Variations in the *Phytophthora infestans* Population in Nepal as Revealed by Nuclear and Mitochondrial DNA Polymorphisms

S. R. Ghimire, K. D. Hyde, I. J. Hodgkiss, D. S. Shaw, and E. C. Y. Liew

First, second, third, and fifth authors: Centre for Research in Fungal Diversity, Department of Ecology & Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, PR China; and fourth author: School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, Wales, UK.
Accepted for publication 9 September 2002.

ABSTRACT

Ghimire, S. R., Hyde, K. D., Hodgkiss, I. J., Shaw, D. S., and Liew, E. C. Y. 2003. Variations in the *Phytophthora infestans* population in Nepal as revealed by nuclear and mitochondrial DNA polymorphisms. Phytopathology 93:236-243.

Phytophthora infestans isolates collected from potato and tomato crops from various parts of Nepal during the 1999 and 2000 crop seasons were characterized for nuclear and mitochondrial DNA polymorphisms using restriction fragment length polymorphism markers. The nuclear DNA probe RG57 detected 11 multilocus genotypes among 280 isolates. Three genotypes were detected 21 times or more, constituting 94% of the total population, whereas frequencies of other genotypes ranged from 0.004 to 0.014. The overall genotypic diversity as estimated by the Gleason index was 1.78. Most of the overall diversity was present at the highest level (i.e., interregional, 46%), indicating limited gene flow among regions. Cluster analysis of multilocus genotypes derived from RG57 and mating type data for Nepalese isolates and representative isolates worldwide showed Nepalese isolates grouping into four clusters. Characterization of 67 isolates for mitochondrial DNA polymorphisms revealed the presence of two mt-haplotypes, Ia and Ib with the proportions of 0.88 and 0.12, respectively. Polymorphisms in nuclear and mitochondrial DNA revealed a moderate level of diversity in this population. Genotype NP3 had an identical RG57 fingerprint to US1 and had mt-haplotype Ib, confirming the presence of an old population in Nepal. Most of the genotypes had a different RG57 fingerprint than that of US1 and mt-haplotype Ia, the common characteristics of new populations. The presence of a new population at high proportions in Nepal was consistent with the global trend of mt-haplotype distribution, and suggests the displacement of old populations. This study indicates at least three possible introductions of *P. infestans* to Nepal.

Additional keywords: genotypic diversity, potato late blight, restriction fragment length polymorphisms.

Phytophthora infestans (Mont.) de Bary causes late blight in potato and tomato and is worldwide in distribution (23). Late blight is a major yield-limiting disease in all potato-growing areas of the world (4,12). *P. infestans* is a heterothallic oomycete, which forms sexual oospores when A1 and A2 mating types interact. There is evidence that essentially a single asexual clone associated with the A1 mating type was distributed worldwide but that A2 and sexual reproduction were confined to highland Mexico, the center of diversity (18). Following migration events over the last 25 years, both mating types and new genotypes have replaced the old A1 clone in most countries (11,19,25,29,35–37). Variants resistant to metalaxyl have arisen wherever this systemic fungicide has been widely used to control the disease and have complicated management of the disease with this curative fungicide (3,5,7,9,13,16,21).

Late blight is one of the most important biotic constraints to potato production in Nepal (1). The disease first was reported in Nepal sometime between 1883 and 1897 (34) and has been appearing in epidemic proportions since the mid-1990s. A nationwide crop failure due to late blight was observed in 1996 (8). Subsequent regional outbreaks have been observed almost annually, and cultivars previously resistant are no longer adequately resistant. Characterization of a limited number of *P. infestans* isolates, mainly from the eastern part of Nepal, revealed the presence of both mating types and both old and new alleles at two allozyme loci in isolates collected during 1996 and 1997 (28). Our studies of 371 isolates collected nationwide during 1999 and 2000 showed the presence of 30 virulence phenotypes, both mating types and both metalaxyl-sensitive and -resistant isolates (14,15). These studies were quite useful for a general understanding of diversity in the Nepalese *P. infestans* population. However, it was not possible to work out the underlying genetic diversity in Nepal.

Therefore, we intend to further characterize the Nepalese *P. infestans* isolates for restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers with the following objectives: (i) to characterize the diversity in the Nepalese population for nuclear and mitochondrial genomes; (ii) to study the relatedness of the Nepalese population to populations worldwide, and to identify possible migration events contributing to the current level of diversity; (iii) to investigate the occurrence of sexual reproduction and its contribution to underlying genetic diversity; (iv) to test for clonality among RG57 genotypes; and (v) to develop a database on genetic variations in the Nepalese population of *P. infestans* to serve as a baseline against which further genetic changes could be measured.

MATERIALS AND METHODS

Source of isolates. A three-level hierarchical sampling scheme was adopted to allow the estimation of genetic diversity to be partitioned within and among agroecological regions. Three potato-growing areas were selected from each of the three agroecological regions across the country (15) (Fig. 1). Samples of diseased foliage were collected from five to eight farms per area and three to seven isolates were established from each farm. This yielded 35 to 50 isolates from each area. Sampled farms were 500 to 2,000 m

Corresponding author: K. D. Hyde; E-mail address: kdhyde@hkucc.hku.hk

Publication no. P-2002-1216-01R

apart and plants from which isolates were obtained were 10 to 20 m apart in the field. Host cultivar was not identified but each farm usually grew a single cultivar. Late blight-infected potato leaves were collected during the 1999 and 2000 growing seasons. Sampling was done once in an area in either the 1999 or 2000 crop-growing season. A total of 371 successful isolations were made from potato and an additional 15 isolates were established from blighted tomato cultivars CL-1131 and BL-410 in the summer of 2000 at the Agriculture Research Station, Lumle, Nepal. Isolations were performed from a single lesion on antibiotic amended rye A agar medium as described by Ghimire et al. (14). A single, hyphal-tip isolate was established from each culture to avoid mixtures of genotypes. All isolates were cryogenically preserved at HKUCC, the University of Hong Kong, and working isolates were maintained by serial subculture every 4 months.

DNA extraction. A total of 280 isolates consisting of 268 isolates from potato (28 to 30 isolates per potato-growing area) and 12 isolates from tomato were chosen at random from 371 and 15 isolates, respectively, for DNA extraction. Isolates were grown in 90-mm pea-broth plates containing rifamycin at 12.5 μg ml^{-1}, ampicillin at 12.5 μg ml^{-1}, and nystatin at 25 μg ml^{-1} for 2 to 3 weeks at 18°C. Genomic DNA was extracted as described by Pipe et al. (30).

Multilocus fingerprinting with probe RG57. Approximately 8 μg of genomic DNA was digested with the restriction enzyme *Eco*RI as per the manufacturer's instruction. The restriction fragments were separated on a 0.8% agarose gel for 18 to 20 h at 35V. The fragments were transferred onto Nylon membranes (Hybond N+ hybridization membrane) using the alkaline transfer method. The probe was labeled using the chemiluminescent digoxigenin-labeling kit (Boehringer) and hybridized to the membrane as described by Pipe and Shaw (31).

RAPD. RAPD analysis was performed for 28 isolates belonging to three different RFLP genotypes to test the clonality of the two most common RG57 genotypes detected in this study, NP1 and NP2. One isolate of genotype NP3 (a genotype having a very similar RFLP pattern to NP1 and NP2) also was included in RAPD analysis. The NP1 isolates were from seven potato-growing areas and NP2 isolates were from three potato-growing areas. Pilot experiments using the polymerase chain reaction (PCR) method of Maufrand et al. (26) were performed on two isolates each of NP1 and NP3 genotypes with 32 primers. Eleven primers (5′-CCTGGGTCCA-3′, 5′-CCTGGGTGGA-3′, 5′-CCGGCCCC-AA-3′, 5′-GTCCCAGAGC-3′, 5′-TTGGCCGAGC-3′, 5′-GGG-CACGCGA-3′, 5′-GAGGGCGAGG-3′, 5′-GAGCACCAGG-3′, 5′-GAGCACGGGG-3′, 5′-GGGCGCGAGT-3′, and 5′-GGGGG-TTAGG-3′) that each produced at least four reproducible and scorable bands in these two genotypes were selected for this study. The master mix for PCR reactions was prepared as follows: 200 μM each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 5 pM 10-mer primer, enzyme buffer (50 mM KCl, 10 mM Tris-HCl, pH 9), and 1 unit of *Taq* DNA polymerase. The PCR conditions were set as follows: one cycle of 94°C for 2 min; 35 cycles of 94°C for 15 s, 35°C for 30 s, and 72°C for 60 s; and one cycle of 72°C for 5 min.

Mitochondrial DNA haplotype. Mitochondrial haplotypes were determined for 67 isolates representing 17 phenotypes based on mating types (A1 or A2), metalaxyl sensitivity (sensitive = S, intermediate = I, and resistant = R), and RG57 fingerprint: A1:S:NP1 (n = 31), A1:I:NP1 (n = 6), A1:R:NP1 (n = 5), A1:S:NP2 (n = 1), A1:S:NP3 (n = 4), A1:I:NP3 (n = 1), A1:R:NP3 (n = 2), A1:S:NP4 (n = 1), A1:I:NP5 (n = 1), A1:S:NP7 (n = 1), A1:S:NP9 (n = 1), A2:S:NP2 (n = 5), A2:I:NP2 (n = 3), A2:R:NP2 (n = 2), A2:I:NP8 (n = 1), A2:R:NP10 (n = 1), and A2:R:NP11 (n = 1). Known polymorphic regions in mitochondrial DNA were amplified by PCR using primer pairs P2 and P4 as described by Griffith and Shaw (22). The PCR product (10 μl) was digested with restriction enzymes *Msp*I and *Eco*RI in a 20-μl volume at 37°C overnight. Restriction fragments were separated on a 1.4% agarose gel for 3 h at 70 V, and visualized under UV light.

Data analysis. Each DNA fingerprint was scored for presence or absence of a band at each of 25 loci. The Gleason index of diversity was used to estimate genotypic diversity (32). The geographical distribution of diversity was assessed at two levels (region and area) following the methods used by Drenth et al. (10). A multimarker phenotype or genotype was constructed by combining mating type and RFLP fingerprint data. Data from the Global Marker Database for *P. infestans* were used to construct a similar multilocus genotype for isolates representing different continents. Similarity between the Nepalese isolates and isolates obtained worldwide were assessed by cluster analysis. A tree was constructed using the unweighted pair group method with arithmetic means (UPGMA) algorithm based on the Jaccard coefficient. Similar analysis was performed on RAPD data to examine the clonality of isolates belonging to the same RFLP genotype. Analyses were performed using the NTSYS-pc statistical package, version 2.1 (33).

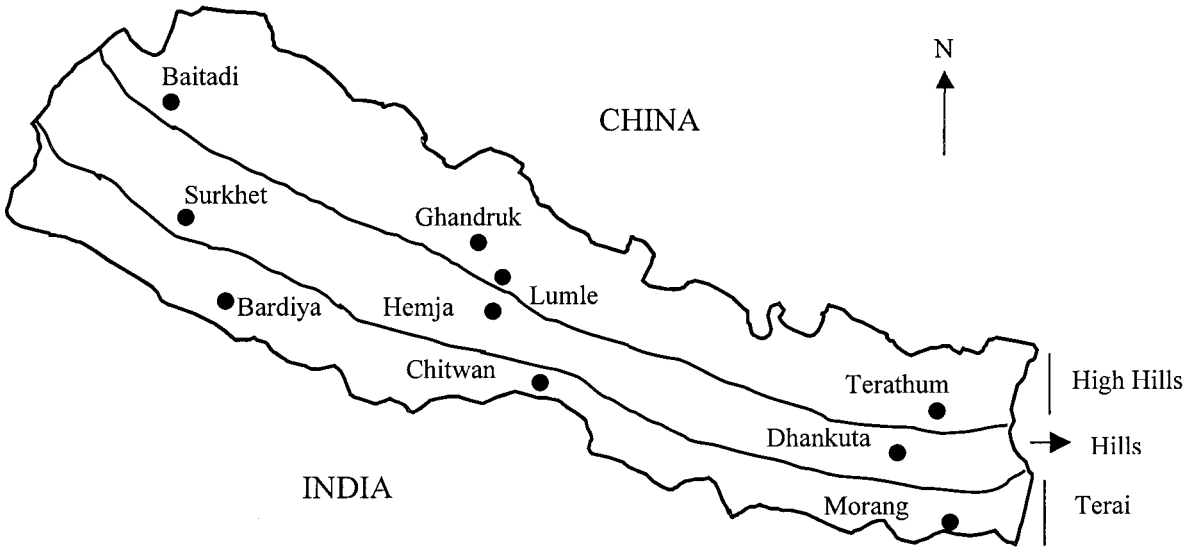

Fig. 1. Map of Nepal showing the agroecological regions (—) and sampling areas (●).

RESULTS

The number of different RG57 genotypes, number of hybridizing and polymorphic fragments, and their relative frequencies are presented in Table 1. Eighteen fragments were identified among 280 isolates at frequencies ranging from 0.003 to 1. All isolates were monomorphic for fragment numbers 3, 5, 7, 13, 14, 20, 21, and 24, while fragment numbers 1, 2, 4, 8, 9, 10, 16, 22, 23, and 25 showed polymorphisms. The number of hybridizing fragments among potato-growing areas ranged from 13 to 16 and the number of polymorphic fragments ranged from 0 to 7. A total of 11 different fragment combinations (RG57 genotypes) were found among 280 isolates (Fig. 2). Of these 11 RG57 genotypes, 3 (NP1, NP2, and NP3) occurred 21 times or more, constituting 94% of the population (Table 2). The number of genotypes per area ranged from one to five. Dhankuta, Hemja, and Terathum had five, four, and three genotypes respectively. All three areas in Terai had a single genotype (i.e., NP1). Genotype NP1, which constituted 68% of the population, was present in all regions and in all study areas except two (Table 3).

The Gleason index of diversity was calculated for all areas and regions and for the overall population. Variation was noted in the diversity levels among subpopulations from areas and regions (Table 3). The highest genotypic diversity was at Dhankuta (H_G = 1.2) and the least at Morang, Chitwan, Lumle, and Bardiya (H_G = 0). Similarly, the regional distribution of diversity was the highest in the Hills (H_G = 1.79) and least in the Terai (H_G = 0). The overall genetic diversity for the Nepalese *P. infestans* population was 1.78. The partition of diversity value into hierarchical components revealed that most of the diversity occurred at the highest level (i.e., interregional differences, 46.14%). The contributions of the intra-area differences and inter-area differences within each region to overall diversity were 20.01 and 33.84%, respectively. Comparisons of bands among the 11 RG57 genotypes showed an overall difference of one to six bands. Sixty percent of the comparisons differed by three or more bands.

UPGMA cluster analysis based on the Jaccard coefficient of multilocus genotypes (RFLP fingerprint and mating type data) from Nepal and different countries is presented in Figure 3. The Nepalese genotypes fell mainly into four different clusters at different positions of the dendrogram. Genotype NP1 was identical to genotypes from Peru, Colombia, and Ecuador, while genotype NP2 was identical to a genotype from Peru but differed in mating type. Genotype NP3 was identical with genotypes from Estonia, China, Korea, and Taiwan. Similarly, NP4 was identical with the

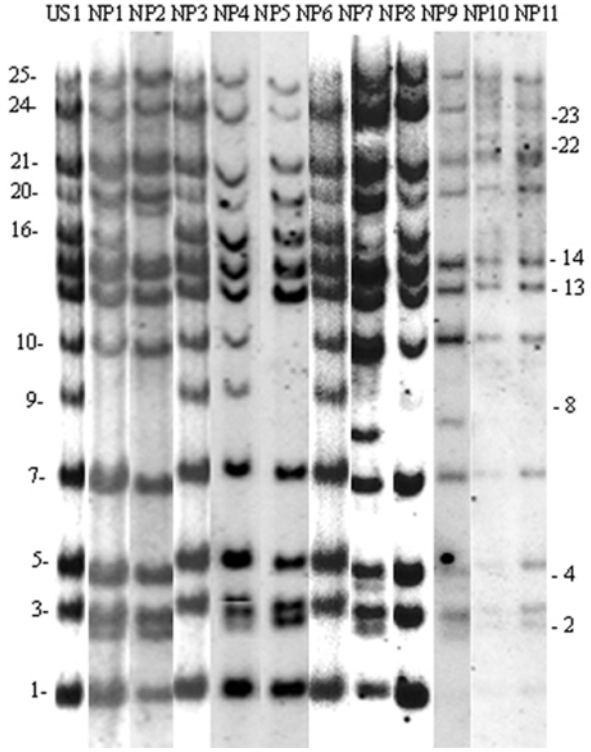

Fig. 2. DNA fingerprints of Nepalese *Phytophthora infestans* isolates obtained with probe RG57. US1 was included as a reference.

TABLE 1. Number of RG57 genotypes, RG57 hybridizing fragments, and polymorphic fragments and their relative frequencies in *Phytophthora infestans* isolates collected from 10 different areas in Nepal in 1999 and 2000

Parameters	Potato-growing area[a]										
	TR	DKT	BRT	GDK	HG	CTN	BTD	SKT	NG	LAC[b]	Average
No. of different RG57 genotypes	3	5	1	2	4	1	2	2	1	1	…
No. of RG57 hybridizing fragments	14	16	13	14	14	13	13	13	13	13	…
No. of polymorphic fragments[c]	2	7	0	1	2	0	1	1	0	0	…
Number of isolates tested	30	28	30	30	30	30	30	30	30	12	…
RG57 fragment number											
1	1	0.96	1	1	1	1	1	1	1	1	0.996
2	1	0.57	1	1	0.96	1	1	1	1	0	0.853
3	1	1	1	1	1	1	1	1	1	1	1.000
4	0	0.07	0	0	0	0	0	0	0	0	0.007
5	1	1	1	1	1	1	1	1	1	1	1.000
7	1	1	1	1	1	1	1	1	1	1	1.000
8	0	0.11	0	0	0	0	0	0	0	0	0.011
9	0	0.43	0	0.07	0.07	0	0	0	0	1	0.057
10	1	1	1	1	1	1	1	0.87	1	1	0.987
13	1	1	1	1	1	1	1	1	1	1	1.000
14	1	1	1	1	1	1	1	1	1	1	1.000
16	0	0.96	1	1	0.37	1	0.87	1	1	1	0.820
20	1	1	1	1	1	1	1	1	1	1	1.000
21	1	1	1	1	1	1	1	1	1	1	1.000
22	0.07	0	0	0	0	0	0	0	0	0	0.007
23	0.03	0	0	0	0	0	0	0	0	0	0.003
24	1	1	1	1	1	1	1	1	1	1	1.000
25	1	1	1	1	1	1	1	1	1	1	1.000

[a] TR = Terathum, DKT = Dhankuta, BRT = Morang, GDK = Ghandruk, HG = Hemja, CTN = Chitwan, BTD = Baitadi, SKT = Surkhet, NG = Bardiya, LAC = Lumle.
[b] All isolates were from tomatoes.
[c] Within-area polymorphisms.

genotypes from Peru and Kenya, NP5 was identical with a genotype from Colombia, and NP7 had an identical DNA fingerprint with PO-45 from Poland. Genotype NP8 was identical to the genotypes from Chile, Ecuador, Kenya, and Peru, and NP12 was identical with one of the genotypes from Peru. All other genotypes (NP6, NP9, NP10, and NP11) were unique in this study.

We found the RAPD pattern to be reproducible, as observed by Maufrand et al. (26). Eleven primers detected a total of 79 reproducible and clearly scorable bands, and 26 of them were polymorphic. RAPD analysis detected polymorphisms within the isolates of identical RG57 genotypes. RAPD markers further differentiated isolates of NP1 and NP2 genotypes into three and two RAPD phenotypes, respectively (Fig. 4). The differentiation in these genotypes was not correlated with any phenotypic characters such as race, mating types, and metalaxyl sensitivity. All isolates of genotype NP1 from Bardiya had distinct RAPD patterns, whereas all tested isolates from Morang, Ghandruk, and Dhankuta had a common RAPD pattern. The isolates from Chitwan, Baitadi, and Surkhet had two different RAPD patterns. Cluster analysis of RFLP fingerprint data showed a similarity coefficient of 0.92 between NP1 and NP2, whereas it was 0.73 based on RAPD data, and the similarity coefficient between three RAPD phenotypes of NP1 genotype was greater than 0.98 (Fig. 4).

Characterization of 67 *P. infestans* isolates with 17 phenotypes belonging to 10 RG57 genotypes for mt-haplotypes showed the presence of two mt-haplotypes (Table 2, Fig. 5). Three bands (387, 361, and 350 bp) were very similar in size in both haplotypes; therefore, they appeared as a single band of high intensity. A band of 79 bp, the smallest fragment expected for haplotype Ib, was inconspicuous due to low gel resolution. Haplotype Ib was associated with A1 mating type isolates (excluding one A2 mating type isolate), whereas haplotype Ia was associated with both mating types. No apparent relationships were observed between mt-haplotype and metalaxyl sensitivity characteristics in this study. Isolates belonging to the same RG57 genotype always had the same mt-haplotype. Out of 10 RG57 genotypes characterized for mt-haplotype, 2 were of haplotype Ib, whereas 8 genotypes were of haplotype Ia (Table 2). Among 67 isolates tested, 12% belonged to haplotype Ib and 88% belonged to haplotype Ia.

DISCUSSION

RFLP fingerprinting revealed 11 RG57 genotypes among 280 Nepalese isolates of *P. infestans*. The same isolates, when characterized previously for mating types, metalaxyl sensitivity, and virulence, differentiated into 2, 3, and 30 phenotypes, respectively (14,15). A combination of mating type, metalaxyl sensitivity, and RFLP markers differentiated the 280 isolates into 20 phenotypes. RG57 has been extensively used to study genotypic diversity in *P. infestans*. A previous study of Goodwin et al. (20) with isolates from Mexico showed a high level of genotypic diversity. Comparison of the Nepalese isolates with these Mexican isolates showed that all the RFLP fragments present in the Nepalese isolates previously had been recorded in Mexico. This supports the view that the Nepalese isolates belong to a subpopulation of the Mexican population. The study of Koh et al. (25) on 124 isolates from China, Japan, Korea, the Philippines, and Taiwan showed the presence of only two lineages, each representing the old and new populations. One of the lineages detected in all these five countries was identical to a genotype found throughout the world previously, designated as US-1 (18). The same lineage, corresponding to genotype NP3, was detected in this study with

TABLE 2. The RG57 genotypes, fingerprint pattern and frequency, mating types, and mt-haplotypes among 280 isolates of *Phytophthora infestans* in Nepal in 1999 and 2000

Genotype	Fingerprint pattern (band 1 to 25)	Frequency[a]	Mating type	Mt-haplotype
NP1	11101 01001 00110 10001 10011	191 (68.2)	A1	Ia
NP2	11101 01001 00110 00001 10011	51 (18.2)	A1 and A2	Ia
NP3	10101 01011 00110 10001 10011	21 (7.5)	A1	Ib
NP4	11101 01011 00110 10001 10011	4 (1.4)	A1	Ia
NP5	11101 01000 00110 10001 10011	4 (1.4)	A1	Ia
NP6	10101 01011 00110 10001 10010	3 (1.1)	A1	…
NP7	11111 01101 00110 10001 10011	2 (0.7)	A1	Ia
NP8	10101 01001 00110 10001 10011	1 (0.4)	A2	Ib
NP9	01101 01101 00110 00001 10011	1 (0.4)	A1	Ia
NP10	11101 01001 00110 00001 11111	1 (0.4)	A2	Ia
NP11	11101 01001 00110 00001 11011	1 (0.4)	A2	Ia

[a] Value in parentheses is the percentage.

TABLE 3. Genotypic diversity and diversity components of the *Phytophthora infestans* population in Nepal in 1999 and 2000

Region and area	Isolates tested	RG57 genotype(s)	Gleason index (H_G)[a]
High Hills			
Baitadi	30	NP1, NP2	0.294
Ghandruk	30	NP1, NP4	0.294
Lumle	12	NP3	0.000
Terathum	30	NP2, NP10, NP11	0.588
Subtotal	102	NP1, NP2, NP3, NP4, NP10, NP11	1.081
Hills			
Dhankuta	28	NP1, NP3, NP6, NP7, NP9	1.200
Hemja	30	NP1, NP2, NP4, NP8	0.882
Surkhet	30	NP1, NP5	0.294
Subtotal	88	NP1, NP2, NP3, NP4, NP5, NP6, NP7, NP8, NP9	1.787
Terai			
Morang	30	NP1	0.000
Chitwan	30	NP1	0.000
Bardiya	30	NP1	0.000
Subtotal	90	NP1	0.000
Total	280	NP1 to NP11	1.775

[a] Intra-area difference (h^-_{area}/h_{total}) = 0.200; inter-area within the region difference ($h^-_{region} - h^-_{area})/h_{total}$ = 0.338; interregional difference ($h_{total} - h^-_{region})/h_{total}$ = 0.461.

isolates occurring in the third highest frequency (7.5%). However, another lineage, JP1 with A2 mating type, had a very different RG57 fingerprint from isolates associated with the A2 mating type in Nepal (25). Based on this evidence, it can be inferred that the former lineage might have established in these east Asian countries and Nepal during an earlier migration of *P. infestans* (18), whereas the A2 isolates in Nepal must have arrived through separate introductions.

The Gleason diversity index of 1.78 for nuclear DNA shows limited genotypic diversity in the *P. infestans* population in Nepal compared with central Mexico, Finland, Poland, the Netherlands, and Norway, where the pathogen is believed to be reproducing sexually (2,10,20,36). However the level of diversity is higher than reported for populations in the United States and Canada in the early 1990s (17). The level of diversity in the Nepalese population revealed by molecular markers is in agreement with those revealed by virulence, mating types, and metalaxyl sensitivity markers (14,15). Partition of genetic diversity at different levels revealed that most of the diversity occurred at the highest level (46%; i.e., interregional differences). Such a high diversity among the regions is due to the presence of unique genotypes in the High Hills (NP10 and NP11) and Hills (NP5, NP6, NP7, NP8, and NP9) (Table 3). Most of these unique genotypes were from areas where mating type A2 was detected as well as from one of the adjoining areas (15). High interregional difference is indicative of limited gene flow among the regional subpopulations. Similarly, a moderate level of genotypic diversity within the areas (20.01%) and within each region (33.84%) indicates limited intra- and inter-area gene flow. Genetic differences among the subpopulations could be due to the timing and nature of the introductions. The usual mechanisms of gene flow in *P. infestans* on potato are dispersal of airborne sporangia and the movement of contaminated seed potatoes. The impact of migration through airborne sporangia among the regions and study areas (except among the sites in Terai) in Nepal appears to be limited. This may be due to various factors, among which the more probable ones are the difference in cropping seasons, presence of hindering mountain ranges, and vast noncropping distances between the study areas. Potato growers in

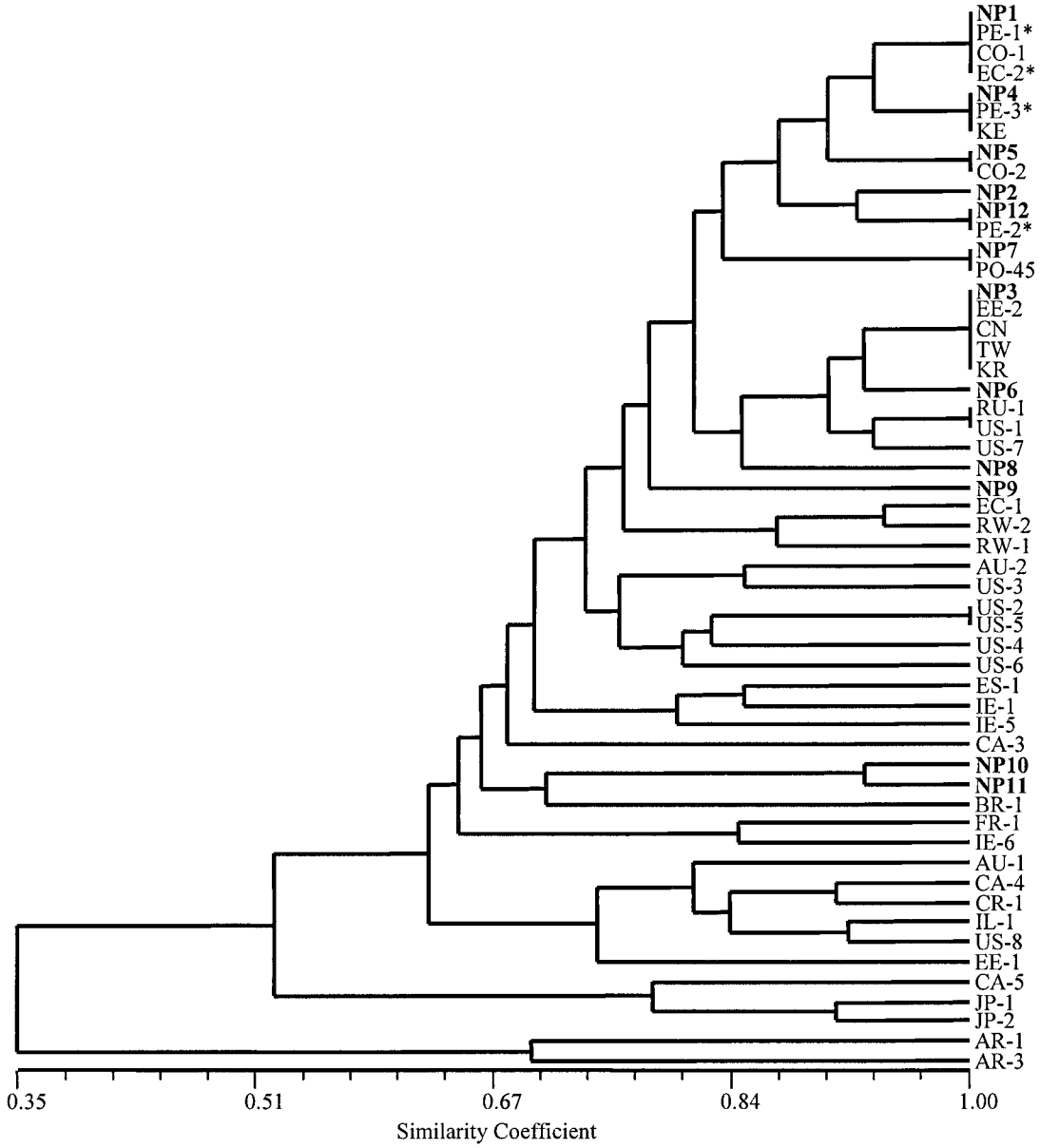

Fig. 3. Unweighted pair group method with arithmetic means cluster analysis on multilocus genotypes (RG57 fingerprint and mating type) of *Phytophthora infestans* genotypes from Nepal and representative worldwide genotypes based on the Jaccard Coefficient. Genotype labels are the International Organization for Standardization (ISO) two-letter country code plus a unique number. Asterisks indicate arbitrary numbers only for this study.

Nepal have a tradition of unidirectional movement of seed potato (from higher to lower altitudes), which also contributes to the limited gene flow in *P. infestans* in the country.

The mt-haplotype distribution in Nepal is consistent with global trend in mt-haplotype distribution among *P. infestans* isolates (6,22,24). If the displacement process operates at the same rate as observed in other parts of the world, isolates with haplotype Ib will be extinct in a few years. Based on the findings from other parts of the world, it can be speculated that the population in Nepal previously was composed only of isolates associated with haplotype Ib, as were the majority of A1 isolates from East Asian countries (25). Almost all isolates with mt-haplotype Ib in Nepal were of A1 mating type and had an RG57 fingerprint identical to US-1. Therefore, they could be from an early immigration. All other genotypes, including the isolates of both mating types and mt-haplotype Ia, could have migrated from Latin America to Nepal in the 1980s when Andean potato plants were being introduced, and thereafter through planting materials. The presence of some identical RG57 genotypes (with haplotype Ib) in Peru, Colombia, Ecuador, and Nepal, and import of planting material from Peru for many years, supports Latin America as a probable source of Ib haplotype in Nepal.

Aggregation of the Nepalese isolates in four different clusters in the UPGMA dendrogram suggests multiple introductions of the pathogen into Nepal (Fig. 3). The first introduction could have been of the NP3 genotype from early migration of *P. infestans*. This genotype has an identical RG57 fingerprint to US1 clone and mt-haplotype Ib, which was detected widely in East Asian countries by Koh et al. (25). Genotype NP6 in the same cluster could be a variant of NP3 by the loss of a single band, 25. Genotypes NP1, NP2, NP4, NP5, NP7, and NP12 could have been introduced in subsequent migrations. The analysis of banding patterns among these genotypes showed that all genotypes except NP7 differed by one to two bands. Most probably, a few genotypes (e.g., NP1 and NP2) were introduced and subsequent sexual reproduction generated other genotypes. Genotypes NP1 and NP2 are genetically very similar, and sexual recombinants from these two genotypes would not be expected to deviate much from either parent. The derivation of these genotypes from NP3 is ruled out by the difference in mt-haplotype. The presence of two bands in NP7 that are not present in NP1 or NP2 suggests that NP7 is unlikely to have been derived from these two genotypes. Therefore, NP7 most probably arrived in Nepal via separate introduction. Most of the Nepalese RG57 genotypes (NP1, NP2, NP4, NP5, NP8, and NP12) are reported in Peru, Colombia, and Ecuador; therefore, this introduction could be directly from these countries through import of planting material. Genotypes NP10 and NP11 could be either a separate introduction or variants of NP2. It requires only one and two mutations in NP2 to produce NP10 and NP11 respectively; therefore, mutation may be the more likely explanation. Genotype NP9, which has a unique banding pattern, could possibly be a sexual recombinant resulted from a cross between NP2 and one of the genotypes of A1 mating type.

Genotype distribution in different study areas is shown in Table 3. The areas where A2 was reported (Hemja and Terathum) had relatively high genotypic diversity compared with the areas with only A1 mating type. This trend was consistent for all areas except for Dhankuta, an area close to Terathum. Although this study on a limited number of isolates in each area did not detect A2 isolate in Dhankuta or A1 isolate in Terathum, the presence of compatible

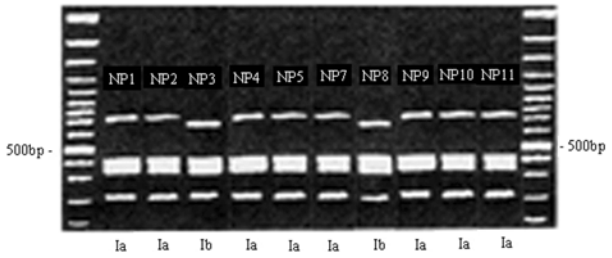

Fig. 5. Distribution of mitochondrial haplotypes Ia and Ib among 10 RG57 genotypes of *Phytophthora infestans* in Nepal. A band of 79 bp in Ib is not distinct due to low gel resolution.

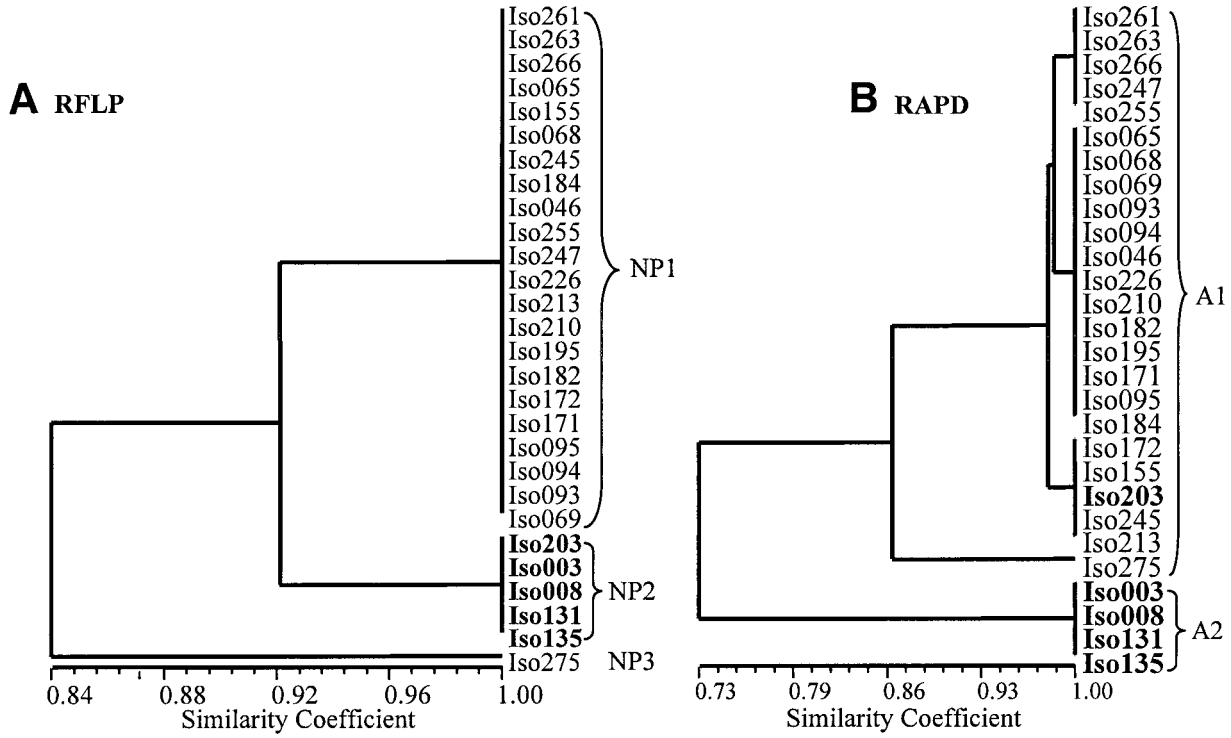

Fig. 4. Differences in polymorphisms revealed by RG57 and random amplified polymorphic DNA (RAPD) markers in some isolates of *Phytophthora infestans* from Nepal. Isolates highlighted in bold are of NP2 genotypes; RFLP = restriction fragment length polymorphism.

mating types in both areas at low proportions is possible. The level of genotypic diversity and the presence of unique genotypes in Nepal may be the outcome of sexual reproduction. The proportion of recombinant genotypes was low in the Nepalese population, whereas it was reported to be high in other sexually reproducing populations in Europe (2,10,37). This difference may reflect the relatively recent onset of sexual reproduction in Nepal. The majority of comparisons between RG57 genotypes differed by three or more bands, indicating that mutation was not the only force responsible for the variation. If mutation alone had been responsible for generating variability, then the genotypes would be expected to differ only by one or two alleles in a short evolutionary time period (27). Therefore, the recent occurrence of sexual reproduction in this population is very likely.

This study detected polymorphisms within RG57 genotypes NP1 and NP2, although identical RG57 genotypes often are considered clonal (20). The similarity coefficient between NP1 and NP2 as revealed by RFLP and RAPD analysis were 0.92 and 0.73, respectively. The RFLP analysis including NP3 showed that NP1 and NP2 are closely related, but this was not the case with RAPD analysis. RAPD analysis further differentiated NP1 into three different RAPD phenotypes with similarity coefficients of about 0.98 indicating a high level of similarity among RAPD phenotypes, which may have arisen due to mutation. One isolate of NP2 with the A1 mating type clustered with one of the NP1 clusters, showing that RAPD can differentiate the mating types within an RG57 genotype. However, it requires further study with a larger number of isolates to confirm this finding.

The molecular characterization of *P. infestans* isolates from Nepal revealed a moderate level of diversity in both nuclear and mitochondrial genomes. As in other parts of the world, the pre-1980 population in Nepal was probably exclusively the US1 clonal lineage, which is still represented by NP3. Subsequent introductions were probably imported from Latin America with planting materials. A high proportion of new genotypes and a low proportion of the old genotype (NP3 with haplotype Ib) suggests that new genotypes are displacing the old ones. The presence of sexual reproduction in this population is suggested by the fact that it contains some unique genotypes, which are very similar to the probable parental genotypes (NP1 and NP2).

ACKNOWLEDGMENTS

This study is part of a collaborative project between The University of Hong Kong and The Nepal Agricultural Research Council. We thank A. Drenth for providing the RG57 probe; the farmers, extension workers, and researchers who provided their precious support in the collection of isolates; and the Hong Kong Research Grants Council and Hill Agricultural Research Project, Nepal, for providing research funding.

LITERATURE CITED

1. Anonymous. 1997. The strategy for potato research. Lumle Agric. Res. Cent. Strat. Pap. No. 97/10. Kaski, Lumle Agricultural Research Centre, Nepal.
2. Brurberg, M. B., Hannukkala, A., and Hermansen, A. 1999. Genetic variability of *Phytophthora infestans* in Norway and Finland as revealed by mating type and fingerprint probe RG57. Mycol. Res. 103:1609-1615.
3. Carter, G. A., Smith, R. M., and Brent, K. J. 1982. Sensitivity to metalaxyl of *Phytophthora infestans* populations in potato crops in southwest England in 1980 and 1981. Ann. Appl. Biol. 100:433-441.
4. CIP 1996. Enhancing the global late blight network. Global initiative on late blight. Centro Internacional de la Papa, Lima, Peru.
5. Davidse, L. C., Looijen, D., Turkensteen, L. J., and Van der Wal, D. 1981. Occurrence of metalaxyl-resistant strains of *Phytophthora infestans* in Dutch potato fields. Neth. J. Plant Pathol. 87:65-68.
6. Day, J. P., and Shattock, R. C. 1997. Aggressiveness and other factors relating to displacement of population of *Phytophthora infestans* in England and Wales, Eur. J. Plant Pathol. 103:379-391.
7. Deahl, K. L., Young, R. J., DeMuth, S. P., and Inglis, D. A. 1992. Virulence of metalaxyl-resistant strains of *Phytophthora infestans.* (Abstr.) Phytopathology 82:719.
8. Dhital, B. K., and Ghimire, S. R. 1996. Blighted potato. In: The Rising Nepal (3 May 1996), Kathmandu, Nepal.
9. Dowley, L. J., and O'Sullivan, E. 1981. Metalaxyl-resistant strains of *Phytophthora infestans* (Mont.) de Bary in Ireland. Potato Res. 24:417-421.
10. Drenth, A., Goodwin, S. B., Fry, W. E., and Davidse, L. C. 1993. Genotypic diversity of Phytophthora *infestans* in the Netherlands revealed by DNA polymorphism. Phytopathology 83:1087-1092.
11. Forbes, G. A., Escobar, X. C., Ayala, C. C., Revelo, J., Ordonez, M. E., Fry, B. A., Doucett, K., and Fry, W. E. 1997. Population genetic structure of *Phytophthora infestans* in Ecuador. Phytopathology 87:375-380.
12. Fry, W. E., and Goodwin, S. B. 1997. Reemergence of potato and tomato late blight in the United States. Plant Dis. 81:1359-1357.
13. Fry, W. E., Goodwin, S. B., Matuszak, J. M., Spielman, L. J., Milgroom, M. G., and Drenth, A. 1992. Population genetics and intercontinental migration of *Phytophthora infestans*. Annu. Rev. Phytopathol. 30:107-119.
14. Ghimire, S. R., Hyde, K. D., Hodgkiss, I. J., and Liew, E. C. Y. 2001. Race diversity and virulence complexity of *Phytophthora infestans* in Nepal. Potato Res. 44:253-263.
15. Ghimire, S. R., Hyde, K. D., Hodgkiss, I. J., and Liew, E. C. Y. 2001. Phenotypes of *Phytophthora infestans* in Nepal: Mating types and response to metalaxyl. Potato Res. 44:337-347.
16. Gisi U., and Cohen, Y. 1996. Resistance to phenylamide fungicides: A case study with *Phytophthora infestans* involving mating type and race structure. Annu. Rev. Phytopathol. 34:549-572.
17. Goodwin, S. B., Cohen, B. A., Deahl, K. L., and Fry, W. E. 1994. Migration from north Mexico as the probable cause of recent genetic changes in populations of *Phytophthora infestans* in the United States and Canada. Phytopathology 84:553-558.
18. Goodwin, S. B., Cohen, B. A., and Fry, W. E. 1994. Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. Proc. Natl. Acad. Sci. USA 91:11591-11595.
19. Goodwin, S. B., Smart, C. D., Sandrock, R. W., Deahl, K. L., Punja, Z. K., and Fry, W. E. 1998. Genetic changes within population of *Phytophthora infestans* in the United States and Canada during 1994 to 1996: Role of migration and recombination. Phytopathology 88:939-949.
20. Goodwin, S. B., Spielman, L. J., Matuszak, J. M., Bergeron, S. N., and Fry, W. E. 1992. Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in northern and central Mexico. Phytopathology 82:955-961.
21. Goodwin, S. B., Sujkowski, L. S., and Fry, W. E. 1996. Widespread distribution and probable origin of resistance to metalaxyl in clonal genotypes of *Phytophthora infestans* in the United States and Western Canada. Phytopathology 86:793-800.
22. Griffith, G. W., and Shaw, D. S. 1998. Polymorphism in *Phytophthora infestans*: Four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. Appl. Environ. Microbiol. 64:4007-4014.
23. Hooker, W. J. 1981. Compendium of Potato Diseases. The American Phytopathological Society, St. Paul, MN.
24. Klimczak, L. J., and Prell, H. H. 1984. Isolation and characterization of mitochondrial DNA of the oomycetous fungus *Phytophthora infestans*. Curr. Genet. 8:323-326.
25. Koh, Y. J., Goodwin, S. B., Dyer, A. T., Cohen, B. A., Ogoshi, A., Sato, N., and Fry, W. E. 1994. Migration and displacement of *Phytophthora infestans* populations in East Asian countries. Phytopathology 84:922-927.
26. Maufrand, R., Archer, S. A., Buck, K. W., Shaw, D. S., and Shattock, R. C. 1995. The use of RAPD markers in genetic studies of *Phytophthora infestans*. Pages 55-63 in: *Phytophthora infestans* 150. L. J. Dowley, E. Bannon, L. R. Cooke, T. Keane, and E. O'Sullivan, eds. Boole Press Ltd., Tegasc, Ireland.
27. Milgroom, M. G., Lipari, S. E., and Powell, W. A. 1992. DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonetria parasitica*. Genetics 131:297-306.
28. Nishimura, R., Sato, K., Lee, W. H., Singh, U. P., Chang, T. T., Suryaningsih, E., Suwonakenee, S., Lumyong, P., Chamswarng, C., Tang, W., Shrestha, S. K., Kato, M., Fuji, N., Akino, S., Kondo, N., Kabayashi, K., and Ogoshi, A. 1999. Distribution of *Phytophthora infestans* populations in seven Asian countries. Ann. Phytopathol. Soc. Jpn. 65:163-170.
29. Perez, W. G., Gamboa, J. S., Falcon, Y. V., Coca, M., Raymundo, R. M., and Nelson, R. J. 2001. Genetic structure of Peruvian population of *Phytophthora infestans*. Phytopathology 91:956-965.
30. Pipe, N. D., Azcoitia, V., and Shaw, D. S. 2000. Self fertility in *Phytophthora infestans*: Heterokaryons segregate several phenotypes. Mycol. Res. 104:676-680.
31. Pipe, N. D., and Shaw, D. S. 1997. Telomere-associated restriction

fragment length polymorphisms in *Phytophthora infestans.* Mol. Plant Pathol. On-Line Publication no. 1997/1124pipe.
32. Rolf, F. J. 2000. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, Version 2.1. Exeter Publishing, Setauket, NY.
33. Sanders, H. L. 1968. Marine benthic diversity: A comparative study. Am. Nat. 102:243-282.
34. Shrestha, S. K. 1976. Study on late blight disease of potato in Nepal. Nepal. J. Agric. 6-11:91-105.
35. Spielman, L. J. 1991. Isozymes and the population genetics of *Phytophthora infestans*. Pages 231-241 in: *Phytophthora*. J. A. Lucas, R. C. Shattock, D. S. Shaw, and L. R. Cooke, eds. Cambridge University Press, Cambridge.
36. Spielman, L. J., Drenth, A., Davidse, L. C., Sujkowski, L. J., Gu, W. K., Tooley, P. W., and Fry, W. E. 1991. A second worldwide migration and population displacement of *Phytophthora infestans*? Plant Pathol. 40:422-430.
37. Sujkowski, L. S., Goodwin, S. B., Dyer, A. T., and Fry, W. E. 1994. Increased genotypic diversity via migration and possible occurrence of sexual reproduction of *Phytophthora infestans* in Poland. Phytopathology 84:201-207.