and U7 (U) in adjacent lanes. The DNA was cut with *Bam*HI (B), *Pst*I (PI), *Eco*RI (EI), *Hin*dIII (H), *Pvu*II (PII), *Xho*I (X), *Kpn*I (K), and *Eco*RV (EV).
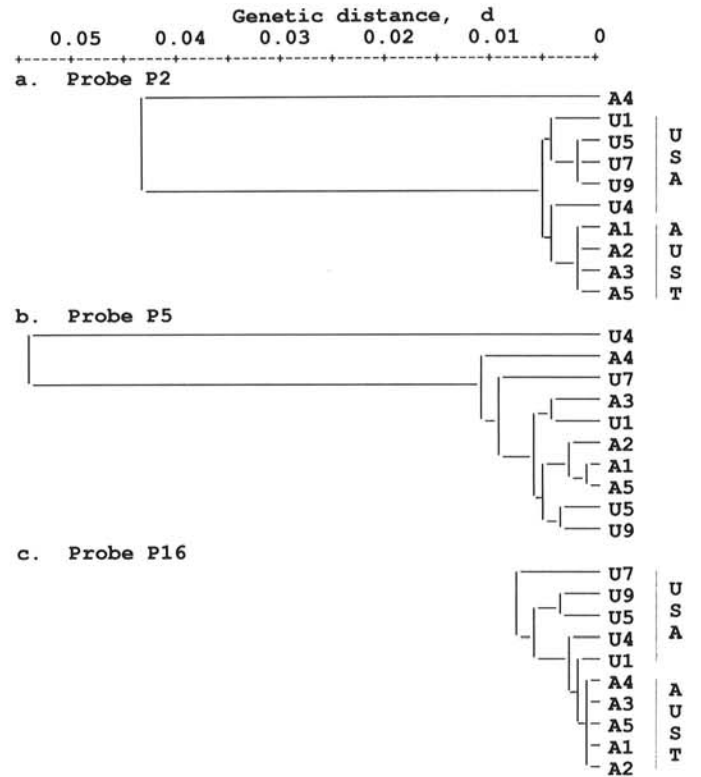
TABLE 3. An example of pairwise comparisons of restriction fragment length polymorphisms between 10 isolates of *Phytophthora megasperma* f. sp. *glycinea*, using isolate A1 as reference[a,b]

Probe	Isolates[c]								
	A2	A3	A4	A5	U1	U4	U5	U7	U9
P2	0.979	0.953	0.523	0.977	**0.888**	0.929	0.909	**0.897**	0.918
P3	0.964	0.964	0.956	0.972	0.968	0.945	0.937	**0.863**	0.942
P4	0.934	0.926	0.951	0.905	0.935	0.927	0.921	**0.882**	**0.894**
P5	0.950	0.919	**0.857**	0.987	0.904	0.407	0.914	**0.864**	0.925
P6	0.930	0.930	0.907	0.907	0.930	0.914	0.914	**0.821**	0.930
P7	0.994	0.988	0.988	0.991	0.988	0.966	0.972	**0.838**	0.963
P8	1.000	0.982	0.952	0.971	0.929	0.932	0.932	0.916	0.932
P16	0.988	0.978	0.972	0.988	0.975	0.952	0.915	**0.850**	**0.892**
P19	0.981	0.968	0.974	0.974	0.961	0.940	0.941	0.922	0.935
P22	0.993	0.959	0.966	0.973	0.973	0.906	0.973	0.932	0.973

[a]Symbols for isolates and probes are explained in Tables 1 and 2, respectively.
[b]F values for the pairwise comparison of A1 and the isolate specified were calculated using the procedure according to Nei (18).
[c]F values less than 0.9 are in bold. Values about 0.5 or less are boxed.

using P2 as probe: these two isolates gave a series of unique fingerprints with eight different restriction endonucleases (Fig. 2), showing how probe P2 can clearly distinguish A4 from U7 (with U7 being a representative of the other isolates).

Cluster analysis of genetic distances between isolates. Cluster analysis by the UPGMA method generated dendrograms (phenograms) of relationships between isolates, e.g., Figure 3 for probes P2, P5, and P16. The phenograms for probes P2 (Fig. 3A) and P5 (Fig. 3B) clearly demonstrated the selectivity of each of these probes for DNA fingerprinting isolates A4 and U4, respectively; these two isolates each differed from the other nine isolates by a d of about 0.05 (large genetic distances, corresponding to the large RFLP differences noted previously). All other isolates differed from each other by a d of about 0.01 or less (small genetic distances, corresponding to the small RFLP differences noted previously).

Isolates within a genetic distance of about 0.01 were scrutinized for finer relationships. With probe P2, for example, the four Australian isolates other than A4 fell into a narrow Australian cluster (average d about 0.002) associated with a wider cluster of five U.S. isolates (average d about 0.005) as indicated in Figure 3A. Similar Australian and U.S. clusters were observed in phenograms generated using probes P16 (Fig. 3C) and P8 (data not presented). However, the other probes, P5 (Fig. 3B), P3, P4, P6, P7, P19, and P22 (data not presented), produced phenograms with mixed clusters of Australian and U.S. isolates, but with U7 frequently being unclustered and most distant from all the other isolates.

To obtain an overall assessment of genetic distance between all 10 isolates, we pooled F values from the eight probes other than P2 and P5 (P2 and P5 gave unrepresentative F values for isolates A4 and U4), to obtain a mean F value for each pairwise comparison of isolates (mean F values had standard errors ranging from 1.6 to 2.6% of the mean). Mean F values were used to estimate mean d values for cluster analysis. Figure 4 shows that this procedure produced a phenogram that clearly separated U7 from all the other isolates (average d of 0.0066). The remaining isolates fell into a discrete cluster of four U.S. isolates (average d within 0.0026) and a discrete cluster of five Australian isolates (average d within 0.0022), with these two clusters being separated by an average d of 0.0031.

DISCUSSION

In this investigation we explored the genetic variation existing in representative isolates from two geographically isolated populations of *P. m. glycinea*. Ten random, medium- to high-copy-number probes and eight six-base restriction endonucleases were used for RFLP analysis of the genomes of five U.S. and five Australian isolates of *P. m. glycinea*. The use of eight restriction endonucleases allowed us to make a reasonable estimate of the frequency with which each probe generated polymorphisms. Analysis of polymorphisms generated by each probe revealed two size classes, small and large, of RFLP difference among isolates of *P. m. glycinea*. The small class was characterized by a genetic distance, d, about 0.01 or less between isolates. The large class was observed with only two probes, each of which distinguished only a single isolate of apparent d of about 0.05 from the other nine isolates.

Genetic variation can occur by a number of different mechanisms, including point mutation and gross sequence rearrangement. Point mutations accumulate continuously within a sequence as a function of time. Gross sequence rearrangements, such as deletions, insertions, or inversions, occur less frequently but create an abrupt difference between closely related isolates. A simple interpretation of our data is that the small genetic distances represent predominantly the time-dependent accumulation of point mutations as a consequence of geographic separation of groups of isolates. However, the two large genetic distances that were observed with only two separate probes and two isolates were probably due to an infrequent sequence rearrangement in regions of the genome adjacent to or within sequences homologous to the respective probe. A more detailed molecular analysis of

sequences homologous to these two probes will be necessary to define the changes in genome structure of these two isolates. Regardless of the cause, the large class served to DNA fingerprint and hence identify particular isolates among the genetically relatively uniform group of *P. m. glycinea* isolates surveyed from the Australian and North American populations. In future population studies, therefore, probes P2 and P5 should be of value in tracking other isolates closely related to isolates A4 and U4, respectively. In addition, these probes will provide useful markers in sexual and parasexual analyses involving these isolates. As isolate-specific diagnostic probes were found at a frequency of two in 100 (Table 3), it should be possible to find specific probes for particular isolates of interest by screening further multicopy probes.

Genetic distance, d, is defined as the expected frequency of nucleotide substitution among isolates of an organism from a common ancestor (18–20). The calculation of d from differences in the size of restriction fragments is dependent on the assumption that polymorphisms arise from nucleotide substitutions rather than from gross sequence rearrangements (18–20). Because RFLP differences were small in the comparison of most isolates of *P. m. glycinea* with most of the probes, we suggest that the small d values are a reasonable estimation of the genetic distance between isolates. However, because the d values in the large class are unlikely to have arisen solely from nucleotide substitutions, they are probably a gross overestimate of genetic distance; nonetheless, the large d values (or their corresponding low F values) represent a convenient numerical difference index for identifying fingerprinting probes when surveying genetically similar isolates.

Cluster analyses using d values obtained with individual probes produced a series of somewhat different phenograms (Fig. 3). Some probes grouped isolates into discrete Australian and U.S. clusters, whereas other probes grouped isolates into mixed clusters containing both Australian and U.S. isolates. With most (but not all) probes, isolate U7 was unclustered and most distant from the other isolates. These analyses suggest that variation in the genome in regions homologous or adjacent to the probes was not uniform, and emphasize the underlying close genetic relationship between the Australian and the U.S. isolates.

Because probes P2 and P5 appeared to overestimate d with some isolates, the other eight probes were used to obtain mean estimates of d for the overall assessment of genetic relationships among all 10 isolates. Cluster analysis (Fig. 4) confirmed that isolate U7 was unclustered and clearly different from the other nine isolates (average d of 0.0066). The remaining four U.S. isolates formed a discrete cluster, and the five Australian isolates formed a separate cluster, with these two clusters being closer to each other ($d = 0.0031$) than to isolate U7.

Figure 4 indicates that, apart from U7, the Australian and U.S. isolates surveyed in this investigation appear to be derived
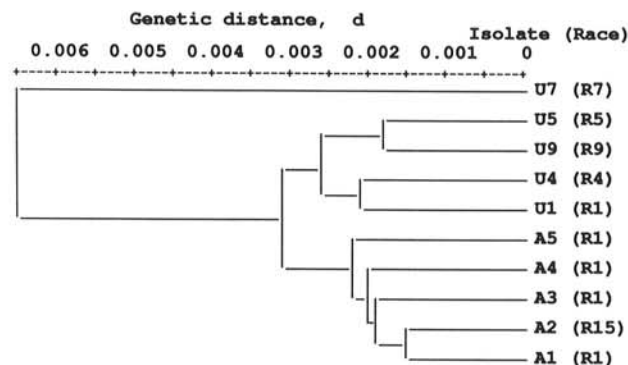
Fig. 4. Computer-generated phenogram of 10 isolates of *Phytophthora megasperma* f. sp. *glycinea*, showing genetic distance, d (18), calculated using mean values of F from eight individual probes (the probes listed in Table 2 less P2 and P5). Values of F for individual probes were determined as described in Table 2; equal weight was given to each probe in obtaining mean values.

from a relatively recent common ancestor. Because U7 was so different from the other isolates, the U.S. population could well be more diverse and have been the source of the present Australian population, but a comprehensive survey of more isolates would be necessary to confirm this hypothesis. In any case, the overall similarity of Australian isolates to U.S. isolates suggests that Australian isolates of *P. m. glycinea* were derived from one or a few closely related introductions of *P. m. glycinea* from overseas, rather than originating from an indigenous Australian source before the introduction of soybean to Australia.

The range of d values among individuals of *P. m. glycinea* described above (Fig. 4) is similar to the range of d values reported for nuclear DNA between individuals and local races of other organisms (33). Our data can also be compared to the restriction analyses of mitochondrial DNA (mtDNA) from 24 North American isolates of *P. m. glycinea*, representing seven races, by Förster et al (7). These workers observed only five distinct RFLP patterns, named Pmg1, Pmg1a, Pmg1b, Pmg2, and Pmg3. Mutation in mtDNA occurs at a relatively constant rate and is believed by some workers to give a more accurate measure of evolutionary relationships than nuclear DNA (4,32). We recalculated the data of Förster et al (7) as genetic distance (d) and obtained values of 0.0008 (Pmg1 vs. Pmg1a), 0.0070 (Pmg1 vs. Pmg2), and 0.0036 (Pmg1 vs. Pmg3); these d values were in the same range as those obtained with repetitive nuclear DNA in the present investigation, if we disregard the infrequent large d values.

Figure 4 shows that race characteristics and genetic distance between isolates of *P. m. glycinea* were independent of each other. Australian isolates A1 and A2 were found to be the two most closely related isolates in this investigation ($d = 0.0015$) but belonged to different races: race 1 and race 15, respectively. However, a U.S. isolate of race 1 (U1) was closer to U.S. isolates of other races (race 4 [U4], race 5 [U5], and race 9 [U9]) than to the four Australian isolates of race 1 (A1, A3, A4, and A5). Furthermore, probe P2 gave a unique DNA fingerprint for one isolate of race 1 (A4), which distinguished it from the other isolates of race 1 listed above. Our analysis of nuclear DNA thus accords with and extends the data of Förster et al (7), which indicated no correlation between race and mtDNA pattern type (for example, different isolates of race 1 gave types Pmg1, Pmg1a, Pmg1b, and Pmg2, and isolates of race 3 gave types Pmg1, Pmg1a, and Pmg3). We suggest that the lack of correlation between race and mtDNA or nuclear DNA restriction patterns indicates either that germ plasm is exchanged frequently between field isolates of *P. m. glycinea* or that new races arise independently on many occasions from a variety of other races. A similar noncorrelation between race phenotype and genetic distance was found among Australian isolates of a closely related fungal pathogen, *Phytophthora vignae* (15).

In summary, our results have identified DNA probes that are diagnostic for particular isolates of *P. m. glycinea*. The isolates A4 and U4 can be DNA fingerprinted by single cloned probes, P2 and P5, respectively. The isolate U7 can be readily distinguished by using a small number of high-copy probes, because of its overall wider genetic distance from the other nine isolates surveyed in this investigation. These probes will be used in future comprehensive surveys of isolates for the detailed genetic characterization of Australian and U.S. populations of *P. m. glycinea*.

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