ABSTRACT

GOMEZ-ALPIZAR, LUIS E. Molecular Evolution of *Phytophthora infestans* (Mont.) de Bary, the late blight pathogen (Under the direction of Jean B. Ristaino).

Phytophthora infestans (Mont.) de Bary causes late blight of potato and tomato and is one of the world's most devastating plant diseases. *P. infestans* left its footprint in human history when, in the 19th century, it was responsible for the Irish Potato Famine. Nuclear and mitochondrial DNA variability was used to examine the population history of *P. infestans*. DNA sequence data from three nuclear regions (Intron Ras, Ras, and β-tubulin) and two mitochondrial regions (P3 and P4) were obtained from ninety isolates from various locations including Brazil, Bolivia, Ecuador, Peru, Costa Rica, Mexico (Toluca Valley), the USA and Ireland. Population summary statistics show that the Mexican population from the presumed center of origin of *P. infestans*, harbored less nucleotide and haplotype diversity than South American populations, and was genetically differentiated from other populations, particularly at the mitochondrial loci. Coalescent-based genealogies of mitochondrial (*rpl14, rpl5, tRNAs, cox1*) and nuclear (Intron Ras+Ras) loci were congruent and demonstrated the existence of two lineages leading to the present day haplotypes of *P. infestans* associated with potatoes. A third lineage, associated with a group of isolates from *Solanum tetrapetalum* collected in the Andean Highlands of Ecuador was also found. In the mitochondrial genealogy the two potato lineages corresponded to the mitochrondrial haplotypes Type I and Type II described elsewhere. Mitochondrial haplotypes were associated with different nuclear backgrounds. Haplotypes found in the Toluca Valley population were derived from only one of the two lineages in both mitochondrial and nuclear genealogies, whereas haplotypes found in South American populations (Peru and Ecuador) were derived from both lineages. Haplotypes

found in USA and Ireland populations were also derived from both lineages and these populations were not genetically differentiated from the Peruvian populations, suggesting a common ancestry among these populations. Evidence for recombination was found for Mexican and USA populations. *Solanum tetrapetalum* isolates were highly polymorphic within the regions analyzed and may be a new species. The results support a South American origin of *P. infestans* and are discussed in relation of previous hypotheses regarding the geographic origin of this plant pathogen.

MOLECULAR EVOLUTION OF *PHYTOPHTHORA INFESTANS* (MONT.) de BARY, THE LATE BLIGHT PATHOGEN

by

LUIS E. GOMEZ-ALPIZAR

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APPROVED BY:

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Biography

Luis Enrique Gómez-Alpízar was born to Lidieth Alpízar and Enrique Gómez on July 2, 1969 in Palmar Norte, Puntarenas, Costa Rica, the youngest of three children. He attended elementary school at Escuela Felix Arcadio Montero, and high school at Colegio Santa Maria de Guadalupe, Santo Domingo, Heredia. In 1979, he entered the University of Costa Rica and earned a degree of Bachelor of Science in Agronomy in 1984 and the degree of Licenciate in Agronomy with a major in Phytotechnics in 1986. After completing his Licenciate degree, he began work at the Agronomy Research Center, Plant Biotechnology Laboratory, University of Costa Rica. In 1990, he received a scholarship from the German Academic Exchange Service (DAAD) to pursued graduate studies at the University of Goettingen, Germany; and earned the degree of Master of Science in Plant Pathology in 1992. His M.Sc. thesis work under the supervision of Dr. Heinrich Lehmann-Danzinger was entitled "Characterization of the resistance to Black Sigatoka disease (*Mycosphaerella fijiensis*) in bananas (*Musa* spp)". From 1993 to 2000 he continued his work at University of Costa Rica and become associate professor. He taught undergraduate and graduate (Master level) students in the Department of Agronomy. In 1994, he was placed in charge of the Plant Biotechnology Laboratory. He participated as national coordinator in the international project funded by the European Union Program for International Cooperation (INCO) "Exploitation of the genetic biodiversity of wild relatives for breeding potatoes with sustainable resistance to late blight" under the general coordination of Dr. Lieselotte Schilde of the Tübingen University, Germany. In addition of the University of Costa Rica and University of Tübingen, other participating institutions were: AZTI - CIMA, Spain; INIAP - PNRT Ecuador; FORTIPAPA, Ecuador; PROINPA, Bolivia; National University of Colombia; and the International Potato Center (CIP), Peru. In 2000, he received a scholarship from Fulbright-

Laspau and began his doctoral work in Plant Pathology under the guidance of Dr. Jean B. Ristaino at North Carolina State University in August 2000. Luis is married to Ruth Villanueva, since 1991, and is the proud father of two boys: Esteban (six years-old) and Samuel (15 months).

Dedication

This work is dedicated to my beloved wife Ruth for her love, devotion, patience, support, and encouragement during our life together, and to my two sons, Esteban and Samuel, who remind me that the future is always bright…

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I am also very thankful to Fulbright-Laspau Foundation, the University of Costa Rica, and the North Carolina State University for their financial support to complete my Ph.D. program.

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POPULATION HISTORY OF *PHYTOPHTHORA INFESTANS* INFERRED FROM NUCLEAR AND MITOCHONDRIAL DNA SEQUENCES

Abstract

Nuclear and mitochondrial DNA variability was used to examine the population history of *P. infestans*. DNA sequence data from three nuclear regions (Intron Ras, Ras, and β-tubulin) and two mitochondrial regions (P3 and P4) were obtained from ninety isolates from various locations including Brazil, Bolivia, Ecuador, Peru, Costa Rica, Mexico (Toluca Valley), the United States (USA) and Ireland. Population summary statistics show that the Mexican population from the presumed center of origin of *P. infestans*, harbored less nucleotide and haplotype diversity than South American populations, and was genetically differentiated from other populations, particularly at the mitochondrial loci. Coalescent-based genealogies of mitochondrial loci (*rpl14, rpl5, tRNAs, cox1*) and nuclear locus (Intron Ras+Ras) were congruent and demostrated the existence of two lineages leading to the present day haplotypes of *P. infestans* associated with potatoes. A third lineage, associated with a group of isolates from *Solanum tetrapetalum* collected in the Andean Highlands of Ecuador was also found. In the mitochondrial genealogy, the two potato lineages corresponded to the mitochrondrial haplotypes Type I and Type II described elsewhere. The same mitochondrial haplotype was associated with different nuclear backgrounds. Haplotypes found in the Toluca Valley population were derived from only one of the two lineages in both mitochondrial and nuclear genealogies,

whereas haplotypes found in South American populations (Peru and Ecuador) were derived from both lineages. Haplotypes found in USA and Ireland populations were also derived from both lineages and these populations were not genetically differentiated from Peruvian population, suggesting a common ancestry among these populations. Evidence for recombination was found for Mexican and the USA populations. *Solanum tetrapetalum* isolates were highly polymorphic within the regions analyzed and may be a new species. The results support a South American origin of *P. infestans* and are discussed in relation of previous hypotheses regarding the geographic origin of this plant pathogen.

Introduction

Phytophthora infestans (Mont.) de Bary causes late blight of potato and tomato and is one of the world's most devastating plant diseases. The annual economic cost in developing countries alone is estimated at $3.25 billion, with approximately $750 million spent on fungicides (GILB 2004). *P. infestans* left its footprint in human history when, in the 19th century, it was responsible for the Irish Potato Famine, which caused the starvation and death of more than one million people, precipitating a massive migration from Ireland to North America. Soon after this catastrophe, the speculation about the origin of *P. infestans* began. Since then, three theories have been proposed to describe the center of origin and/or the source of inoculum of *P. infestans*: the Andean theory (Abad and Abad 1997), the Mexican theory (Niederhauser 1991, Goodwin *et al*. 1994) and the Three-Step or Hybrid theory (Tooley et al. 1989; Andrivon 1996).

The Andean theory considers the northern part of the South American Andes (currently Peru and Bolivia), the center of origin of the cultivated potato (Volkov *et al*. 2003), as both the center of origin of *P. infestans* and the source of inoculum for the 19th century late blight epidemics (Abad and Abad, 1997). Berkeley (1846) and de Bary (1861), pioneers in the discipline of plant pathology, also believed that the pathogen originated in the center of origin of the host. However, difficulties in proving the ancient presence of the disease in the Andes (Niederhauser 1991) along with observations that South American wild species did not possess major genes (R) for race-specific resistance to late blight (Reddick, 1939, Niederhauser 1991), and the low genetic variability found in a limited sample of isolates of *P. infestans* from potatoes growing in the central highlands of Peru (Tooley *et al*. 1989) weakened this theory.

The Mexican theory proposes the central highlands of Mexico, which includes the Toluca Valley, as the center of origin and the place from where the pathogen spread to other parts of the world (Niedarhauser 1991, Goodwin *et al*. 1994). This theory is the most commonly accepted because both mating types (A1 and A2) are present; there is a high genetic diversity for *P. infestans*, and wild potatoes show high levels of resistance, mainly qualitative resistance, in that region. Other species closely related to *P. infestans*: *P. mirabilis*, *P. phaseoli*, and *P. ipomoae* have been found there. According to the Mexican theory the first migration from Mexico to the United States (USA) occurred in infected tubers of wild potato species (Goodwin *et al*. 1994). No commercial potato production was known in Mexico in the 1840s (Bourke 1964). The pathogen was then speculated to migrate from the USA to Europe and from Europe to the rest of the world

(Goodwin *et al*. 1994.). Apparently, as a consequence of a strong bottleneck a single clonal lineage, called the US-1, became dominant in the populations of *P. infestans* outside central Mexico. The US-1 is the A1 mating type and a Ib mitochondrial DNA (mtDNA) haplotype (Goodwin et al. 1994, Gavino and Fry 2002).

The Three-Step or Hybrid theory proposes that Mexico represents the center of origin of the late blight pathogen, but the source of inoculum for the 19th century epidemics in Europe and the USA originated from the South American Andes (Tooley *et al*. 1989; Andrivon 1996). According to this theory, *P. infestans* migrated first from Mexico to South American Andes centuries ago. This theory was presented to reconcile the fact that cultivated potatoes were not grown in Mexico in the 1840s and the US-1 genotype was found panglobally distributed (Andrivon 1996). Peru and other Andean countries exported bat guano for fertilizer and potato seed on steamships to the USA and Europe in the 1840s.

Some researchers (Erselius *et al*. 1999, Adler *et al*. 2004) have noted that the above theories are based primarily on studies, with isolates of *P. infestans* from potato and to a lesser extent tomato. Both are important crops in temperate zones where most of the research has been conducted. A recent body of information, however, has challenged the present knowledge of *P. infestans* population history (Abad *et al*. 2002). First, until recently, most isolates of *P. infestans* outside Mexico belonged to the US-1 clonal lineage (Goodwin *et al*. 1994). This led to the hypothesis that the US-1, considered as a migrant from Mexico, represented the ancestral type of *P. infestans* and caused the original epidemics in Europe in the 19th century (Goodwin *et al*. 1994). However, sequence

analysis of mtDNA of *P. infestans* specimens taken from historical herbarium samples collected around the time of the famine showed that the Ib mtDNA was not present in the samples (Ristaino *et al*. 2001, May and Ristaino 2004). The Ia haplotype was found in a worldwide collection of the 19th century samples and in the oldest samples from the famine epidemics (May and Ristaino 2004). More recent samples from the Andean region in Ecuador and Bolivia were the Ib haplotype (May and Ristaino, 2004). In addition, surveys in the Toluca Valley and other parts of Mexico failed to find the US-1 genotype in extant Mexican populations of *P. infestans* (Gavino and Fry 2002; Flier *et al*. 2003). The Ia mtDNA haplotype was the only haplotype found in the Toluca Valley (Flier *et al*. 2003). This haplotype is also present in South American populations of *P. infestans* (Perez *et al*. 2001). Second, evidence has been presented (Ordoñez *et al*. 2000, Adler *et al*. 2004) that the Ecuadorian highlands harbor a wide genetic diversity within *P. infestans sensu lato* that infect wild *Solanum* and had not been detected in studies performed elsewhere on pathogen populations that attack potato and tomato. Both mating types (A1 and A2) were found in a common host, pear melon (*S. muricatum*), and the presence of a new mtDNA was also found there (Ordoñez *et al*. 2000, Adler *et al*. 2002, 2004). New genotypes of *P. infestans* have been also found in Peru in a nationwide survey, although only the A1 mating type was detected (Perez *et al*. 2001). Recent studies indicate that *P. infestans* populations in the main potato late blight areas in Bolivia are entirely A2 mating type and complex, with an average of seven virulence genes (Navia *et al*. 2001; Fernandez-Northcote and Plata, 1998). Third, evidence is growing that R genes, each one of them conferring resistance against only a few strains of the pathogen, also

occur in potatoes indigenous to South America (Perez *et al*. 2000; Trognitz *et al*. 1996). Taken together the new evidence indicates that the genetic variability of P. *infestans* in South America is higher than previously realized. The possibility still exists that the pathogen originated in the Andean region, perhaps in co-evolution with potato's wild relatives among the *Solanum* species (CIP, 2002).

Over the past decade, studies of DNA sequence variability have been applied to study the geographic origin and population history of diverse taxa including plants, animals, fungi, and humans. This has been most notable within the field of phylogeography, a discipline concerned with the demographic, behavioral, and historical factors influencing the geographic distributions of gene lineages (Avise *et al*. 1987; Hare and Avise, 1998, Avise 2000). Rapidly evolving and non- recombining cytoplasmic DNA (mitochondrial and chloroplastic) has been used as markers of choice, but recently nuclear genes have been studied (Hare 2001; Emerson *et al*. 2001). There is a general agreement that the combined use of both mitochondrial and nuclear DNA sequences may provide a better understanding of the phylogeographic structure of species as well as populations. Principles of genealogical concordance have been developed as conceptual guides to relate significant partitions in gene trees to significant historical partitions at the level of organismal phylogeny (Avise and Ball, 1990, Hare and Avise, 1998). This approach has not been yet applied to study the population history of *P*. *infestans*, although recently the first multi-gene based phylogeny of *Phytophthora* species, including nuclear and mitochondrial genes, was published (Kroon *et al*. 2004).

In this article we describe a population genetic and phylogeographic analysis using mitochondrial and nuclear genes to investigate the origin of *Phytophthora infestans*. Two questions are addressed: i) Does mitochondrial and nuclear DNA evidence justify the specific hypothesis that a common ancestor of *P. infestans* originated in South American or Mexican populations? and ii) What can be inferred about the geographic origin of the inoculum that caused the early epidemics of the 19th century?

Materials and Methods

Isolates

Ninety-one isolates of *Phytophthora infestans* were obtained from eight geographical locations (populations), including countries associated with the origin and migration theories. The isolates were collected by different scientists from 1984 to 2003 (Table 1.1). Six to eighteen isolates per region were used. Most of the isolates have been characterized for mating type; mitochondrial DNA (mtDNA) haplotype, allozyme genotypes, *glucose-6-phosphate isomerase* (*Gpi*) and *Peptidase* (*Pep*); and DNA fingerprint using the RG-57 probe by the collectors and represent the degrees of genetic and phenotypic diversity found in each location based on these phenotypic and molecular markers. In this study isolates were examined for mtDNA haplotype and allozyme genotype *Gpi* using standard protocols (Griffith and Shaw, 1998; Goodwin *et al*. 1995). The isolates were obatained predominately from potato (*Solanum tuberosum*). Isolates from Ecuador were obtained from different species of *Solanum* (Adler *et al*. 2004). The Mexican sample was collected in the Toluca Valley (Grundwald *et al*. 2001), the

presumed center of origin of *P. infestans* according to two theories (Niederhauser 1991; Goodwin *et al*. 1992, Andrivon 1996). Isolates were maintained on rye B agar medium (Caten and Jinks, 1968) or rye-V8 agar medium at 18 °C.

DNA isolation, amplification and sequencing

Isolates were grown in 90-mm plates containing pea broth (300 g frozen garden peas boiled for 10 min in 1 liter distilled water) for 2 to 4 weeks at 18 °C. Total DNA was extracted from dried mycelium by using a cetyltrimethylammonium bromide (CTAB, Sigma Chemical Co., St. Louis, MO, USA) protocol (Ristaino *et al*. 2001). DNA was diluted 1:10 or 1:100 (3 to 10 ng/ µl) for all PCR reactions.

Two regions of the mitochondrial genome (P3 and P4) and portions of two single-copy nuclear genes, RAS and B-tubulin, were amplified via polymerase chain reaction (PCR). P3 region includes the genes *rp114*, *rp15*, and *tRNAs*. P4 region includes a part of the *cox 1* gene. Two regions of the RAS gene were amplified, intron 1 (223 bp) located in the 5' untranslated region of the gene and a 600 bp portion covering part of exon 3, exon 4, exon 5, part of exon 6 and introns 3 and 4 (Figure 1.1). The PCR primers, their location on the GenBank accession on which the primer is based and the source are shown in Table 1.2. Two 50-µl reactions were carried out per primer set and isolate. Each reaction contained 1X PCR buffer (Invitrogen, Carlsbad, CA), 2.75mM $MgCl_2$, 1 unit *Taq* polymerase (Invitrogen), 200 µM each dNTP, 0.4 µM each forward and reverse primer, 160 µg/µl BSA (20 mg/ml, Roche, Mannheim, Germany) and ~ 5-10 ng genomic DNA. Cycling conditions were, for mitochondrial gene regions, 94˚ (1.5 min); then 40

cycles of 94˚ (40 sec), 55˚ (1 min), 72˚ (1.5 min); and a final extension of 72˚ (10 min). For nuclear genes, 96˚ (1 min); then 35 cycles of 96˚ (1 min), 55˚ (1 min), 72˚ (2 min); and a final extension of 72˚ (10 min). The two PCR products were pooled and purified with QIAquick PCR Purification Kits (QIAGEN, Valencia, CA). Purified fragments were sequenced directly on both strands by using the same primers as those used in the initial amplification. Sequencing reactions were prepared using the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed on an ABI PRISM® 377 automated sequencer (Applied Biosystem, Foster City, CA).

Sequence analysis

All sequences were aligned manually and edited using BioEdit (Hall 1999). Multiple sequence aligment was also performed in Clustal X (Thompson *et al*. 1997). All polymorphims were rechecked from the chromatograms. For Ras and B-tubulin genes, sites showing the presence of two coincident peaks in the forward and reverse sequence chromatograms were observed (Figure 1.2), indicating heterozygous positions, as a result of the coamplification and simultaneous sequencing of two complementary loci. Isolates of *P. infestans* are diploid (Brasier 1992) and show disomic inheritance (Tooley *et al*. 1985; Spielman, 1991), such that individuals can be either homozygous (single peak) or heterozygous (double peak) at some loci. Scoring of most heterozygotes was accomplished by reading double-peaked variable sites directly from the chromatograms. The identity of the two haplotypes within the heterozygote was then inferred by using Clark's "haplotype subtraction" algorithm (Clark 1990), whereby the heterozygote sequence is compared with pairs of homozygotes until the observed combination of

double-peaked sites can be accounted for. Most of the isolates examined were either homozygous or heterozygous at a single site, yielding unambiguous haplotype solutions. When the isolates were heterozygous at more than one site, preference was given to combinations involving known haplotypes of higher regional population frequency (Le Jossec *et al*. 2004).

Sequence analysis showed that some heterozygosities in the amplified PCR products of RAS and B-tubulin genes resulted in the creation of a *Nru I* and of an *Nco I* restriction sites in one of the alleles, respectively. No *Nru I* or *Nco I* restriction sites were found anywhere else in each of the whole gene fragment. To ascertain the accuracy of determination of heterozygosity, the correspondent gene fragment was digested for 4 h at 37°C with *Nru I* (Promega, Madison, WI) or *Nco I* (New England Biolabs) in a 30-µl reaction volume containing 10 µl PCR product, 3 µl of 10 x buffer (supplied with the enzyme), and 1.5 ul of 12 U/µl *Nru I* or 10 U/µl *Nco I*, respectively. Digestion products were loaded onto a 1.6% agarose gel containing ethidium bromide (1.0 µg/ml). TBE (45 mM Tris Borate, 1 mM EDTA, pH 8.3) was used as the running buffer, and a 100-bp ladder (Invitrogen) was used as molecular size markers. DNA bands were visualized by UV transilumination.

Phytophthora infestans and assumptions for phylogenetic inference

Several assumptions are key in estimating the evolutionary history from DNA sequence data: no selection, a lack of recombination, random mating within a single population, and random sampling (Emerson *et al*. 2001). These assumptions have been taken into account for developing different programs to estimating gene genealogies and demography histories. Violations of the assumptions can affect the results and their interpretations. Due to recombination, for example, different segments within a haplotype will have independent evolutionary histories. Ignoring recombination can undermine phylogeny reconstruction by introducing homoplasy (Emerson *et al*. 2001, Hare, 2001, Hey and Machado, 2003).

Phytophthora infestans is heterothallic and requires two compatibility types (=mating types), designated A1 and A2, to complete the sexual stage (Brasier 1992). *P. infestans* regularly completes the sexual stage (outcross) only in central Mexico (Toluca Valley), and there is evidence of sexual reproduction in some European countries and the USA in recent years. Anywhere else populations of *P. infestans* reproduce clonally and one or several clonal lineages are present. Therefore the assumption of random mating within a single population does not apply for populations of *P. infestans* outside central Mexico.

Data analysis

Data analysis was performed using the Java program SNAP Workbench (Price and Carbone, 2003) and following several steps outlined by Carbone *et al*. (2004). SNAP Workbench manages and coordinates a series of other programs used for phylogenetic as well as migration analyses. An important functionality of the SNAP Workbench is the implementation of protocols consisting of a defined set of programs, assumptions, and parameter settings for following different paths during the analysis (Carbone *et al*. 2004). After aligning the DNA sequences of each locus, sequences were collapsed into unique haplotypes removing insertions and deletions (indels) and excluding infinite-sites violations using SNAP Map (Aylor and Carbone 2003). Base substitutions were categorized as phylogenetically informative or uninformative, transitions vs transversions, and replacement vs. synonymous amino acid changes in the coding region. A phylogenetic analysis with unweighted parsimony was performed using PHYLIP (Felsenstein 1993); if the heuristic searches yielded two or more equally parsimonious trees, a strict consensus tree was inferred.

To examine the overall support or conflict among the variable sites in the DNA sequence alignment, compatibility matrices were generated for the entire alignment of each locus as well as for all subsets of haplotypes that share a common ancestor in the consensus tree using SNAP Clade (Markwordt *et al*. 2003). Compatibility matrices were graphically illustrated using SNAP matrix (Markwordt *et al*. 2003) and examined for the existence of two or more identical pattern of compatibility/incompatibility. Clusters of two or more identical sites in a matrix define a recombination block. If recombination

blocks were evident two different paths were followed. In one path, recombination block boundaries and the minimum number of recombination events were identified using RecMin (Myers and Griffiths 2003). The putative recombination boundaries identified within each recombining clade from RecMin were used as a guide to identify the common or shared recombination block boundaries among all clades. The nonrecombining blocks were collapsed again into unique haplotypes using SNAP Map. When detectable recombinants were few, they were removed from the analysis. In the second path, RecPars (Hein, 1990) was used to infer the number of recombinant segments (i.e. recombination blocks) in each locus data set. Two non-recombinant blocks were defined, one formed by the mitochondrial regions P3 and P4 and other by the two segments of the Ras gene (Intron Ras + Ras) and were used for reconstructing gene genealogies. The nuclear locus did not include the variable site found in B-tubulin gene because of its incompatibility with other sites.

Neutrality tests and population subdivision

After no recombining blocks were defined, the DNA sequences were analyzed in DnaSP 4 (Rozas and Rozas, 1999). For each locus in each population the population mutation parameter per nucleotide site θ using Watterson's (1975) estimate θ_w, based on the number of segregating sites, *s*, and the average pairwise nucleotide diversity, π (Tajima, 1983) were estimated, and different tests of neutrality (Tajima, 1989; Fu & Li, 1993; Fu 1997) were performed in order to determine whether the data were consistent with the expectations of the neutral model of molecular evolution. These neutrality tests assume a

constant population size, no recombination and no migration, that each sample is taken from a single randomly mating population and random sampling. The latter assumption is violated in this study, particularly for the USA sample, since different previously characterize US genotypes were included in equal number. The consequences of violating this assumption are considered in the Results and the Discussion. The tests were performed on the two non-recombining blocks. Departures from neutrality may be caused by demographic forces such as population growth, contraction, and subdivision (Fu 1996, 1997; Simonsen *et al*. 1995) or by the effect of diversifying or directional selection on linked sites (genetic hitchhiking, Fu 1997). Carbone et al. (2004) distinguished among population growth and background selection by comparing Tajima's D, Fu and Li's D* and F* and Fu's *Fs* tests. If only Fu and Li's D* and F* are significant background selection is assumed (Fu 1997). If only Tajima's D and Fu's *Fs* are significant there is evidence for population growth or genetic hitchhiking. Small sample size of individual populations limits the power of these tests (Simonsen *et al*. 1995). To increased the sample size the eight initial populations were also analyzed as a POOLED (all populations combined); as two populations South America (SA: Brazil, Bolivia, Peru, and Ecuador) and non South America (NSA: Costa Rica, Mexico, the USA, and Ireland); and as a four populations: Brazilian and Bolivian isolates (BRABO), Peruvian and Ecuadorian isolates (PEECU); Mexican and Costa Rican isolates (MECO); and USA and Irish isolates (USIR). The criteria for amalgamating were geographical location (sampling areas), sharing of genotypes (Table 1.1), and relation with migration theories (supposed ancestral or no ancestral condition of the population).

Genetic differentiation among populations (individual and as a group) was analyzed using SNAP Map and Permtest (Hudson *et al*. 1992a,b) as implemented in the SNAP Workbench (Carbone *et al*. 2004). This analysis was also performed using DnaSP4 (Rozas and Rozas, 1999). The Permtest was done using a nonparametric permutation method based on Monte Carlo simulations. Hudson's test statistics (K_{ST}, K_S, and K_T) were calculated for each recombining block and again for the entire data set. Testing the hypothesis of no genetic differentiation is an important first step and determines whether a panmictic or a subdivided population model is assumed for examining population divergence using Genetree and recombination parameter estimation using Recom58 (Griffiths and Marjoram 1996).

Coalescent analysis

If evidence for geographic subdivison was found, the observed patterns of genetic divergence were further explored to determine whether there was evidence for constant migration among populations or shared ancestral polymorphisms followed by population divergence but no migration. Two migration models were used for this analysis. First, MDIV was used to test for equilibrium migration vs. shared ancestral polymorphisms between two subdivided populations (Nielsen and Wakeley 2001). The approach of Nielsen and Wakeley (2001) uses either an infinite sites or a finite sites model without recombination and implements both likelihood and bayesian methods using Markov chain Monte Carlo coalescent simulations for jointly estimating the population mean mutation rate (θ), divergence time (T*)*, migration rate (M), and time since the most recent

common ancestor (TMRCA) between two subdivided populations. This approach assumes that both populations descended from one panmictic population that may (or may not) have been follow by migration. Second, if MDIV showed evidence of equilibrium migration, MIGRATE was used to estimate migration rates assuming equilibrium migration rates (symmetrical or asymmetrical) in the ancestral history of the populations (Beerli and Felsenstein 2001). Both MDIV and MIGRATE assume neutrality and no recombination and therefore these analyses were restricted to the neutral nonrecombining blocks in the data set.

The ancestral history of the sample for each nonrecombining block, mitochondrial (P3+P4) and nuclear (IntronRas and IntronRas+RAS), was reconstructed using Genetree version 9.0 (Griffiths and Tavaré 1994) as implemented in SNAP Workbench. The genealogy with the highest root probability, the ages of mutations, and the TMRCA of the sample were estimated from coalescent simulations.

Results

DNA sequence variability

A total of 3266 nucleotides were sequenced, 1256 nucleotides corresponding to portions of two single-copy nuclear genes, RAS (Intron Ras and Ras) and B-tubulin, and 2010 nucleotides corresponding to the two regions of the mitochondrial genome, P3 (*rpl14, rpl5, tRNAs*) and P4 (part of *cox 1*), described by Griffith and Shaw (1998). Though sequence data were collected from 91 isolates, complete sequence data for all five loci were not available for all isolates. Complete sequence data were available for 76 and 66 isolates for the three nuclear loci and for the two-mitochondrial regions, respectively. The same isolates were present in the two data sets.

Sequence diversity, estimated as the proportion of polymorphic nucleotides, was 2.23 % for Intron Ras (five variable sites), 1.29% for RAS (seven variable sites), 0.20 % for B-tubulin (one variable site), 0.76% for P3 (nine variable sites), and 0.48% for P4 (four variable sites) (Table 1.3). Overall sequence diversity estimates were 1.03% and 0.65% for single-copy nuclear genes and mitochondrial regions respectively. Four nucleotide substitutions leading to amino acid change were found in P3 region (*rpl14* gene). No amino acid changes were observed in the other genes examined. Two and one synonymous substitutions were detected for Ras and B-tubulin genes respectively (Table 1.3). For each polymorphic site only two nucleotide changes were observed. Isolates from *S. tetrapetalum* from Ecuador were highly variable across all loci and when included in the analysis; sequence diversity estimates were higher 6.69% for Intron Ras

and 1.44% for both mitochondrial regions, respectively (Table 1.3). Seven nucleotide substitutions leading to amino acid changes were also found in the P3 region (Table 1.3).

P. infestans is diploid and evidence has been obtained for heterozygosity from analysis of dimeric enzyme patterns (Tooley et al. 1985, Spielman 1991). Therefore, amplification of genomic DNA is expected to result in PCR products that arise from both chromosomes and that upon direct sequencing yield profiles that show two coincident peaks in the sequence chromatogram resulting from the coamplification of both alleles. In the two nuclear loci investigated in this study, sequence results showed either one of the two bases or the heterozygous combination of the same two bases. However, sequencing errors rather than true heterozygosities could have also generated the observed patterns. Sequence analysis showed that some heterozygosities in the PCR products of RAS and B-tubulin genes create a unique *Nru I* or *Nco I* restriction site in one of the alleles, respectively. To investigate whether the double peaks (Figure 1.2) represented true heterozygosities or sequencing errors, since no *Nru I* or *Nco I* restriction sites were found anywhere else in the corresponding gene fragment, the PCR products were digested with *Nru I* or *Nco I*, respectively. As shown in Figures 2b and 2c, the digested products confirmed that the allele with the restriction site gave two DNA fragments, while the other one remained undigested. The size of the two fragments added to the size of the undigested fragment. Therefore, sequencing errors were unlikely to account for the polymorphims observed in the nuclear genes of this diploid organism (Brasier 1992). Recently, Dr. D.E.L. Cooke, (Scottish, Crop Research Institute, Invergowrie, Dundee, DD2 5DA, United Kingdom) sequenced a portion of the Ras gene from two Scottish

isolates and found the same heterozygous sites reported in this study, confirming that both chromosomal alleles were amplified. Although the results do not preclude the possibility that some isolates might be aneuploid or polyploid (Tooley and Therrien 1991), interpretation of the sequence data from this study is most consistent with isolates being diploid. These results agree with other studies that demonstrated typical diploid heterozygous patterns for dimeric enzyme markers in *P. infestans* (Tooley et al. 1985, Spielman, 1991).

Nucleotide diversity

Summary statistics describing the sequence diversity in the pooled and individual populations for both nuclear and mitochondrial loci are presented in Table 1.4. Nucleotide diversity (π) for the pooled (total) sample was 0.00220 for mitochondrial loci and 0.00329 for nuclear loci. The average per-nucleotide expected heterozygosity, θ_w, for the pooled sample was 0.00139 and 0.00280 for mitochondrial and nuclear loci, respectively. When *S. tetrapetalum* isolates were included (Pooled Tetra), π and θ_w increased to 0.00282 and 0.00351 in the mitochondrial loci, for which these isolates were sequenced. Among population samples and the mitochondrial locus, the Ecuadorian Tetra population, which includes isolates from *S. tetrapetalum*, had the highest nucleotide diversity, followed by the USA, Irish, Peruvian, Ecuadorian, and Mexican populations. No nucleotide diversity was found in the Brazilian and Bolivian populations, since only one haplotype was found in this population. The Mexican population had low nucleotide diversity and accounted for only 20% of the nucleotide diversity of the Pooled

population. The South American (SA) and Non-South American (NSA) populations showed similar levels of nucleotide diversity in the mitochondrial loci. Brazilian and Bolivian populations had the highest nucleotide diversity for the nuclear loci, followed by the USA, Irish, Peruvian, Mexican and Ecuadorian populations. The Costa Rican population was monomorphic for the nuclear loci. The Mexican population again showed a relatively low level of nucleotide diversity compared to other populations, however nuclear diversity was 42% of the Pooled sample. The South American population showed higher nucleotide diversity compared to the Non-South American population. The low nucleotide diversity found in the Mexican population, in both mitochondrial and nuclear loci, is quite surprising since central Mexican populations of *P. infestans* are considered the most diverse in the world based on RFLP, AFLP and isozyme analyses (Goodwin 1997, Flier et al. 2003). The high nucleotide diversity found in the USA population might reflect the non-random selection of the USA sample. The equal frequency of highly differentiated haplotypes causes an artificial increase in polymorphic sites (Ballard, 2000) rendering high values of π and θ. Isolates from *S. tetrapetalum* formed a highly divergent haplotype, which explains the highest nucleotide diversity obtained for the Ecuadorian Tetra and Pooled Tetra populations.

Tests of neutrality and population subdivision

In order to determine whether the data departured from an equilibrium neutral model, four neutrality tests were performed. No significant values were observed for the Pooled (total) sample for both mitochondrial and nuclear loci (Table 1.4), so the equilibrium model of neutral evolution could not be rejected. The values of the tests tend to be positive. Positive values might be the result of the observed predominance of two classes of haplotypes in the mitochondrial loci and only four classes of haplotypes in the nuclear loci. In the mitochondrial loci the two-haplotype classes were evenly represented in the sample whereas in the nuclear loci one haplotype class dominated the sample, whereas the other three-haplotype classes were at similar frequencies (Table 1.5).

The pattern observed for different populations was informative and showed some significant values, especially for the mitochondrial loci, suggesting departure from neutrality in some populations. Positive significant values were observed for the Peruvian sample at the mitochondrial loci, whereas the values were significantly negative for the Ecuadorian sample. In the Peruvian sample, the two-haplotype classes were present at similar frequencies, but in the Ecuadorian sample one haplotype class dominated. When all South American isolates were pooled, the South American and South American Tetra populations, Fu's F statistic was positive and significant, indicating that selection might be acting on these loci in these populations. Significant positive values were observed for all tests in the USA sample. These results are most likely related to the sampling strategy used for this population that included an equal subsample of differentiated US genotypes. Negative values for Tajima's and Fu and Li's test statistics were found in the Mexican

sample indicating an excess of low-frequency polymorphisms, which is expected under a model of population growth from its ancestral size or a recent selective sweep. Two of the three variable sites found in the Mexican sample had a minor allele frequency of 10% compared to the most common allele, implying that the rare alleles are derived. However the most common allele had a frequency in the Mexican sample of 50%. This pattern in the Mexican population is consistent with drift and a strong founder effect. Positive significant values were observed in Brazilian and Bolivian populations at the nuclear loci. Two nuclear haplotype classes were evenly represented in these samples. It should be noted that small sample sizes and the presence of population subdivision limited the power of the neutrality tests (Simonsen et al. 1995; Fu 1996).

Alternative tests for population subdivision (Hudson et al. 1992) were used to avoid inferring false positives from neutrality tests (Carbone et al. 2004). Tables 1.6 and 1.7 summarize the values of Hudson's test statistics for each population (eight geographical regions) for nuclear and mitochondrial loci, respectively. Pairwise comparisons between South American (SA) and non-South American (NSA) samples showed genetic differentiation for both nuclear (P =0.0265, Kst= 0.0175, Ks=2.5082) and mitochondrial (P=0.0000, Kst=0.2143, Ks=3.8625) loci. The differentiation level was similar within the non South American group (P=0.0001, Kst=0.1162, Ks=1.6608) and within the South American group (P=0.0001, Kst=0.2347, Ks=2.4530 for nuclear loci, whereas for the mitochondrial loci, the differentiation level was higher within the non South American group (P=0.0013, Kst=0.31364, Ks=2.8229) than within the South American group with (P=0.0237, Kst=0.1292, Ks=4.7311) or without (P=0.0190,

Kst=0.2303, Ks=2.7602) *Solanum tetrapetalum* isolates. Among South American populations, the Brazilian and Bolivian population were not different from each other but were different from the Peruvian and Ecuadorian populations in the nuclear loci. Among non South American populations, the Mexican population was genetically different from the USA and Irish populations for both nuclear and mitochondrial loci. The USA and Irish populations were not genetically differentiated. Mexican population was genetically differentiated from all South American populations in the mitochondrial loci, whereas was not differentiated from Peruvian and Ecuadorian populations in the nuclear loci. The USA and Irish populations were more closely related to South American populations, particularly to the Peruvian population.

When the analysis was performed grouping the isolates in four populations, Hudson's tests for population subdivision showed the same trend. Genetic differentiation was detected between BRABO and USIR (P = 0.0438, Kst = 0.0800, Ks= 3.6543, Kt = 3.9720), BRABO and PEECU (P = 0.0000, Kst = 0.2649, Ks= 2.3557, Kt = 3.2051); and BRABO and MECO (P = 0.0000, Kst = 0.3403, Ks= 1.8029, Kt = 2.7331) for the nuclear locus. For the mitochondrial locus, significant population differentiation was found between BRABO and MECO (P = 0.0093, Kst = 1.0000, Ks= 0.0000, Kt = 1.8461), PEECU and MECO (P = 0.0000, Kst = 0.4567, Ks= 2.1698, Kt = 3.9938), MECO and USIR (P = 0.0040, Kst = 0.2075, Ks= 2.4776, Kt = 3.1264).

Coalescent analysis

Coalescent analysis using Genetree was carried out to infer the mutational history, time scale of the origin and evolution of polymorphic variation, of the mitochondrial (P3/P4, Figure 1.3) and nuclear (Intron Ras and Intron Ras + RAS, Figures 1.4 and 1.5) loci, separately. Recombining haplotypes were excluded from the analysis (Table 1.5). The analysis was performed assuming an infinite-sites model, constant population size, random mating within a single population, and population subdivision. The topology of the coalescent-based genealogy inferred for the mitochondrial loci (Figure 1.3) was essentially the same as those of the coalescent-based genealogy inferred for the nuclear loci (Figure 1.4 and Figure 1.5). The distribution of haplotypes among populations is indicated below the trees. The isolates associated with each haplotype are shown in Table 1.8. Three independent ancient lineages were present in the mitochondrial (Figure 1.3) and nuclear coalescent-based genealogies (Figure 1.4). One of the lineages included the isolates from *Solanum tetrapetalum* from Ecuador. The other two ancient lineages gave rise to the extant haplotypes found in isolates from potato, some wild *Solanum* species and to a lesser extent tomato (Table 1.1). The lineage associated with *S. tetrapetalum* isolates was highly differentiated from the other two lineages and may be a separate species. The ancestral lineages that gave rise to the present day haplotypes of *P. infestans* from potato were derivated from a common ancestor at T= 0.4 (mitochondrial) and T= 0.6 (nuclear). Mutations on these ancestral lineages gave rise to the extant haplotypes of *P. infestans*.

In the mitochondrial genealogy (Figure 1.3), the two ancestral lineages associated with potato correspond to the mtDNA haplotypes Type I and II, respectively described for *P. infestans* (Carter et al. 1990,1991). Mutations 23 and 16, found in P4 and P3 regions respectively, create the EcoRI restriction sites used in the PCR-RFLP method developed by Griffith and Shaw (1998) to differentiate Type I and II. Since these mutations occured in the ancient lineage corresponding to Type II, Type I can be inferred as the ancestral mitochondrial haplotype since no mutation occurred after the splitting event from the common ancestor (T= 0.4) earlier than mutations 23 and 16. The fact that the lineage associated with *S. tetrapetalum* is also Type I, and can be considered as a closely related outgroup, also supports the Type I as the ancestral lineage. No mutations were found in the mitochondrial regions that separate mitochondrial haplotype Ia from mitochondrial haplotype Ib to determine which is the ancestral to each other. However, several recent mutations have given rise to other rare haplotypes among the Type I.

Within the ancestral Type II lineage, haplotype IIa was inferred as ancestral to haplotype IIb, due to mutation 4, which arose more recently. The two most common haplotyes of each lineage (H1 and H2) contain isolates of both A1 and A2 mating types (Figure 3). The Type I haplotype was globally distributed, with the exception of Brazilian and Bolivian populations. The Type II haplotype was also found widely distributed, although it was not found in the Toluca Valley and Costa Rican populations (Table 1.8).

Haplotype H7 (Type IIb) contains members of the US6, US11, US12, and US13 genotypes, and haplotype H8 members of the US-14 and US-17 genotypes. These

genotypes previously had been considered either tomato specific isolates or sexual recombinants (Goodwin *et al*. 1998).

The two ancestral potato lineages observed for the nuclear loci (Figure 1.4 and 1.5) did not exactly match the two-mitochondrial lineages, indicating the nuclear haplotypes contain isolates with distinct mitochondrial haplotypes. In other words, the same mtDNA haplotypes were associated with different nuclear backgrounds, as has been observed by others using different molecular markers (Gavino and Fry, 2002). The two most common nuclear haplotypes (H1 and H2) of each ancient lineage contained isolates of both mtDNA lineages (Types I and II) and of both mating types (A1 and A2). Two other common nuclear haplotypes (H4 and H6) also contained isolates from the two mtDNA lineages. H1 haplotype contained mainly isolates of the Type II mitochondrial lineage and A2 mating type. The Peruvian isolates PCZ 007 and PCZ 0050 and the Irish isolate 16/94 were the only isolates of the H1 haplotype that were of the Type I lineage and A1 mating type.

In general, the Type I mtDNA lineage showed more nuclear diversity than the Type II mtDNA lineage. All eight nuclear haplotypes contained isolates of Type I mtDNA lineage, but only four of the nuclear haplotypes contained isolates of the Type II mtDNA lineage (Figure 1.5).

Several unique haplotypes were found in the Mexican population (Toluca Valley), but they were descendents from only one of the ancient lineages in both mitochondrial (Figure 1.3) and nuclear (Figure 1.4 and 1.5) genealogies, whereas South American haplotypes descended from both ancestral lineages. In the mitochondrial genealogy one

isolate from Peru (PHU006) represented a unique haplotype (H9) and an Ecuadorian isolate (3252) was inferred as recombinant haplotype (H10) (Table 1.6). The USA and Irish haplotypes were also derived from both ancestral lineages and unique haplotypes were also observed for these populations.

Discussion

This is the first time that DNA sequence variability analyses from both mitochondrial (mtDNA) and nuclear loci have been used to study the evolutionary history of *Phytophthora infestans*. Gene genealogies inferred from the mtDNA data (P3+P4 regions) and the autosomal data (Ras gene) were congruent. Both mitochondrial and nuclear coalescent-based genealogies showed the existence of two ancient lineages leading to the present day haplotypes of *P. infestans* associated with potato (*Solanum tuberosum*), some wild *Solanum* species; and to a lesser extent tomato (three isolates included) (Table 1.1). The ancient lineages from the mitochondrial genealogy corresponded to the known Type I and Type II mitochondrial haplotypes (Carter *et al*. 1990, 1991) and contained individuals of both A1 and A2 mating types. The two ancient lineages in the nuclear genealogy included individuals of the two mitochondrial lineages and both mating types. A third lineage, associated only with a group of isolates from *Solanum tetrapetalum* collected in the Andean Highlands of Ecuador was also found. These results are unlikely to be due to a sampling bias. Gavino and Fry (2002) assessed the diversity in mtDNA in a worldwide collection of 596 isolates of *P. infestans* using the PCR-RFLP method developed by Griffith and Shaw (1998). Only four mtDNA haplotypes, namely Ia, Ib (Type I) and IIa, IIb (Type II) were identified among the

isolates. They also found that the two-mtDNA haplotypes were associated with different nuclear backgrounds. Purvis *et al*. (2001) fingerprinted ninety-eight isolates from England and Wales using both the RG-57 probe and AFLP. Both techniques distinguished isolates of mtDNA haplotype Ia from those of haplotype IIa. No other mtDNA haplotypes were found in their samples.

Geographic origin of the ancestral population

We were interested in two questions. First, does mitochondrial and nuclear DNA evidence justify the specific hypothesis that a common ancestor of *P. infestans* originated in South American or Mexican populations? In other words, what is the geographic origin of the ancestral population? Population genetic theory predicts that ancestral populations will have increased polymorphisms compared to more recent, non-ancestral populations. Greater genetic diversity in central Mexico (Toluca Valley) than other areas of the world, based on allozymes, RG-57 and AFLP markers, has frequently been taken as evidence of this population's antiquity (Spielman 1991, Goodwin 1997; Flier *et al*. 2003). The fact that the genetic diversity of *P. infestans* outside central Mexico can be interpreted as being a subset of the central Mexican one has been seen as even more compelling and overwhelming evidence for the Mexican origin of the pathogen (Goodwin 1997). However, greater diversity in a place may be due to a particular history of founder effects, extinctions, and expansions of local populations (Takahata, 1994; Jaruzelska *et al*. 1999). Surprinsingly, data presented here show that the nucleotide diversity in the central Mexican (Toluca Valley) population was low for both mitochondrial and nuclear

loci compared to South American populations (Table 1.4). The nucleotide diversity in central Mexico population was five-fold less than the Peruvian sample for the mitochondrial loci and nearly half for the nuclear loci. Globally, nucleotide diversity in central Mexican populations represented 19% and 42% of the mitochondrial and nuclear diversity of the pooled (total) sample. In contrast, the genetic variability found in South American populations accounted for almost 90% of the variability found in non South American populations for the mitochondrial loci and was almost twice of that of the Non South American populations for the nuclear loci. This indicates that central Mexican populations of *P. infestans* harbor less genetic diversity than populations in South America, suggesting an ancestral condition for South American populations.

The haplotype structure also indicates less ancestral diversity in the Toluca Valley Mexican population compared with South American populations. Haplotypes found in the Toluca Valley of central Mexico, even rare haplotypes, were always derived from only one of the ancient lineages for the mitochondrial (Figure 1.3) and nuclear (Figure 1.4 and 1.5) loci. The Type II mitochondrial haplotype class was not found in the Toluca Valley population. Both mitochondrial haplotype classes (Type I and Type II) were found in South American populations. If we assume that the accepted central Mexican origin of *P. infestans* is correct, the following two scenarios are possible. One is that mutations leading to Type II haplotypes occurred before the separation of Type II haplotypes and their closest Type I haplotypes, but the former became very low in frequency or extinct in the Toluca Valley populations. In other words, both haplotype classes originated in the Toluca Valley, but haplotype class Type II went extinct there. The second possibility is

that mutations occurred in a lineage that was outside of Toluca Valley. Given the fact that Type II haplotypes, particularly Type IIa, have a high frequency among populations of *P. infestans* outside the Toluca Valley (Gavino and Fry, 2002), the first scenario is less likely. In addition, mutations leading to the present day Type II haplotypes occurred after and not before the splitting of the two ancient lineages from their common ancestor (Figure 1.3), supporting the second scenario. The Toluca Valley population is unlikely to be the center of origin of the mitochondrial Type II haplotype class. Gavino and Fry (2002) and Flier *et al*. (2003) also reported a monomorphic condition for the mitochondrial haplotypes for the Toluca Valley population. Only mtDNA haplotype Ia was found in this population.

The pattern of genetic variability in the Toluca Valley Mexican population for the mitochondrial locus was also consistent with a strong founder effect, not seen in other populations (Table 1.4). In addition, Hudson's tests for population subdivision (Table 1.6 and 1.7) showed that Mexican population is genetically differentiated from Peruvian and Ecuadorian populations in the mitochondrial locus, but not in the nuclear locus. Levels of differentiation estimated from mitochondrial and autosomal nuclear loci are expected to differ at equilibrium because of their effective population size differences. Mitochondrial locus with a lower effective size often has its diversity more strongly affected by historical events such as founder effects or bottlenecks than do autosomal nuclear genes (Arnaud-Haond *et al*. 2003).

A third lineage associated with *S. tetrapetalum* from Ecuador was found. This lineage fits the morphological description for *P. infestans* (Adler *et al*. 2004) but was

genetically different to the other two ancient lineages associated with potatoes. This shared morphology also supports the Andean Highlands of South America as ancient center of *P. infestans* diversity (Brasier and Hansen 1992).

The existence of three related ancestral lineages also suggests that the ancient *P. infestans* population was subdivided and the extant haplotypes of this pathogen were derived from different ancient lineages. Possibly, limited gene flow occurred between the ancient lineages; and the relatively high nuclear diversity observed was present in the ancient population from which these lineages originated. A similar hypothesis has been proposed to explain patterns of nucleotide diversity and haplotype distributions in modern humans (Zietkiewicz *et al*. 1998; Yu *et al*. 2002; Toomajian and Kreitman 2002).

If ancient lineages of *P. infestans* diverged a long time ago, it is expected that they would show some other characteristics that tend to distinguish them, for example host-specificity, patterns of geographical distribution or population subdivision. Recently, Adler *et al*. (2004) presented evidence of a high level of diversity and the existence of host-defined sub-populations in the *P. infestans* populations from the Ecuadorian highlands. Three clonal lineages (US-1, EC-1, EC-3) and one heterogeneous group (EC-2) were found in association with different host species in the genus *Solanum* and others (Adler et al. 2004). In general a one-host/one-clonal lineage relationship was observed. There appeared to be little or no gene flow among the clonal lineages. Host specificity is therefore the driving force for maintaining genetic isolation between sub-populations (clonal lineages) of *P. infestans* in Ecuador. This scenario is consistent with an ancient population in a divergent process and agrees with the data presented here. This situation

suggests that sympatric speciation; or the origin of two or more new species from a single local ancestral population in the same geographical region (Bolnick 2002; Losos and Glor 2003) may be possible in *P. infestans* (Flier *et al*. 2003). The initiation of sympatric speciation occurs presumably when the ancestral population is exposed to two or more ecological niches, commonly host-defined niches (Gudelj *et al*. 2004), however the occurrence of sympatric speciation remains contentious (Bolnick, 2002; Losos and Glor, 2003). Nonetheless the host-adapted groups described in Ecuador indicate a long history of association between host and the pathogen in South America and could represent several stages of differentiation for the *P. infestans*-*Solanum* spp pathosystem. With more time and the accumulation of more mutations, it is possible that populations of *P. infestans* will evolve into clearly distinct genetic lineages with more pronounced host specificities, as seems to be the case for the lineage associated with *S. tetrapetalum*. In the central Toluca Valley of Mexico, *P. infestans* also attacks several wild tuber-bearing species of *Solanum*, however, evidence from recent studies in Toluca Valley indicates that these wild *Solanum* spp. are all attacked by *P. infestans* and that limited gene flow exists between the subpopulations of the pathogen on wild *Solanum* spp. and cultivated potatoes (Flier *et al*. 2003).

The data presented here support the South American Andes as the center of origin of *P. infestans* instead of the Toluca Valley in central Mexico. What could be alternative explanations to the support of out-of-South America hypothesis? Dean and Ballard (2004) outlined several alternate explanations for DNA data indicating a geographic

origin of a species over other. These explanations include: i) locus specificity; ii) sampling bias; iii) population admixture, and iv) selection.

Locus specificity seems unlikely to explain the results presented here since both nuclear (biparentally inherited) and mitochondrial (uniparentally inherited) loci were included and both genealogies were concordant. Genealogical concordance between independent (unlinked and nonepistatic) nuclear and mitochondrial genes offers strong support to the out-of-South America hypothesis.

Sampling bias also seems unlikely to explain the results since each isolate from the Toluca Valley is an unique genotype (Grundwald *et al*. 2001, Goodwin *et al*. 1992, Flier *et al*. 2003) and the isolates used in this study included different allozymes pattern and mating type (Table 1.1). In addition, a similar sampling scheme was applied to each population. If *P. infestans* isolates immigrated from differentiated populations into South America, rather than the reverse scenario, a potential bias due to population admixure could occur.

Admixure may lead to elevated polymorphisms that might be discerned as ancestral. Under such a scenario, the sequences from South America may actually have originated elsewhere else. It is accepted that migration has played an important role in the spread of *P. infestans* (Fry *et al*. 1992, 1993; Goodwin 1997; Goodwin *et al*. 1998). Although, evidence suggests that Mexico is the source of recent migrations of *P. infestans* into other areas of the world (Niederhauser, 1991, Goodwin 1997) early migrations are more likely to be from South America (May and Ristaino, 2004) and

modern *P. infestans* populations in other countries resembled those in South America (Tooley *et al*. 1989, Ghimire *et al*. 2003).

Selection may have removed polymorphisms from the ancestral population and/or promoted variation in derived populations. Selection has been proposed to explain the presence of only one mtDNA haplotype (Type Ia) in *P. infestans* populations in the Toluca Valley, contrasting with their higher nuclear diversity (Gavino and Fry, 2002). According to Gavino and Fry (2002) one possible mechanism might be that domestic potatoes provide a different environment and therefore selected different mtDNA haplotypes than wild *Solanum* spp. However, so far the same mtDNA haplotype (Type Ia) has been found associated with wild *Solanum* and cultivated potatoes in the Toluca Valley (Flier *et al*. 2003). No indication of selection in either mitochondrial or nuclear loci for Mexican population or the pooled sample was found (Table 1.4). The neutrality tests were consistent with a model of neutral evolution. However, it is important to note that non-significant results do not completely rule out the action of natural selection (Simonsen *et al*. 1995; Wayne and Simonsen 1998). Significant values for neutrality tests were observed for South American populations and could indicate that selection might be operating in the South American populations, especially for the mitochondrial locus. If this is the case, the selection mechanism involving host–pathogen interaction between *P. infestans* haplotytpes and hosts in the genus *Solanum* fits the scenario of the host-adapted clonal lineages found in the Andean Highlands of Ecuador (Adler *et al*. 2004).

Source of origin of the isolates of *P. infestans* that caused early epidemics

The second question addressed here was the source of origin of the isolates of *P. infestans* that caused the potato famine. The geographic origin of isolates in Ireland and the USA can be estimated using as a criterion the number of shared haplotypes between the areas considered non-ancestral and the putative ancestral areas (in our case central Mexico or South America) (Baudry *et al*. 2004). Populations of *P. infestans* in the South American Andes are derived from the two ancestral lineages found in both mitochondrial (Figure 1.3) and nuclear genealogies (Figure 1.4 and 1.5). Populations in USA and Ireland contained members derived from both ancestral lineages and were more similar to South American populations than to central Mexico population, indicating that the USA and Ireland populations shared a common ancestor with the South American populations as has been previously suggested by others based on race composition, allozyme markers and nuclear DNA content (Tooley *et al*. 1989). Populations from the USA and Ireland were not genetically different from the Peruvian population for both mitochondrial and nuclear loci. The most common haplotypes corresponding to each ancient lineage in each genealogy were always found in South American populations within the present day haplotypes. In addition, the Ib mtDNA haplotype, once found panglobally distributed (Goodwin *et al*. 1994) has a South American origin (May and Ristaino 2004; Fry and Gavino, 2002).

A recent analysis of mtDNA of *P. infestans* in herbarium material showed that Ia mtDNA haplotype was the most common haplotype in the 1800's, therefore it was present in Europe (May and Ristaino, 2004) long before the 1970's, the year associated

with the first migration of this mtDNA haplotype along with the IIa mtDNA haplotype from Mexico to Europe. Early migrations of *P. infestans* from South America are more likely, since potato, tomato and other Solanaceous crops originated there and were used by ancient cultures. During 19th century, for example, South America exported guano as fertilizer and potato tubers in steamships to USA and Europe (Berkeley, 1846). Inhabitants of North and Central America did not cultivate potatoes until very recently. In central Mexico the production of domestic potatoes started in the latter half of the 20th century (Salaman, 1949, Gavino and Fry, 2002). Several groups, however, collected and ate wild tuber-bearing *Solanum* species in these regions since remote times (Andrivon, 1996), but tuber blight was virtually nonexistent on wild potato species growing in the Toluca Valley (Rivera Peña 1990). Even today, some genotypes found in other parts of the world can be traced back to South America. Ghimire *et al*. (2003), for example, found that the genotypes of *P. infestans* in Nepal, including those of both mating types and mtDNA Ia haplotype, and mtDNA Ib haplotype present identical RG-57 patterns found in Andean countries of South America. Nepal has imported planting material from Peru for many years. It is also interesting that the Irish isolate 3/99 has the same RG-57 pattern as isolates belonging to the clonal lineage PE-3 (mtDNA Ia) from Peru. South America is the most likely source of many isolates found around the world. The USA and Irish population were not different from the Peruvian populations, also strong suggests that Peru was the source of origin of the population that spread to these two countries.

Evolution of mtDNA haplotypes of *P. infestans*

The evolution of mitochondrial diversity in *P. infestans* has been the subject of much research and debate (Carter *et al*. 1990, Gavino and Fry 2002; Flier *et al*. 2003). Four mitochondrial haplotypes have been previoulsly described in *P. infestans*: Ia, Ib, IIa and IIb (Carter *et al*. 1990, 1991). Gavino and Fry (2002) proposed that haplotype Ib is ancestral to the other known haplotypes, although they did not completely rule out the hypothesis that haplotype Ia might be the ancestral haplotype since in their study haplotypes Ia and Ib differed from each other by only a single nucleotide. Type II haplotypes (IIa and IIb) were considered derived from Type I and appear to be closely related (Carter *et al*. 1990, 1991). Recently, Flier *et al*. (2003) suggested that the tight association between haplotype Ia and the sexual recombining population of *P. infestans* in the Toluca Valley was strong circumstantial evidence that Ia rather than Ib represents the ancestral type of mtDNA haplotype in *P. infestans*. Mitochondrial genealogy (Figure 1.3) shows that Type I and Type II lineages split from a common ancestor. The Type I haplotypes are ancestral to Type II haplotypes, since the mutations occur early in the ancient lineage Type II after splitting from the common ancestor. In addition, Type I haplotypes share the same nucleotide states (C, sites 17 and 24 Table 7) with the closely related lineage associated to *S. tetrapetalum*, indicating that the common ancestor had this nucleotide in both sites. Nucleotide T present in Type II haplotypes is considered derived, not present in the common ancestor. The same nucleotide state found in the P4 region of Type I haplotypes of *P. infestans* was recently found in three other species closely related to *P. infestans*: *P. mirabilis*, *P. ipomoeae*, and *P. phaseoli* (Kroon *et al*.

2004), supporting the ancestral condition of the nucleotide state present in Type I haplotypes. Additionally, more variants were found in Type I haplotypes compared to Type II haplotypes. No mutations were found that subdivided Type I haplotypes into Type Ia and Type Ib. Thus, it is not possible to infer which of these was ancestral to each other. The Ib mtDNA haplotype presents an extra *Msp*I restriction site (CCCG) that helps to differentiate it from mtDNA haplotype Ia (Carter *et al*. 1990, 1991; Griffith and Shaw, 1998). Sequence analysis showed that the restriction site was not present in the other closely related species (Kroon *et al*. 2004), consistent with a derived condition. This supports the Ia mtDNA haplotype as the ancestral haplotype within Type I haplotype. Work using herbarium samples also showed that the Ia mtDNA haplotype was the most common haplotype in older samples (May and Ristaino, 2004). Type IIa is ancestral to Type IIb, since no mutations occured in Type IIa after the splitting event (Figure 3). The Type IIa mtDNA haplotype has a wide geographical distribution whereas the Type IIb mtDNA haplotype has a limited distribution (Gavino and Fry, 2002). Recently, our group sequenced the complete mitochondrial genome of the four extant mtDNA haplotypes of *P. infestans*. A preliminary phylogenetic analysis based on the complete sequences agrees with the results presented here (Figure 1 Appendix, Avila-Adame *et al*. Manuscript in preparation).

Sexual reproduction and variability in *P. infestans*

The existence of a sexually reproducing population of *P. infestans* in the Toluca Valley in central Mexico was based on the presence of the two known compatibility groups (Gallegly and Galindo, 1958, Smoot *et al*. 1958) and oospores found in potato crops (Gallegly and Galindo, 1958) and recently in *S. demissum* (Flier *et al*. 2001). DNA sequences analysis from single-copy nuclear genes confirmed for the first time that recombination was occurring. Recombination was detected in Mexican and US populations (Table 1.5). Genotypes US11 and US15 were considered possible sexual recombinants based on RFLP analysis (Goodwin *et al*. 1998). US-11 was first identified in the Columbia Basin of Oregon and Washington and western Washington during 1993 and 1994. Goodwin *et al*. (1998) proposed that US-11 arose as a sexual recombinant, possibly between US-6 and US-7 or US-8 and laboratory crosses between US-6 and US-7 provided evidence for this (Gavino *et al*. 2000). Outside of Mexico, evidence for sexual reproduction of *P. infestans* has been found in the USA and the Netherlands using RFLP and AFLP markers. No isolates from the Netherlands were included in our study. Sexual reproduction seems to be an important part of the life history of *P. infestans*, although asexual reproduction is predominant in many populations.

Thus, our data suggest that the high level of genetic variability found in central highlands of Mexico is the result of sexual reproduction and not of ancestry. Several lines of evidence support this hypothesis. First, sexual recombination by itself generated high variability as measured by the traditional markers (Goodwin *et al*. 1992b, Mayton *et al*. 2000, Oliva *et al*. 2002, Knapova *et al*. 2002, Flier *et al*. 2003). Second, the detection

of the A2 mating type in Europe (Hohl and Iselin, 1984), a non ancestral region for *P. infestans*, resulted in a shift from low to high genotypic diversity, particularly in places where both mating types, A1 and A2, are found together, mirroring the diversity found in central Mexico (Sujkowski *et al*. 1994, Drenth *et al*. 1993, Bruberg *et al*. 1999, Elansky *et al*. 2001, Zwankhuizen *et al*. 2000, Kapanova and Gisi, 2002, Day *et al*. 2004). Third, reduced mitochondrial variability in Toluca Valley populations indicates the predominance of one maternal lineage. Isolates from Toluca Valley share a common haplotype. Recombination only impacts the nuclear genome. And finally, some markers (as RG-57 and AFLP) could allow differentiating even very closely related strains but face difficulties for inferring evolutionary patterns. By contrast, sequence variation of slow evolving loci will lead to a lack of discrimination between very closely related strains, but can provide more reliable information concerning evolutionary relationships (Cooper and Feil 2004).

Host-adaptation

Strong evidence for the existence of host-adaptation of *P. infestans* to potatoes and tomatoes has been reported (Legard *et al*. 1995; Oyarzún *et al*. 1998; Lebreton *et al*. 1999; Vega-Sanchez *et al*. 2000; Suassuna *et al*. 2004). Although *P. infestans* can infect both hosts, isolates are generally more aggressive on the host of origin (Adler *et al*. 2004). This type of host adaptation is apparently determined by quantitative differences in pathogenic fitness. Although difficult to measure under laboratory conditions, in the field a pathogen genotype is rarely found on anything but its primary host (Adler *et al*.

2004). Isolates of *P. infestans* from potato and tomato showed different responses to several fungicides even within a single genotype (Daayf and Platt, 2003). Tomato isolates were in general more sensitive to the fungicides. It has been suggested that adaptation to tomato is associated with loss of fitness on potato. Therefore tomato-aggressiveness may be a trait that evolved recently in *P. infestans*. Some evidence for host-specificity was found in the mitochondrial genealogy. Haplotype H7 (Figure 3) included only the US genotypes: US-6, US-11, US-12 and US-13, which are considered tomato-aggressive isolates (Goodwin *et al*. 1998). These isolates belong to mtDNA haplotype IIb (Table 1.1). Haplotype IIb has a restricted geographical distribution in North America: western Canada, western United States and north–western Mexico and until now was only associated with the clonal lineages US-6 and US-11 (Gavino and Fry, 2002). Our data indicate that genotypes US-12 and US-13 are also the IIb mtDNA haplotype (Table 1.1). In Europe, haplotype IIb has occassionally been reported, but associated with different allozyme patterns, including both mating types A1 and A2. Recently, US-11 was found in Taiwan attacking tomatoes (Deahl *et al*. 2002).

The isolates from *S. tetrapetalum* collected in Ecuador (Table 1.1) were genetically different from the other isolates across all loci analyzed suggesting that a new species might be present in the highlands of South American Andes (Table 1.3, Figure 1.3 and 1.4). Recently, Kroon *et al*. (2004) named a group of isolates from *S. muricatum* collected in Ecuador *Phytophthora andina*. Wattier *et al*. (2003) also found that two Ecuadorian isolates from *S. brevifolium* were different from the isolates of *P. infestans* from potato based on sequence variation of five inter-genic mitochondrial DNA spacers.

S. tetrapetalum, *S. muricatum*, and *S. brevifolium* are hosts of the hetergeneous EC-2 group (Adler *et al*. 2004). A phylogenetic analysis, based on nuclear and mitochondrial loci, places *S. andina* in the same clade as *P. infestans*, *P. mirabilis*, *P. ipomoeae*, and *P. phaseoli* and all are closely related species showing host-specificity (Waterhouse 1963, Brasier and Hansen 1992, Cooke *et al*. 2000). The existence in a geographic region of a group of closely related species, suggests that this region is the place of origin of that group (Brasier and Hansen, 1992). *P. mirabilis* causes leaf blight on *Mirabilis jalapa*, *P. ipomoeae* leaf bight on *Ipomoea longipedunculata*, and *P. phaseoli* attacks beans. Toluca Valley is thought to be also the center of origin for these three species (Flier *et al*. 2002). Interestingly, *Mirabilis jalapa* is native from South America and is known as "four o' clock" or Marvel of Peru. It seems likely that this plant was brought to Mexico.

Genetic diversity in *Ipomoea* and *Phaseolus* has shown the existance of two major domestication sites for these genera: Mesoamerica or Middle America (e.g., Mexico and countries of Central America) and Andean South America (e.g. Peru, Bolivia, and northern Argentina). This lead to the emergence of two genepools: Andean and Middle American (Templar *et al*. 2003, Rajapakse *et al*. 2004, Beccara Velasquez 1994). Therefore the possibility exists that *Phytophthora* species associated with *Ipomoea* and *Phaseolus* originated also in South America and were brought to Mexico in the ancient times. *P. ipomoeae* presents the same haplotype, based on restriction digest of the P4 region, described for Ecuadorian isolates belonging to the EC-2 group (Flier *et al*. 2002; Ordoñez et al. 2000). South America is also the center of diversity of tomatoes (Nesbitt *et al*. 2002).

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Figure legends

Figure 1. 1 *Phytophthora infestans* Ras gene organization showing location of sequenced regions and the *NruI* restriction site.

Figure 1. 2. A) Intron Ras sequence chromatograms of *Phytophthora infestans* showing sites with double bands (peaks) indicating heterozygotes, F: Forward; R: Reverse; B) *NruI* restriction digestion of RAS PCR products from *P. infestans* isolates. 1) 100 bp ladder, 2) CR-51, 3) CR-52, 4) PER 800, 5) PER 832, 6) 100bp ladder. Lanes 2 and 3) CR-51 and CR-52 (homozygotes). Lanes 4 and 5) PER 800 and PER 832 (heterozygotes) Lanes 1 and 6) 100 bp ladder. C) *NcoI* restriction digestion of β-Tubulin PCR products from *P. infestans* isolates. Lane 2) Undigested PCR product. Lanes 3, 4, and 5) BOL -3, BOL-6, BOL-14 (heterozygotes). Lanes 6 and 7) US-13 and US-14 (heterozygotes). Lanes 8 and 9) PIC97620 and PIC97224 (homozygotes). Lanes 10 and 11) CR-52 and CR-61 (heterozygotes). Lanes 1 and 12) 100bp ladder

Figure 1. 3 Coalescent-based genealogy with the highest root probability showing the distribution of mutations for the mitochondrial (P3+P4) region of *Phytophthora infestans*. The inferred genealogy is based on 10 different starting random number seeds, using 5 million simulations of the coalescent for each run. All runs converged to the same genealogy. The time scale is in coalescent units of effective population size. In the gene genealogy, the direction of divergence is from the top of the genealogy (oldest, *i.e*, the past) to the bottom (youngest, *i.e.*, the present); coalescent is from the bottom (present) to the top (past). Since the gene genealogy is rooted, all the mutations and bifurcations are also time ordered from top to bottom. The numbers below the tree from top to bottom designate each distinct haplotype and its frequency (i.e., the number of occurrences of the haplotype in the sample), the frequency of each haplotype in each population, the mating type of the isolates in the haplotype, and the Carter's mtDNA haplotypes determined by PCR-RFLP method (Griffith and Shaw, 1998). Circles designated replacement substitutions.

Figure 1. 4 Coalescent-based genealogy with the highest root probability showing the distribution of mutations for the Intron Ras region of *Phytophthora infestans*. The inferred genealogy is based on 10 different starting random number seeds, using 5 million simulations of the coalescent for each run. All runs converged to the same genealogy. The time scale is in coalescent units of effective population size. In the gene genealogy, the direction of divergence is from the top of the genealogy (oldest, *i.e*, the past) to the bottom (youngest, *i.e.*, the present); coalescent is from the bottom (present) to the top (past). Since the gene genealogy is rooted, all the mutations and bifurcations are also time ordered from top to bottom. The numbers below the tree from top to bottom designate

each distinct haplotype and its frequency (i.e., the number of occurrences of the haplotype in the sample), the frequency of each haplotype in each population, the mating type of the isolates in the haplotype, and the Carter's mtDNA haplotypes determined by PCR-RFLP method (Griffith and Shaw, 1998).

Figure 1. 5 Coalescent-based genealogy with the highest root probability showing the distribution of mutations for the Intron Ras + Ras (IRRAS) region of *Phytophthora infestans*. The inferred genealogy is based on 10 different starting random number seeds, using 5 million simulations of the coalescent for each run. All runs converged to the same genealogy. The time scale is in coalescent units of effective population size. In the gene genealogy, the direction of divergence is from the top of the genealogy (oldest, *i.e*, the past) to the bottom (youngest, *i.e.*, the present); coalescent is from the bottom (present) to the top (past). Since the gene genealogy is rooted, all the mutations and bifurcations are also time ordered from top to bottom. The numbers below the tree from top to bottom designate each distinct haplotype and its frequency (i.e., the number of occurrences of the haplotype in the sample), the frequency of each haplotype in each population, the mating type of the isolates in the haplotype, and the Carter's mtDNA haplotypes determined by PCR-RFLP method (Griffith and Shaw, 1998).

Figure 1. 6. A Neighbor-joining phylogenetic unrooted tree based on the complete mtDNA sequence from the four extant mitochondrial haplotypes of *P. infestans*. Boostrap values based on 1000 replicates are shown at branches.

Table 1.1 Isolates of *Phytophthora infestans* used in this study

Geographical Origin/Isolate Code	Host	Year Collected	Mating Type	mtDNA Haplotype[a]	Allozyme Genotype[b] *Gpi*	Allozyme Genotype[b] *Pep*	RG-57 Lineage	Source
Bolivia								
BOL 3	Potato	__[c]	A2	IIa	100/100	__	BR-1	PROINPA
BOL12	Potato	__	A2	IIa	100/100	__	BR-1	PROINPA
BOL13	Potato	__	A2	IIa	100/100	__	BR-1	PROINPA
BOL14	Potato	__	A2	IIa	100/100	__	BR-1	PROINPA
BOL15	Potato	__	A2	IIa	100/100	__	BR-1	PROINPA
BOL16	Potato	__	A2	IIa	100/100	__	BR-1	PROINPA
Brazil								
B040	Potato	1998	A2	IIa	100/100	__	BR-1	Dr. Mizubuti
B124	Potato	1998	A2	IIa	100/100	__	BR-1	Dr. Mizubuti
B189	Potato	1998	A2	IIa	100/100	__	BR-1	Dr. Mizubuti
B193	Potato	1998	A2	IIa	100/100	__	BR-1	Dr. Mizubuti
B217	Potato	1998	A2	IIa	100/100	__	BR-1	Dr. Mizubuti
B219	Potato	1998	A2	IIa	100/100	__	BR-1	Dr. Mizubuti
Peru								
PER800	Potato	1985	A1	Ib	86/100	92/100	US-1	Dr. Tooley
PER802	Potato	1985	A1	Ib	86/100	92/100	US-1	Dr. Tooley
PER804	Potato	1984	A1	Ib	86/100	92/100	US-1	Dr. Tooley
PER808	Potato	1985	A1	Ib	86/100	92/100	US-1	Dr. Tooley
PER812	Potato	1985	A1	Ib	86/100	92/100	US-1	Dr. Tooley
PER832	Potato	1986	A1	Ib	86/100	92/100	US-1	Dr. Tooley
PCZ007	Potato	1997	A1	Ia	100/100	100/100	PE-3	CIP-PERU
PCZ050	Potato	1997	A1	Ia	100/100	100/100	PE-3	CIP-PERU
PCZ026	Potato	1997	A1	IIa	90/100	96/100	PE-6	CIP-PERU
PCZ033	Potato	1997	A1	IIa	90/100	96/100	EC1.1	CIP-PERU
PCZ098	Potato	1997	A1	IIa	90/100	96/100	EC1.2	CIP-PERU
PCZ118	Potato	1997	A1	IIa	90/100	96/100	EC1.2	CIP-PERU
PHU006	Potato	1996	A1	IIa	90/100	96/100	EC-1	CIP-PERU
POX004	Potato	1997	A1	IIa	90/100	96/100	EC-1	CIP-PERU
PCO038	Potato	1997	A1	IIa	90/100	96/100	EC-1	CIP-PERU
PPA008	Potato	1998	A1	IIa	__	__	__	CIP-PERU
PPU003	Potato	1997	A1	IIa	__	__	__	CIP-PERU
PPU100	Potato	1997	A1	Ib	__	__	__	CIP-PERU
Ecuador								
3090	*Solanum phureja*	__	A1	IIa	90/100	96/100	EC-1	CIP-ECUADOR
3092	*S. phureja*	__	A1	IIa	90/100	96/100	EC-1	CIP-ECUADOR
3094	*S. phureja*	__	A1	IIa	90/100	96/100	EC-1	CIP-ECUADOR
3152	*S. andreanum*	__	A1	IIa	90/100	96/100	EC-1	CIP-ECUADOR
3153	*S. andreanum*	__	A1	Ib	86/100	92/100	US-1	CIP-ECUADOR
3154	*S. andreanum*	__	A1	IIa	90/100	96/100	EC-1	CIP-ECUADOR
3163	*S. tetrapetalum*	__	A2	Ic	100/100	76/100	EC-2	CIP-ECUADOR
3164	*S. tetrapetalum*	__	A2	Ic	100/100	76/100	EC-2	CIP-ECUADOR
3165	*S. tetrapetalum*	__	A2	Ic	100/100	76/100	EC-2	CIP-ECUADOR
3166	*S. tetrapetalum*	__	A2	Ic	100/100	76/100	EC-2	CIP-ECUADOR
3167	*S. tetrapetalum*	__	A2	Ic	100/100	76/100	EC-2	CIP-ECUADOR
3198	*S. tuquerense*	__	A1	IIa	90/100	96/100	EC-1	CIP-ECUADOR
3253	*S. columbianum*	__	A1	IIa	90/100	96/100	EC-1	CIP-ECUADOR
3300	*S. paucijugum*	__	A1	IIa	90/100	96/100	EC-1	CIP-ECUADOR

Table 1.1 Continued

Costa Rica								
CI	Potato	2000	A1	Ia	100/100	94/100	CR-1	Mr. Paez
DR4	Potato	2001	A1	Ia	100/100	94/100	CR-1	Mr. Paez
F7	Potato	2001	A1	Ia	100/100	94/100	CR-1	Mr. Paez
GB-2	*S. longiconicum*	2001	A1	Ia	100/100	94/100	CR-1	Mr. Paez
ZB	Potato	2000	A1	Ia	100/100	94/100	CR-1	Mr. Paez
ZE	Potato	2001	A1	Ia	100/100	94/100	CR-1	Mr. Paez
51	*S. longiconicum*	2003	A1	Ia	100/100	__	CR-1	Dr. Brenes
52	*S. longiconicum*	2003	A1	Ia	100/100	__	CR-1	Dr. Brenes
61	Potato	2003	A1	Ia	100/100	__	CR-1	Dr. Brenes
141	Potato	2003	A1	Ia	100/100	__	CR-1	Dr. Brenes
151	Potato	2003	A1	Ia	100/100	__	CR-1	Dr. Brenes
152	Potato	2003	A1	Ia	100/100	__	CR-1	Dr. Brenes
Mexico								
PIC97189	Potato	1997	A1	Ia	86/122	92/100	__	Dr. Grundwald
PIC97207	Potato	1997	A1	Ia	100/122	100/100	__	Dr. Grundwald
PIC97224	Potato	1997	A2	Ia	100/122	100/100	__	Dr. Grundwald
PIC97322	Potato	1997	A1	Ia	100/122	78/100	__	Dr. Grundwald
PIC97370	Potato	1997	A2	Ia	86/100	100/100	__	Dr. Grundwald
PIC97388	Potato	1997	A2	Ia	100/122	92/100	__	Dr. Grundwald
PIC697605	Potato	1997	A1	Ia	86/100	96/100	__	Dr. Grundwald
PIC97620	Potato	1997	A1	Ia	100/122	96/100	__	Dr. Grundwald
PIC97630	Potato	1997	A2	Ia	86/122	92/92	__	Dr. Grundwald
PIC97652	Potato	1997	A2	Ia	100/122	100/100	__	Dr. Grundwald
PIC98301	Potato	1998	A2	Ia	100/100	100/100	__	Dr. Grundwald
PIC98366	*S. demissum*	1998	A1	Ia	86/100	100/100	__	Dr. Grundwald
PIC98369	*S. demissum*	1998	A1	Ia	100/122	100/100	__	Dr. Grundwald
PIC98372	*S. demissum*	1998	A2	Ia	86/122	100/100	__	Dr. Grundwald
PIC98388	*S. demissum*	1998	A1	Ia	100/100	100/100	__	Dr. Grundwald
PIC98392	*S. demissum*	1998	A2	Ia	86/122	100/100	__	Dr. Grundwald
USA								
94-1	Potato	1994	A1	Ib	86/100	92/100	US-1	Dr. Fraser
94-52	Potato	1994	A1	IIb	100/100	92/100	US-6	Dr. Fry
94-53	Potato	1994	A2	Ia	100/111	100/100	US-7	Dr. Fry
94-8-4	Potato	1994	A2	Ia	100/111/122	100/100	US-8	Dr. Fraser
980059	Potato	1998	A1	IIb	100/111	100/100	US-11	Dr. Fry
940494	Tomato	1994	A1	IIb	100/111	92/100	US-12	Dr. Fry
940504	Unknown	1994	A2	IIb	100/100	100/100	US-13	Dr. Fry
940502	Potato	1994	A2	Ia	100/122	100/100	US-14	Dr. Fry
80787-94L	Tomato	1994	A2	Ia	100/100	92/100	US-15	Dr. Kim
97001	Tomato	1996	A1	Ia	100/122	100/100	US-17	Dr. Fry
Ireland								
21A/93	Potato	1993	A2	IIa	100/100	100/100	__	Dr. L. Cooke
12/94	Potato	1994	A2	Ia	100/100	100/100	__	Dr. L. Cooke
15/94	Potato	1994	A2	Ia	100/100	100/100	IE-3	Dr. L. Cooke
18/94	Potato	1994	A1	IIa	__	__	__	Dr. L. Cooke
6/95	Potato	1995	A1	IIa	100/100	100/100	__	Dr. L. Cooke
31/95	Potato	1995	___	IIa	__	__	__	Dr. L. Cooke
3/99	Potato	1999	A1	IIa	100/100	100/100	V-3	Dr. L. Cooke
12/99	Potato	1999	A1	IIa	100/100	100/100	IE-1b	Dr. L. Cooke
15/99	Potato	1999	A1	IIa	100/100	100/100	IE-1	Dr. L. Cooke
16/99	Potato	1999	A1	Ia	100/100	100/100	V-1	Dr. L. Cooke

[a] mtDNA haplotype was determined by the methods of Griffith and Shaw (1998)

[b] Allozyme Genotype: *Gpi* (Glucose-6-phosphate isomerase) and *Pep* (Peptidase) were determined by cellulose-acetate gel electrophoresis (Goodwin et al 1995)

[c] Not determined or in Mexican population the lineage concept is not applied because is a sexual recombinant population

Table 1.2 Primers used in this study

Target DNA	Primer	Primer Sequence	Primer Length (bp)	Primer Position[a]	Source
Mitochondrial					
P3	F3	5` ATGGTAGAGCGTGGGAATCAT 3`	21	2893 - 2913	Griffith and Shaw (1998)
	R3	5` AATACCGCCTTTGGGTCCATT 3`	21	4178 - 4198	Griffith and Shaw (1998)
P4	F4	5` TGGTCATCCAGAGGTTTATGTT 3`	22	9379 - 9400	Griffith and Shaw (1998)
	R4	5` CCGATACCGATACCAGCACCAA 3`	22	10321 -10342	Griffith and Shaw (1998)
Nuclear					
Intron Ras	IRF	5` TTGCAGCACAACCCAAGACG 3`	20	442 - 461	Gomez et al (2003)
	IRR	5` TGCACGTACTATTCGGGGTTC 3`	21	768 - 789	Gomez et al (2003)
Ras	RASF	5` CGTGTCTGCTTCTCCGTTTCG 3`	21	916 - 936	Gomez et al (2003)
	RASR	5` CCAGGCTTTCGGCAAATTCC 3`	20	1496 - 1515	Gomez et al (2003)
β- Tubulin[b]	TUB901	____	____	____	A.C. Leveque
	TUB1401	____	____	____	A.C. Leveque

[a] Location of the primer within the original DNA sequence, GeneBank accession U30474 for Ras gene and U17009 for the mitochondrial regions

[b] Primer sequences for β-Tubulin gene are available from C. Andre Levesque, Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Summerland, British Columbia, Canada.

Table 1.3 Characteristics of the loci studied

Fragment	Gene	Genomic Location	n	Size of sequenced fragment (bp)	Size of unambiguosly aligned region (bp)	No (%) of polymorphic nucleotide sites		No (%) of polymorphic aminoacid sites	
						Potato[a]	S. *tetrapetalum*	Potato	S. *tetrapetalum*
Intron Ras	Non-coding	Nucleus	88	349	223[b]	5 (2.23)	15 (6.69)	0	------[c]
Ras	*PiYpt1*	Nucleus	79	600	542	7 (1.29)	------	0[d]	------
B-Tubulin	*β-tubulin*	Nucleus	78	542	490	1 (0.20)	------	0[e]	------
P3	*rpl14, rp15 tRNAs*	Mitochondria	66	1308	1180	9 (0.76)	17 (1.44)	4	7
P4	*cox 1*	Mitochondria	79	964	830	4 (0.48)	12 (1.44)	0	0

n = number of isolates sequenced

[a] Includes also wild *Solanum* species and tomato isolates (Table 1.1)

[b] Total length of Intron Ras

[c] Not determined

[d] Two synonymous sustitutions were found (Glutamine and Asparagine)

Table 1.4 Diversity estimates and neutrality tests

Locus/Population	Sample summaries					Parameter estimates x 10^{-3}		Test of neutrality			
	l	*n*	*s*	*h*	*k*	π (SE)	θw	Tajima's D Statistic	Fu and Li's D* Statistic	Fu and Li's F* Statistic	Fu's Fs Statistic
Mitochondrial *(P3 + P4)*											
BRAZIL	2011	4	0	1	0	0.00	0.00	ND	ND	ND	ND
BOLIVIA	2011	3	0	1	0	0.00	0.00	ND	ND	ND	ND
PERU	2011	12	8	3	4.35	2.16 (6.9X10^{-5})	1.32	2.562**	1.383*	1.914**	4.958 (NS)
ECUADOR	2011	8	9	2	2.25	1.12 (2.8X10^{-4})	1.73	-1.723*	-1.885*	-2.045*	3.887 (NS)
ECUADOR (TETRA)	2011	10	28	3	10.46	5.20 (5.9X10^{-4})	5.10	0.101 (NS)	1.173 (NS)	1.022 (NS)	8.296*
COSTA RICA	2011	7	0	1	0	0.00	0.00	ND	ND	ND	ND
MEXICO	2011	10	3	4	0.87	0.43 (3.5X10^{-5})	0.53	-0.657 (NS)	-0.805 (NS)	-0.859 (NS)	-1.176 (NS)
USA	2011	10	9	3	4.91	2.44 (1.0X10^{-4})	1.58	2.381**	1.435*	1.876**	4.806 (NS)
IRELAND	2011	5	8	2	4.80	2.39 (3.1X10^{-4})	1.91	1.761 (NS)	1.761*	1.844 (NS)	4.717 (NS)
SOUTH AMERICA	2011	27	9	4	3.29	1.64 (6.5X10^{-5})	1.16	1.315 (NS)	0.805 (NS)	1.118 (NS)	4.364 (NS)
S. AMERICA (TETRA)	2011	29	28	5	6.17	3.07 (1.7X10^{-4})	3.67	-0.598 (NS)	1.492*	0.966 (NS)	6.783*
NON-S. AMERICA	2011	32	12	7	3.72	1.85 (5.8X10^{-5})	1.48	0.806 (NS)	0.514 (NS)	0.711 (NS)	1.811 (NS)
POOLED	2011	59	13	9	4.41	2.20(9.1X10^{-6})	1.39	1.686 (NS)	-0.082 (NS)	0.618 (NS)	2.422 (NS)
POOLED (TETRA)	2011	61	32	10	5.67	2.82 (5.4X10^{-5})	3.51	-0.636 (NS)	1.106 (NS)	0.554 (NS)	3.153 (NS)
Nuclear *(IRRas)*											
BRAZIL	766	12	8	2	4.36	5.70 (1.8X10^{-4})	3.46	2.584**	1.383*	1.921**	7.657**
BOLIVIA	766	12	8	2	4.36	5.70 (1.8X10^{-4})	3.46	2.584**	1.383*	1.921**	7.657**
PERU	766	36	10	4	1.76	2.29 (1.2X10^{-4})	3.15	-0.827 (NS)	1.404 (NS)	0.823 (NS)	2.258 (NS)
ECUADOR	766	14	2	3	0.97	1.26 (5.6X10^{-5})	0.82	1.459 (NS)	0.935 (NS)	1.215 (NS)	0.783 (NS)
COSTA RICA	766	24	0	1	0	0.00	0.00	ND	ND	ND	ND
MEXICO	766	23	3	4	1.04	1.36 (4.2X10^{-5})	1.06	0.723 (NS)	-0.174 (NS)	-0.087 (NS)	0.240 (NS)
USA	766	12	9	3	4.04	5.28 (1.6X10^{-4})	3.89	1.451 (NS)	0.976 (NS)	1.247 (NS)	4.684 (NS)
IRELAND	766	16	10	6	2.21	2.88 (3.0X10^{-4})	3.93	-0.996 (NS)	0.535 (NS)	0.130 (NS)	-0.423 (NS)
SOUTH AMERICA	766	74	10	4	3.21	4.18 (6.6X10^{-5})	2.68	1.504 (NS)	1.386 (NS)	1.688 (NS)	6.916**
NON-S. AMERICA	766	75	11	7	1.70	2.22 (6.2X10^{-5})	2.94	-0.662 (NS)	0.804 (NS)	0.354 (NS)	0.301 (NS)
POOLED	766	149	11	7	2.49	3.25 (3.4X10^{-5})	2.57	0.650 (NS)	0.696 (NS)	0.811 (NS)	2.863 (NS)

l , consensus sequence length; *n* , sample size (number of isolates for mitochondrial locus and number of chromosomes for nuclear locus);
s, segregating nucleotide sites; *h* , haplotypes; *K* , average number of pairwise nucleotide differences; π, average number of base differences per site;
SE, standard error; θw, population mean mutation rate or Watterson's theta estimator.
Tetra= Isolates from Ecuador isolated from *Solanum tetrapetalum*
N.D., not determine because no polymorphism; ns, not significant; *, 0.01<P<0.05; **, 0.001<P<0.01; ***, P<0.001

Table 1. 5 Distribution of haplotypes

Locus	*Mitochondrial (P3+P4)*[a]		*Nuclear (IRRAS)*[b]		*(IntronRas)*	
	111111111					
Position in	233333333333333339999999000000000		1111111			
Genbank data	900013444455588995678991111111111		5566670011233		5555555666677	
Base Accession[c]	807770027716935672039343488889999		6901602678105		1568999016800	
	802306420698493649198438046890l3		1650018487331		9119368500816	
Position	11111111111111111					
in combined	112344555668800234666889999999		11123444567		1111122	
consensus	341114461161389016373677711222222		6901601467983		2569990016900	
	024528642810615862421761379123 46		3852222821775		1331580722027	
Site Number	1111111111222222222333		1111		1111	
	123456789012345678901234567890l2		1234567890123		1234567890123	
Site Type	ttttvvvtvvvttvttvtvttttvttttttv		tvvtttvtvtttv		tttvtvvvttttt	
Character Type	i-----i---ii-iiii------i--------		i-iiiiiiiiiii		--i----ii--i-	
Substitution Type	rsrssrrrsrrsssssss		ss			
Consensus	CGTGTATTCATAGTAACGGGTCACATCACGAC		CGAAAGGGGTGTT		GCCCAGACGACGG	
Haplotype(Frequency)[d]						
H1 (24)	......A...GC.C.GT......T........	H1	(19)	TTCG.A....ACA	H1	(19) ..T..T.....A.
H2 (24)	T.............C..................	H2	(94)	....	H2	(5) AT.AG.C...T.A
H3 (1)	T........C....C..................	H3	(2)	......TA.C...	H3	(138) ....AA....
H4 (1)	T.............C..C..............	H4	(15)	.......A.....	H4	(3) ....AAG...
H5 (3)	T.............C.......G.........	H5	(1)	.......ATC...	H5	(1) ..T........A.
H6 (2)	.AC.CTAGT...A.....ATCT..GGTGTAGA	H6	(20)	.......A.C...		
H7 (4)	...A..A...GC.C.GT......T........	H7	(1)	...........C.		
H8 (2)	.............C..................	H8	(1)	T.CG.A....ACA		
H9 (1)	..........G..C.GT......T........	H9	(1)	....G........		
H10 (1)	......A...GC.C..T......T........	H10	(1)	....G..A.C...		
		H11	(1)	....G..AT....		
		H12	(2)	......T......		

t, transitions; v, transversions; i, phylogenetically informative sites; -, uninformative sites; r, nonsynonymous (i.e., replacement) substitutions; s, synonymous substitutions.

[a]Site number 7 was incompatible with other sites and excluded from the analyses. Site 12 and 16 where incompatible with each other; removing haplotype 10 (3253) eliminated the incompatibility; therefore haplotype 10 was inferred as recombinant.

[b] Inferred recombinant haplotypes: H9 (MEX4); H11 (MEX21); and H12 (US11 and US15) were excluded from the analyses that assume no recombination in the history of the sample.

[c] Numbering in vertical columns is that of GeneBank accession No U17009 for mitochondrial and No U30474 for nuclear loci respectively

[d] Haplotype number and frequency (i.e., the number of the haplotype in the sample) is shown in parentheses. The number of haplotype in the sample refers to the number of chromosomes for the nuclear loci, and the number of strains (isolates) for the mitochondrial loci since *P. infestans* is a diploid organism.

Table 1. 6 Population subdivision at nuclear loci according to Hudson's test statistics Ks (upper right matrix) and Kst (lower left matrix)

	Bolivia	Brazil	Peru	Ecuador	Costa Rica	Mexico	USA	Ireland
Bolivia		**4.363ns**	**2.4088*** **	**2.5347*** **	1.4546***	2.2811***	4.2463ns	3.1320*
Brazil	**-0.0454ns**		**2.4088*** **	**2.5347*** **	1.4546***	2.2811***	4.2463ns	3.1320*
Peru	**0.2947*** **	**0.2947*** **		**1.5359ns**	1.0543***	1.5738ns	2.4959*	1.8959ns
Ecuador	**0.2947*** **	**0.2947*** **	**-0.0088ns**		0.3563***	1.1965ns	2.6691*	1.6291*
Costa Rica	0.3636***	0.3636***	0.1075***	0.2364***		**0.6864*** **	**1.6633*** **	**0.8833** **
Mexico	0.2920***	0.2929***	-0.0007ns	-0.0155ns	**0.1829*** **		**2.4012*** **	**1.6584** **
USA	0.0348ns	0.0348ns	0.0484*	0.0851*	**0.1251*** **	**0.0971*** **		**3.1833ns**
Ireland	0.1275*	0.1275*	0.0202ns	0.0513*	**0.0444** **	**0.0631***	**0.0007ns**	

Comparisons within populations of South America and within non-South American populations are in bold
ns, not significant; *, 0.01<0.05; **, 0.001<P<0.01; ***, P<0.001

Table 1.7 Population subdivision at mitochondrial loci according to Hudson's test statistics Ks (upper right matrix) and Kst (lower left matrix)

	Bolivia	Brazil	Peru	Ecuador	Ecuador Tetra	Costa Rica	Mexico	USA	Ireland
Bolivia			**3.4438ns**	**1.5357ns**	**6.8250ns**	0.0000***	0.5778***	3.6444*	2.7000ns
Brazil			**3.4438ns**	**1.5357ns**	**6.8250ns**	0.0000***	0.5778***	3.6444*	2.7000ns
Peru	**0.2219ns**	**0.2219ns**		**3.8117ns**	**7.2933ns**	3.0813*	3.0551**	5.1460ns	5.0321ns
Ecuador	**0.0089ns**	**0.0089ns**	**0.1299ns**		**6.5350ns**	1.3437***	1.5877***	4.0088ns	3.4643ns
Ecuador Tetra	**0.0488ns**	**0.0488ns**	**0.0624ns**	**0.0177ns**			5.6127***	7.8032ns	8.5125ns
Costa Rica	1.0000***	1.0000**	0.2319*	0.7267***			**0.5098ns**	**3.2157***	**2.2500***
Mexico	0.8819***	0.8819***	0.2500**	0.6949***	0.3524***	**0.0631ns**		**3.1667** **	**2.3778***
USA	0.2126*	0.2126*	-0.0177ns	0.1189ns	0.0549ns	**0.2831***	**0.2963** **		**4.8741ns**
Ireland	0.1562ns	0.1562ns	-0.0561ns	0.0055ns	0.0034ns	**0.3889***	**0.3565***	**-0.0402ns**	

Comparisons within populations of South American and within non-South American populations are in bold
Ecuador Tetra: Ecuadorian population including isolates from *S. tetrapetalum*
ns, not significant; *, 0.01<0.05; **, 0.001<P<0.01; ***, P<0.001

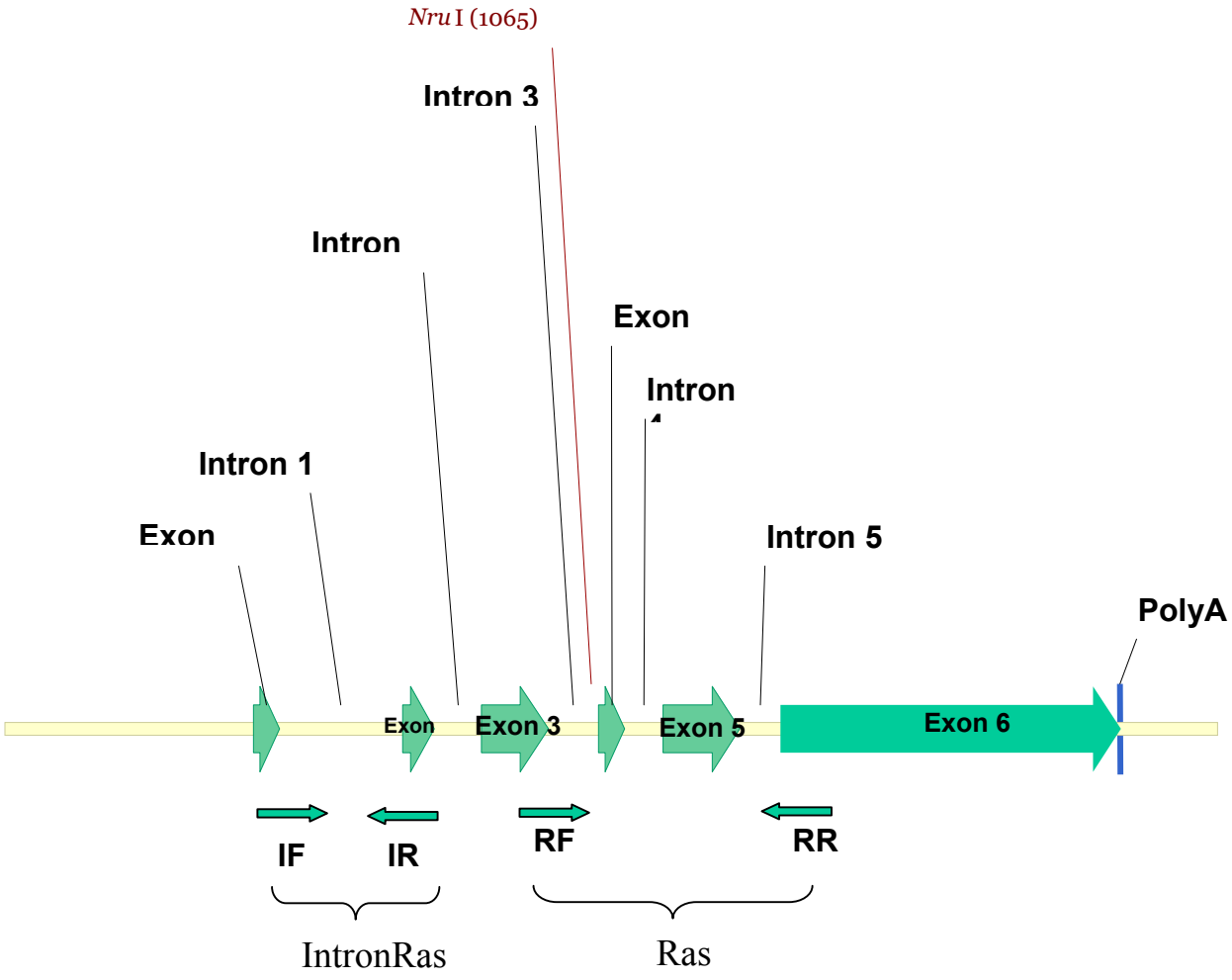
Table 1.8 Identity of haplotypes

Locus	Haplotype (Frequency)	Isolates	Population
Mitochondrial *(P3+P4)*	H1 (24)	B40, B124,B189, B193, B212, BOL3, BOL12, BOL13, 3090, 3094, 3152, 3154, 3198, 3199, 3298, 3300, PCZ026, PCZ098, PCZ118, PPA008, PPU003, 21A93, 15/99, 3/99	Brazil, Bolivia, Ecuador Peru, Ireland
	H2 (24)	3153, PER802, PER808, PER812, PCZ007, PCZ050, PPU100, F7, ZE, 51, 52, 141, 151, 152, Pic97224, Pic97322, Pic97370, Pic97605, Pic97620, US1, US7, US15, 12/94, 16/99	Ecuador, Peru, Costa Rica Mexico, USA, Ireland
	H3 (1)	Pic97207	Mexico
	H4 (1)	Pic97388	Mexico
	H5 (3)	Pic97180, Pic97652, Pic98305	Mexico
	H6 (2)	3165, 3167	Ecuador
	H7 (4)	US6, US11, US12, US13	USA
	H8 (2)	US14, US17	USA
	H9 (1)	PHU006	Peru
	H10 (1)	3253	Ecuador
Nuclear *(IntronRas+RAS)*	H1 (19)	B40, B124, B189, B193, B217, B219, BOL3, BOL12, BOL13, BOL14, BOL15, BOL16, PCZ007, PCZ050, US6, US8, US11, US14, 3/99	Brazil, Bolivia, Peru, USA Ireland
	H2 (94)	B40, B124, B189, B193, B217, B219, BOL3, BOL12, BOL13, BOL14, BOL15, BOL16, PER800, PER802, PER804, PER808, PER812, PER832, PCZ007, PCZ050, PCZ026, PCZ033, PCZ098, PCZ118, PHU006, POX004, PCO038, PPA008, PPU003, PPU100, 3090, 3092, 3094, 3152, 3153, 3154, 3198, 3300, Pic97620, Pic97224, Pic97322, Pic97605, Pic97370, Pic97630, Pic97652, Pic97180, Pic98372, Pic98301, Pic98366, CI, DR4, F7, GB-2, ZB, ZE, 51, 52, 61, 141, 151, 152, US1, US6, US7, US8, US14, US15, US17, 6/95, 31/95, 18/94, 21A93, 12/94, 12/99	All
	H3 (2)	US7, US15	USA
	H4 (15)	PER800, PER802, PER804, PER808, PER812, PER32, PPU100, 3090, 3094, 3153, Pic97180, Pic97207, Pic98366, US1, 3/99	Ecuador, Peru, Mexico, USA, Ireland
	H5 (1)	Pic97207	Mexico

Table 1.8 Continued

	H6 (20)	PCZ026, PCZ033, PCZ098, PCZ118, PHU006, POX004, PCO038, PPA008, PPU003, 3092, 3152, 3198, 3300, Pic97224, Pic97370, Pic97388 Pic97630, Pic98372, 12/94	Peru, Ecuador, Mexico, Ireland
	H7 (1)	16/94	Ireland
	H8 (1)	16/94	Ireland
	H9 (1)	Pic97322	Mexico
	H10 (1)	Pic97605	Mexico
	H11 (1)	Pic98301	Mexico
	H12 (1)	US11, US15	USA
Nuclear *(IntronRas)*	H1 (19)	B40, B124, B189, B193, B217, B219, BOL3, BOL12, BOL13, BOL14, BOL15, BOL16, PCZ007, PCZ050, US6, US8, US11, US14, 3/99	Brazil, Bolivia, Peru, USA Ireland
	H2 (5)	3163, 3164, 3165, 3166, 3167	Ecuador
	H3 (138)	B40, B124, B189, B193, B217, B219, BOL3, BOL12, BOL13, BOL14, BOL15, BOL16, PER800, PER802, PER804, PER808, PER812, PER832, PCZ007, PCZ050, PCZ026, PCZ033, PCZ098, PCZ118, PHU006, POX004, PCO038, PPA008, PPU003, PPU100, 3090, 3092, 3094, 3152, 3153, 3154, 3163, 3164, 3165, 3166, 3167, 3198, 3300, CI, DR4, F7, GB-2, ZB, ZE, 51, 52, 61, 141, 151, 152, Pic97180, Pic 97207, Pic97224, Pic97322, Pic97370, Pic97388, Pic97605, Pic97620, Pic97630, Pic97652, Pic98301, Pic98366, Pic98372, US1, US6, US7, US8, US11, US14, US15, US17, 6/95, 31/95, 18/94, 3/99, 21A93, 12/94, 12/99, 16/94	All
	H4 (3)	Pic97322, Pic97605, Pic98301	Mexico
	H5 (1)	16/94	Ireland

[a] Haplotype number and frequency (i.e., the number of the haplotype in the sample) is shown in parentheses. For nuclear locus the number of haplotype in the sample refers to the number of chromosomes, since *P. infestans* is a diploid organism. The same isolate can carry two different haplotypes. For the mitochondrial locus refers to tl number of isolates.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Intron
Exon
Intron
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PolyA
Exon
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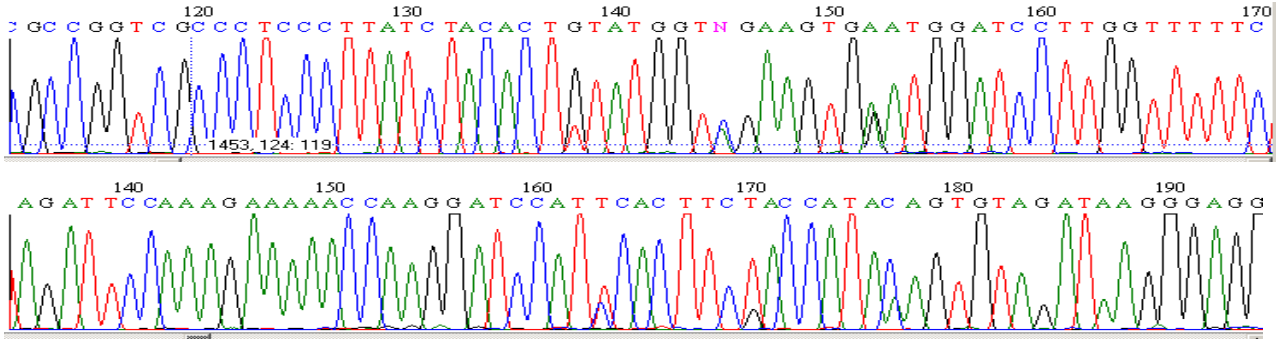
Exon 6
IF
IR
RF
RR
IntronRas
Ras

A)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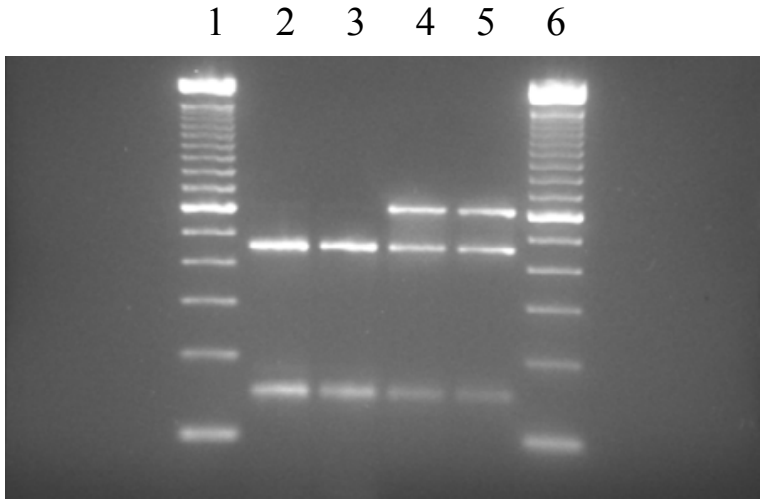
150
160
170
180
190

B)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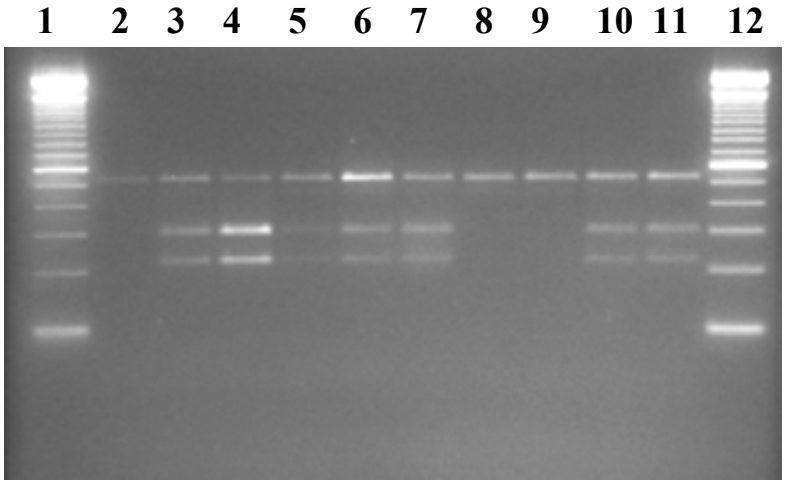
C)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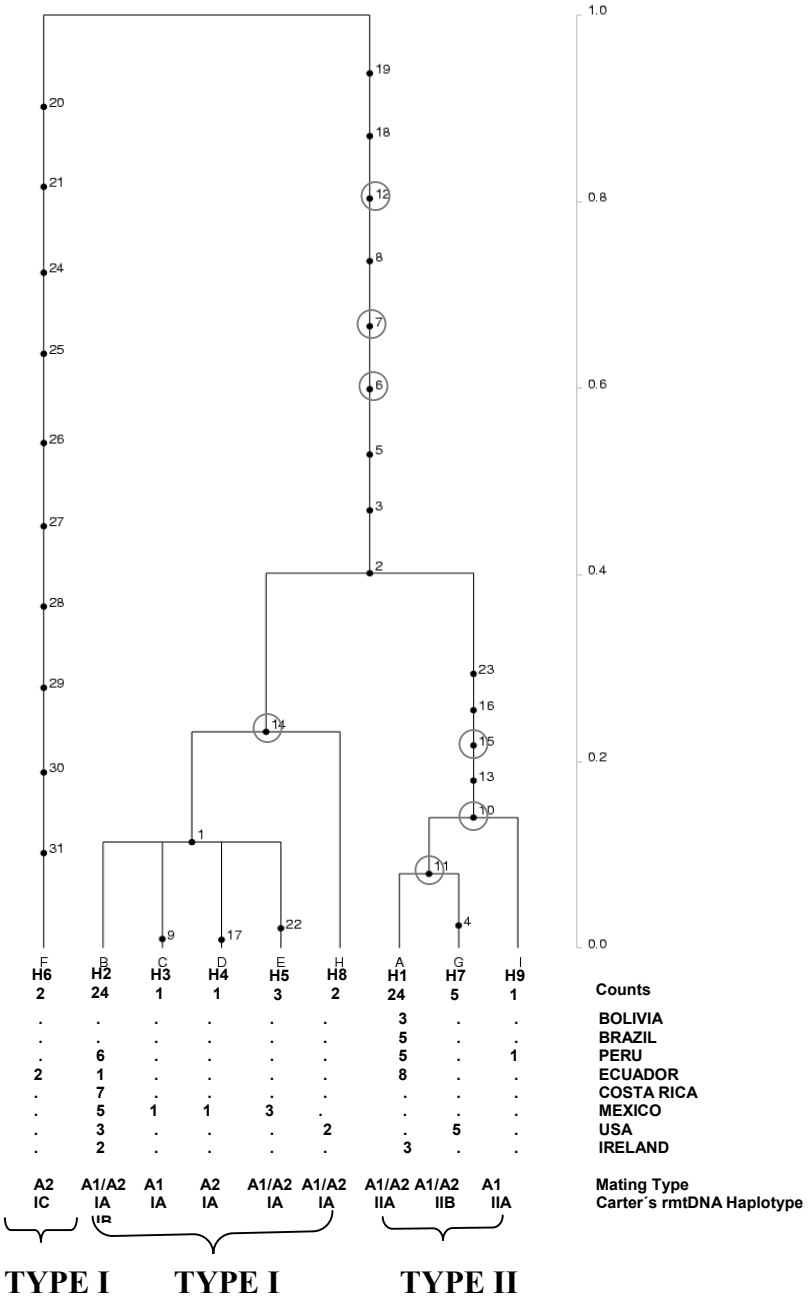
600bp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5
3
27
2
28
23
29
16
14
15
30
13
10
1
31
11
22
9
17
4
F B C D E H A G I
H6 H2 H3 H4 H5 H8 H1 H7 H9
2 24 1 1 3 2 24 5 1
. . . . . . 3 . .
. . . . . . 5 . .
. 6 . . . . 5 . 1
2 1 . . . . 8 . .
. 7 . . . .
. 5 1 1 3 . . . .
. 3 . . . 2 . 5 .
. 2 . . . . 3 . .
A2 A1/A2 A1 A2 A1/A2 A1/A2 A1/A2 A1/A2 A1
IC IA IA IA IA IA IIA IIB IIA
IB
TYPE I
TYPE I
TYPE II
Counts
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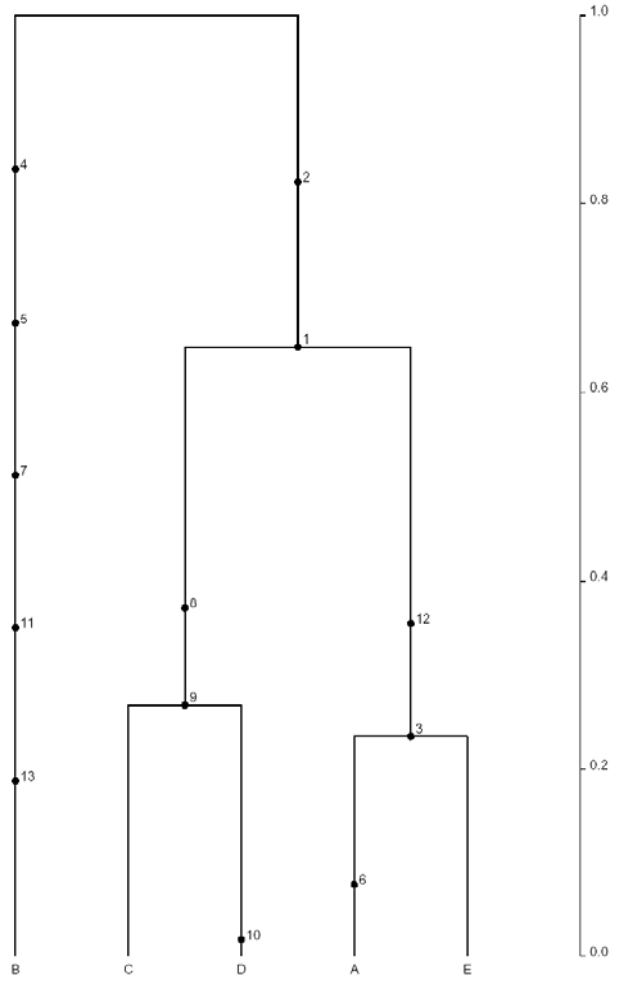
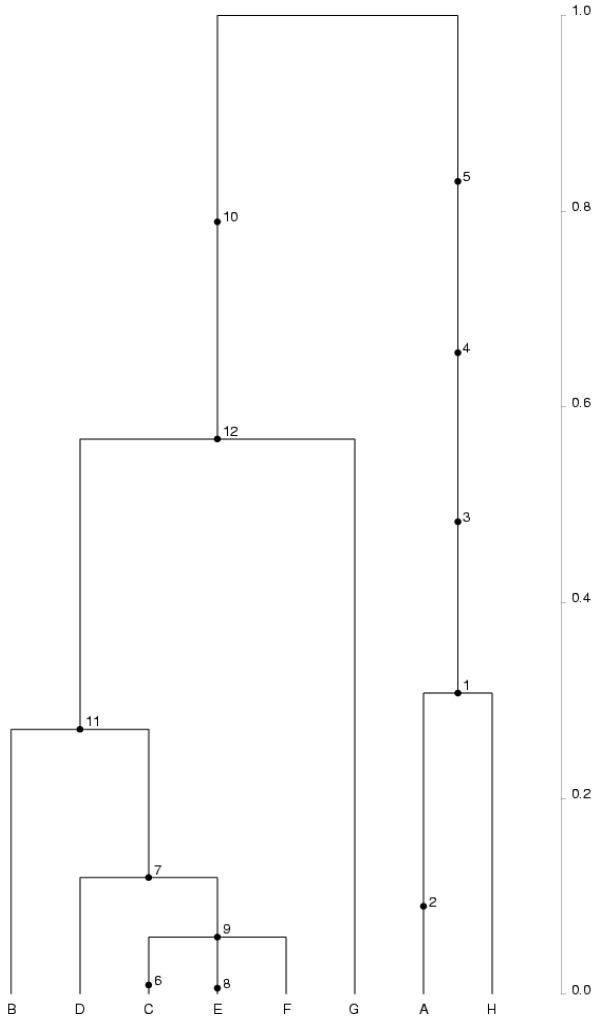
PERU
ECUADOR
COSTA RICA
MEXICO
USA
IRELAND
Mating Type
Carter´s rmtDNA Haplotype

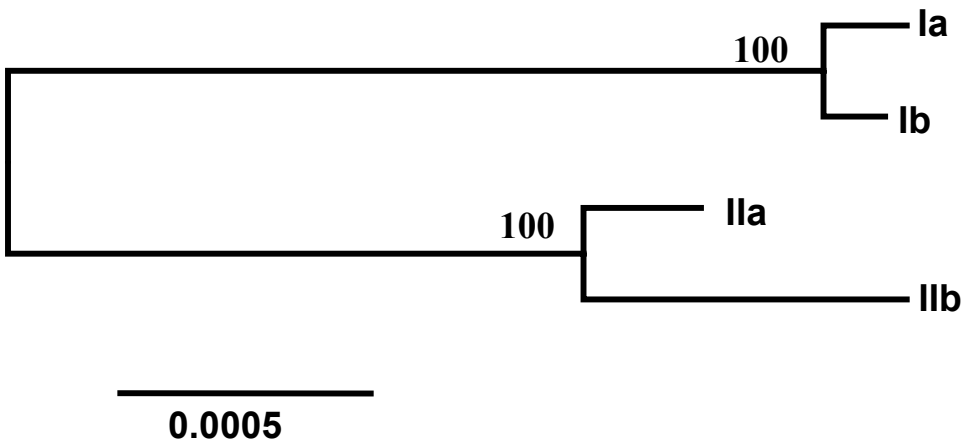


H2	H3	H4	H1	H5	Haplotype
5	138	3	19	1	Counts
.	6	.	6	.	Brazil
.	6	.	6	.	Bolivia
.	34	.	2	.	Peru
5	19	.	.	.	Ecuador
.	24	.	.	.	Costa Rica
.	23	3	.	.	Mexico
.	12	.	4	.	USA
.	14	.	1	1	Ireland
A2	A1/A2	A1/A2	A1/A2	A1	Mating Type
Ic	I / II	Ia	I / II	Ia	Carter's mtDNA Haplotype



H2	H4	H3	H5	H6	H7	H1	H8	Haplotype
94	15	2	1	20	1	19	1	Counts
6	.	.	.	.	.	6	.	Bolivia
6	.	.	.	.	.	6	.	Brazil
18	7	.	.	9	.	2	.	Peru
8	3	.	.	4	.	.	.	Ecuador
24	.	.	.	.	.	.	.	Costa Rica
13	3	.	1	6	.	.	.	Mexico
7	1	2	.	.	.	4	.	USA
12	1	.	.	1	1	1	.	Ireland
A1/A2	A1	A1/A2	A1	A1/A2	A1	A1/A2	A1	Mating Type
I/II	I/II	I	I	I/II	I	I/II	I	Carter's mtDNA Haplotype

Appendix



CHAPTER TWO

MOLECULAR EVIDENCE SUPPORTING THE PRESENCE OF A NEW SPECIES CLOSELY RELATED TO *PHYTOPHTHORA INFESTANS* IN THE HIGHLANDS OF ECUADOR

Abstract

Recent evidence indicates that the genetic variability of *Phytophthora infestans* sensu lato in the Andean Highlands of South America is higher than previously realized. In Ecuador, three clonal lineages (US-1, EC-1, EC-3) and one heterogeneous group (EC-2) were found in association with different host species in the genus *Solanum*. The EC-2 group harbors two mitochondrial (mtDNA) haplotypes, Ia and Ic. Isolates of *P. infestans* sensu lato EC-2 and EC-3 groups fit the morphological description of *P. infestans* but are quite different from any genotypes of *P. infestans* described to date, raising questions about their taxonomic status and relatedness to isolates of *P. infestans* associated with potato and tomato. The level of intraspecific variation and the relatedness of Ecuadorian isolates of *P. infestans sensu lato* (EC-1 and EC-2) and other *Phytophthora* species was examined using the *P. infestans*-specific primers PINF, restriction fragment length polymorphim of the internal transcribed spacer (ITS) region of the ribosomal DNA, and DNA sequences of a portion of the mitochondrial-encoded *cox 1* gene and the intron 1 of the nuclear single-copy gene *Ras*. The PINF specific primer amplified isolates of *P. infestans* sensu lato from Ecuador. The restriction fragment lenght patterns were identical in isolates of P

infestans sensu lato, EC-1 and EC-2, and to *P. infestans*. Phylogenetic analysis of the mitochondrial and nuclear loci indicated a unique position for the isolates from *S. tetrapetalum* (EC-2 Ic mtDNA). They formed a distinct branch in the same clade with *P. infestans* and other closely related species: *P. mirabilis*, *P. phaseoli* and *P. ipomoeae*, and were identical to the newly described species *P. andina* from *S. muricatum*. These results suggest that the EC-2 (Ic mtDNA haplotype) group is identical to *P. andina*. Ecuadorian isolates of *P. infestans* sensu lato from S. *columbianum*, *S. paucijugum*, *S. phureja*, and *S. tuquerense* (EC-1 clonal lineage) were confirmed as *P. infestans* based on molecular and phylogenetic analyses.

Introduction

The genus *Phytophthora* is classified within the diploid, algae-like Oomycetes in the Straminopile clade of the Chromista (Cavalier-Smith, 1986; Guderson *et al*. 1987; Yoon *et al*. 2002). This genus contains about 60 to 70 species that occupy a variety of terrestrial and aquatic habitats (Martin and Tooley, 2003; Kroon *et al*. 2004) and many are responsible for economically important diseases of a wide range of agronomic and ornamental crops (Erwin and Ribeiro, 1996).

Identification of *Phytophthora* species has been traditionally based on the taxonomic scheme developed by Waterhouse (1963) and later improved by Newhook *et al*. (1978) and Stamps *et al*. (1990). This taxonomic scheme for the genus *Phytophthora* is based on differences in sporangium and oospore morphology. Key characters used include the degree of papillation of the sporangia and the nature of the antheridial attachment to the oogonium. Six morphological

groups (I to IV) were defined based on these characters and contain many of the better-known or biologically well-characterized species. Phylogenetic analyses based on nuclear and mitochondrial DNA sequence data indicate that the six taxonomic groups do not represent natural assemblages or reflect true genetic relationships within the genus *Phytophthora* (Cooke *et al*. 2000; Kroon et al. 2004, Martin and Tooley, 2003).

In addition to morphological approaches, other methods have been used to identify *Phytophthora* species, including protein patterns, isozymes, serology, restriction fragment length polymorphims (RFLP) analysis of nuclear and mitochondrial DNA, and more recently single-strand-conformation polymorphism (SSCP) and DNA sequence analyses of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) (Forster *et al*. 1990; Benson, 1991; Mills *et al*. 1991; Ersek et al. 1994; Ristaino *et al*. 1998; Kong *et al*. 2003; Martin and Tooley 2004) .

P. infestans (Mont.) de Bary, the causal agent of late blight disease, is one of the most devastating pathogens of potato and tomato worldwide and was responsible for the Irish potato famine in the 1840's. *P. infestans* is included in Waterhouse Group IV, which comprises homothallic and heterothallic species with amphigynous antheridia and semipapillate sporangia. Other members of this group are: *P. colocasiae*, *P. hibernalis*, *P. ilcis*, *P. mirabilis*, *P. phaseoli*, and *P. eriugena*. Recently, Cooke *et al*. (2000) reported a phylogenetic analysis of 50 *Phytophthora* species, based on sequence analysis of the internal transcribed spacer regions (ITS1 and ITS2) of the rRNA genes. This phylogeny is considered the most comprehensive to date (Martin and Tooley 2004). The genus is monophyletic comprising eight main lineages designated Clades 1-8. Clade 1 was further subdivided into Clade 1a, 1b and 1c. *P. infestans*

clustered in Clade 1c along with *P. mirabilis* and *P. phaseoli*, indicating that these species share a most recent common ancestor. The phylogenetic relationship among species in Clade 1c has been further confirmed based on sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes (Martin and Tooley 2003) and by a multi-gene based phylogeny using nuclear and mitochondrial genes (Kroon et al. 2004).

New *Phytophthora* species have been recently described and their taxonomic status has been clarified on the basis of morphology, physiology, and molecular relatedness (Cooke *et al*. 1999; Brasier *et al*. 2003; Jung *et al*. 2003). Flier *et al*. (2002) described a new species, *Phytophthora ipomoeae*, as the causal agent of leaf blight disease on *Ipomoea longipedunculata*, a morning glory native to the highlands of central Mexico (Toluca Valley). Phylogenetic analyses based on ITS (Flier *et al*. 2002) and nuclear and mitochondrial genes (Kroon *et al*. 2004) showed that is a member of the Clade 1c species. The Toluca Valley in central Mexicon is thought to be the center of origin of *P. infestans*, *P. mirabilis*, and *P. phaseoli*, and *P. ipomoeae* (Niederhauser 1991; Goodwin *et al*. 1992; Brasier and Hansen 1992, Flier *et al*. 2002). Flier *et al*. (2002) hypothesized that central highlands of Mexico are the center of origin of Waterhouse group IV *Phytophthora* species.

Recent evidence indicates that the Andean region of South America is more likely to be the center of origin of *P. infestans* (Chapter, I, Abad *et al*. 1995, 1997, 2002; Ristaino *et al*. 2001; May and Ristaino 2004; Adler *et al*. 2004) and that other lineages morphologically related to *P. infestans* are associated with wild *Solanum* (tuber-bearing and non tuber-bearing) species there (Chapter I, Ordoñez et al 2000; Adler *et al*. 2004). Three clonal lineages (US-1, EC-1, EC-3) and one heterogeneous group (EC-2) were found in association with different host species in

the genus *Solanum* and other plant genera (Table 2.1) in the Andean Highlands of Ecuador. Due to taxonomic uncertainties the populations of *P. infestans* from Ecuador are referred as *P. infestans* sensu lato. The EC-2 and EC-3 lineages fit the morphological description of *P. infestans*, but are quite different from any genotypes of *P. infestans* described to date, and are considered indigenous to the Andean highlands of South America (Adler *et al*. 2004).

The diversity in *P. infestans* sensu lato coming from the non tuber-bearing hosts is higher (Table 2.1), occurring principally among and not within host-specific groups. One exception is the group of isolates associated with the *Anarrhichomenum* complex (EC-2 lineage). Plants belonging to the *Anarrhichomenum* complex include *S. brevifolium*, *S. tetrapetalum*, *S. oblongifolium* and few other plants that do not fit the description of these species, although all are vines with roots growing from nodes and belong to the section *Anarrhichomenum* of the genus *Solanum* (Adler *et al*. 2004). Two distinct pathogen groups have been described for the EC-2 lineage. One has generally been associated with the *Anarrhichomenum* complex (particularly S. *brevifolium*, and S. *tetrapetalum*) and *S. muricatum* (pear melon), whereas the other is associated with the *Anarrhichomenum* complex (*S. oblongifolium*) and *Brugmansia sanguinea*. The first EC-2 group is characterized by the A2 mating type, Ic mtDNA haplotype and allozyme genotypes for glucose phosphate isomerase (*Gpi*) and peptidase (*Pep*), 100/100 and 76/100 respectively. The second EC-2 group is the A1 mating type, Ia mtDNA haplotype, Gpi 100/100 and Pep 76/100. Isolates in the EC-3 lineage attack cultivated tree tomato (*S. betaceum*) and this clonal lineage is characterized by A1 mating type, Gpi (86/100) and Pep (76/100) allozyme genotypes and the Ia mtDNA haplotype (Table 2.1).

The taxonomic status of the isolates assigned to the *Anarrhichomenum* complex (EC-2 lineage) and EC-3 lineage has not yet been cleraly resolved (Adler *et al*. 2004). Molecular and phylogenetic analyses can help to clarify and elucidate the relatedness of these isolates to *Phytophthora infestans* and closely related species. Some evidence has already been presented that members of the EC-2 group might belong to a new *Phytophthora* species. Wattier *et al*. (2003) included two isolates of *P. infestans sensu lato* from *S. brevifolium* in a study of sequence variation of five intergenic mitochondrial DNA spacers of *P. infestans* and related species (*P. mirabilis*, *P. phaseoli*, *P. ipomoeae*). These two isolates from *S. brevifolium* were different from the isolates of *P. infestans* from potato. The isolates presented identical nucleotide sequences but were polymorphic for 45 nucleotides within the five intergenic spacers when compared to the reference sequence from the US-1 (Ib mtDNA haplotype) (Lang *et al*. 1993). Both isolates from *S. brevifolium* were also highly polymorphic when compared to *P. ipomoeae*, *P. mirabilis*, and *P. phaseoli*.

Kroon *et al*. (2004) included a group of isolates from *S. muricatum* from Ecuador in a phylogenetic study of 48 *Phytophthora* species based on nuclear (Translation Elongation Factor 1α and β-Tubulin) and mitochondrial (*cox 1* and NADH dehydrogenase) genes. Kroon *et al*. (2004) named the Ecuadorian isolates *P. andina* and an official description of the species is in preparation (personal communication). The Ecuadorian isolates formed a new branch on both nuclear and mitochondrial phylogenies and clustered together with Clade 1c species: *P. infestans*, *P. ipomoeae*, *P. mirabilis*, and *P. phaseoli*. Kroon *et al*. (2004) also suggested that *P. andina* could be of hybrid origin and that *P. infestans* might be one of the parental species.

In our study of the population history of *P. infestans* (Chapter I), we included five isolates of *P. infestans sensu lato* from *S. tetrapetalum* from Ecuador. These isolates were highly polymorphic across the nuclear (Ras and B-tubulin genes) and mitochondrial (*rpl14, rpl5, tRNAs and cox 1*) loci analyzed. These isolates formed an independent lineage and were different from the lineages associated with potato and tomato isolates in the mitochondrial and nuclear genealogies. The objective of this research was to study the phylogenetic relationship of this group of isolates of *P. infestans sensu lato* from *S. tetrapetalum* with other *Phytophthora* species, particularly with species in the Clade 1c.

Materials and Methods

Isolates

Ecuadorian isolates of *P. infestans sensu lato* from different hosts and clonal lineages used in this study (Table 2.2) were kindly provided by Drs. Nicole Adler, Wilber Flier and Gregg A. Forbes, International Potato Center, Quito Ecuador. *Phytophthora* species used for the phylogenetic analysis, their origins and collection number are given in Table 2.2. Cultures were grown and maintained on Rye-V8 medium at 18?C.

Preparation of DNA

Isolates were grown in 2 ml Eppendorf tubes containing pea broth (120 g frozen garden peas boiled for 10 min in 1 liter distilled water) for 1 to 2 weeks at 18?C. The mycelium was harvested by centrifugation at 13000 rpm, rinsed in sterile distilled water, dried, and either stored

at -20?C or used immediately for DNA extraction. Total DNA was extracted from the mycelium by using a cetyltrimethylammonium bromide (CTAB, Sigma Chemical Co., St. Louis, MO, USA) protocol (Ristaino et al. 2001). DNA was diluted 1:10 or 1:100 and stored at -20?C until use.

Identification of Ecuadorian isolates

Two polymerase chain reaction (PCR) assays were used to determine the relatedness of the isolates of *Phytophthora infestans* sensu lato from Ecuador with other isolates of *P. infestans* and the closely related species *P. mirabilis*. One involved the use of a *P. infestans*–specific primer, PINF, (Trout *et al*. 1997), which in combination with the ITS5 primer (White *et al*. 1990) yields an approximately 600-bp product specific to *P. infestans* (Trout *et al*. 1997). The second PCR assay consisted of a PCR and restriction fragment length polymorphim (RFLP) analysis of ribosomal DNA (rDNA) amplified with ITS4 and ITS 5 (Ristaino *et al*. 1998) for species identification in the genus *Phytophthora*. Both protocols were conducted according to the methods described by Trout *et al*. (1997) and Ristaino *et al*. (1998). Briefly, PCR was conducted in a 50-µl reaction volume using thin-walled 0.2-ml tubes. One microliter of DNA template (1:10 or 1:100 dilution) was added to a 49-µl master reaction mixture containing 5 µl of 10x PCR buffer (100 mM Tris-HCl, 15mM $MgCl_2$, and 500mM KCl, pH 8.3), 36.6 µl of sterile distilled H_2O, 1 µl of 10 mM $MgCl_2$, 2 µl of 2mM dNTPs, 2 µl of 10 µM of each forward and reverse primers (PINF and ITS5 or ITS4 and ITS5, respectively), and 0.4 µl of *Taq* polymerase (5 U/µl; Invitrogen, Carlsbad, CA). All reactions were overlaid with sterile mineral oil prior to

thermal cycling. Thermal cycling parameters were initial denaturation at 96?C for 2 min, followed by 35 cycles consisting of denaturation at 96?C for 1 min, annealing at 55?C for 1 min, and extension at 72?C for 2 min. A final extension at 72?C for 10 min followed. Electrophoresis of amplified products was conducted on 1.6% agarose gels containing ethidium bromide at 0.5 μg/ml with 1x Tris-borate EDTA (TBE) running buffer. A 100-bp DNA ladder was included in each gel as a molecular size standard. Gels were visualized under UV light. The 946-bp PCR product obtained was digested using the restriction enzymes *AluI*, *MspI* and *RsaI*. Ten μl of the PCR product was digested with 1 unit of the restriction enzyme for 4 h at 37?C and then at 65?C for 10 min. Individual digested products were resolved by electrophoresis in 1.6% agaraose gels containing ethidium bromide as described above.

Mitochondrial DNA (mtDNA) haplotypes of the isolates of *P. infestans* sensu lato from Ecuador, EC-1 and EC-2 groups, were determined according to Griffith and Shaw (1998) method. PCR and digested products were resolved by electrophoresis in 1.6% agarose gel containing ethidium bromide in TBE buffer.

DNA amplification and sequencing

Portions of one single copy gene, *Ras*, and one mitochondrial gene (*cox1*) were amplified and sequenced. The portion of the *Ras* gene amplified and sequenced consists of intron 1 (223-bp) located in the 5' untranslated region of the gene (Figure 1.1 Chapter I, Chen and Roxby, 1996). Intron Ras primer pair sequences were as follows: IntronRas F 5' TTGCAGCACAACCCAAGACG 3' and IntronRas R 5' TGCACGTACTATTCGGGGTTC 3'. Primer pair F4/R4 (Griffith and Shaw 1998) were used to amplify and sequence the P4 region

containing a portion of *cox 1* gene. Primers IntronRas F/R and F4/R4 produce PCR fragments of 964 bp and 349 bp, respectively. For each primer set, two 50-µl reactions were carried out per individual. Each reaction contained 1x PCR buffer (Invitrogen, Carlsbad, CA), 2.75 mM $MgCl_2$, 1 unit *Taq* polymerase (Invitrogen), 200 µM each dNTP, 0.4 µM each forward and reverse primer, 160 µg/µl BSA (20 mg/ml, Roche, Mannheim, Germany) and 5-10 ng genomic DNA. Cycling conditions for the P4 region were as follows: 94?C (1.5 min); then 40 cycles of 94?C (40 sec), 55?C (1 min), 72?C (1.5 min); and a final extension of 72?C (10 min); and for the Intron Ras, 96?C (1 min); then 35 cycles of 96?C (1 min), 55?C (1 min), 72?C (2 min); and a final extension of 72?C (10 min). The PCR products were pooled and purified with QIAquick PCR Purification Kits (QIAGEN, Valencia, CA). Purified fragments were sequenced directly on both strands by using the same primers as those used in the intial amplification. Sequencing reactions were prepared using the ABI PRISM? BigDye Terminator Cycle Sequencing ready Reaction Kit and analyzed on an ABI PRISM? 377 automated sequencer (Applied Biosystem, Foster City, CA).

Sequence data for the P4 region (including part of *cox 1*) and Intron Ras were obtained from all the Ecuadorian isolates (Table 2.2). Intron Ras sequence data were also obtained for different *Phytophthora* species (Table 2.3). Sequences from the c*ox 1* from various *Phytophthora* species and *Pythium aphanidermatum* (outgroup) were obtained from GenBank (Kroon et al. 2004; Martin and Tooley 2003).

Phylogenetic analysis

All sequences were aligned using Clustal X (Thompson *et al*. 1997) with subsequent visual adjustment and edition in BioEdit (Hall, 1999). All polymorphisms were rechecked from the chromatograms. Sites showing the presence of two coincident peaks in the forward and reverse sequence chromatograms were observed for Intron Ras, indicating heterozygous sites and were labeled according to the IUPAC coding system.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar *et al*. 2001). Phylogenetic relationships among *Phytophthora* species were inferred by maximum-parsimony (MP) analysis with a heuristic tree search and Neighbor-joining (NJ) tree reconstruction, using the Kimura 2-K parameter correction method (Kimura 1980). Bootstrapping (1000 replicates) was performed to obtain a relative measure of node support for the resulting trees (Felsenstein 1985).

Results

The five Ecuadorian isolates from *Solanum tetrapetalum* were of the Ic mtDNA haplotype (Table 2.1) and confirmed that the isolates belonged to the EC-2 lineage (Ordoñez *et al*. 2000). Ecuadorian isolates from other hosts were of the IIa mtDNA haplotype (Table 2.1) and belonged to the EC-1 clonal lineage (Forbes *et al*. 1997).

Ecuadorian isolates of *P. infestans sensu lato*, *P. infestans* and *P. mirabilis* yielded the expected product of approximately 600 bp in size when amplified with PINF/ITS5 primers (Figure 2.1). Restriction digests of ITS + 5.8S rDNA amplified with ITS 4 and ITS5 primer pair from Ecuadorian isolates, including isolates from *S. tetrapetalum*, yielded identical PCR-RFLP patterns with restriction enzymes *RsaI* (Figure 2.2A), *MspI* (Figure 2.2B), and *AluI* (Figure 2.2C) and were identical to those found in isolates of *P. infestans* and *P. mirabilis*.

The nuclear (Intron Ras) and mitochondrial (*cox 1*) sequences of the five Ecuadorian isolates from *S. tetrapetalum* were identical and clearly different from those of isolates of *P. infestans* from *S. tuberosum*, and isolates of *P. infestans sensu lato* from *S. phureja*, *S. columbianum*, *S. paucijugum* and *S. tuquerense* from Ecuador and all other species of *Phytophthora* examined. Nine heterozygous sites were found in the Intron Ras sequence (223-bp) of isolates of *P. infestans sensu lato* from *S. tetrapetalum*. No heterozygous sites were observed for Ecuadorian isolates of *P. infestans sensu lato* from other hosts or from the isolates of *P. infestans* from *S. tuberosum* or *P. mirabilis* used here. Six of the nine heterozygous sites found in the Intron Ras of *S. tetrapetalum* isolates may have originated from hybridization between *P. infestans* and *P. mirabilis* (Table 2.4). Four heterozygous sites were observed in the Intron Ras sequence of isolates of *P. infestans* from Brazil, Bolivia, Peru, USA, and Ireland

(Chapter I) and two of them (sites 605 and 619 in Genbank accession number U30474) were found in the Ecuadorian isolates from *S. tetrapetalum*.

Both Intron Ras (Figure 2.3) and *cox 1* (Figure 2.4) phylogenies support the designation of the Ecuadorian isolates from *S. tetrapetalum* as a new species closely related to *P. infestans* and the other members of the Clade Ic (Cooke *et al*. 2000). The Ecuadorian isolates from *S. tetrapetalum* were in the same branch with the newly described species *P. andina* found in Ecuador (Kroon *et al*. 2004) for *cox 1* phylogeny (Figure 2.4). Isolates of *P. infestans sensu lato* from other *Solanum* species from Ecuador were identical to and clustered with isolates of *P. infestans* for both nuclear and mitochondrial phylogenies, indicating that they are true isolates of *P. infestans*.

Phylogenetic relationships among *Phytophthora* species based on Intron Ras (Figure 2.3) reflected the groupings previously reported based on ITS (Cooke et al. 2000) and showed minor differences with the phylogeny based on the combined analysis of nuclear encoded genes B-tubulin and Elongation Factor-1 (Kroon *et al*. 2004).

Discussion

Isolates of *P. infestans* sensu lato in the EC-2 (Ic mtDNA haplotype) clonal lineage from *S. tetrapetalum* formed a divergent lineage from *P. infestans* and were distinct from true isolates of *P. infestans* based on mitochondrial and nuclear DNA sequences (Chapter I). The lineage formed by isolates from *S. tetrapetalum* fits the morphological description of *P. infestans* raising questions about its taxonomic status (Chapter I; Adler *et al*. 2004)

PCR amplification with the PINF/ITS5 primer combination did not differentiate the Ecuadorian isolates from *S. tetrapetalum* (EC-2) and other *Solanum* species (EC-1) with *P. infestans* from potato or *P. mirabilis* (Figure 2.1). The PINF primer is considered a *P. infestans*-specific primer on potatoes (Trout *et al*. 1997). In addition to *P. infestans* only isolates of *P. mirabilis* and *P. cactorum* were amplified by the PINF/ITS5 primer combination, previously (Trout *et al*. 1997). *P. mirabilis* is closely related to *P. infestans* species and a member of Clade Ic, which includes also *P. andina*, *P. ipomoeae* and *P. phaseoli* (Goodwin *et al*. 1999; Cooke *et al*. 2000; Kroon et al. 2004). *P. mirabilis* was described as a new species in 1985 (Galindo and Hohl, 1985). There has been controversy around the two species designations until recently when isozyme and RFLP demonstrated that *P. mirabilis* and *P. infestans* were unique and distinguishable species (Goodwin *et al*. 1999, Martin and Tooley, 2004). Trout *et al*. (1997) did not include other members of the Clade Ic (e.g. *P. phaseoli*) in the PINF/ITS5 specificity tests.

Restriction digest of the internal transcribed spacer (ITS + 5.8S rDNA) region of the ribosomal DNA (rDNA) also did not separate isolates of *P. infestans* sensu lato from Ecuador from *P. infestans* or *P. mirabilis* (Figure 2.2), indicating high ITS-sequence similarity. (Trout *et al*. 1997). ITS-sequence similarity between *P. infestans* and closely related species *P. mirabilis*,

P. phaseoli, *P. ipomoeae*, and *P. andina* has also been reported by others to be 99.9% (Kroon et al. 2004).

Nuclear and mitochondrial phylogenies (Figure 2.3 and 2.4) support the designation of Ecuadorian isolates from *S. tetrapetalum* (EC-2 Ic mtDNA haplotype) as a new species closely related to *P. infestans* and the other species of the Clade Ic (Cooke et al. 2000). Isolates of *P. infestans sensu lato* from *S. phureja*, *S. columbianum*, *S. paucijugum* and *S. tuquerense* from Ecuador, EC-1 clonal lineage (Table 2.1), were in the same branch with the isolate of *P. infestans* (US-1 genotype) from *S. tuberosum*, meaning that they are true isolates of *P. infestans*.

S. tetrapetalum isolates clustered with *P. andina*, a recently described species (Kroon et al. 2004), with a boostrap support value of 97%, in the *cox 1* phylogeny (Figure 2.4). It is therefore likely that these isolates are *P. andina*. The Ecuadorian isolates used by Kroon *et al*. (2004) to name *P. andina* were from *S. muricatum*. Wattier *et al*. (2003) sequenced an intergenic region found in the P4 region (*cox 1* gene) from Ecuadorian isolates from *S. brevifolium*. Their sequence data also matched our data (100%) for that region. *S. tetrapetalum*, *S. muricatum* and *S.brevifolium* are members of the *Anarrhichomenum* complex attacked by the EC-2 group (Table 2.1). It is possible that the other EC-2 lineage (Ia mtDNA, A1; and *Gpi* (100/100) and *Pep* (76/100)) that has been associated with other members of the *Anarrhichomenum* complex and *B. sanguinea* (Adler *et al*. 2004) is another species but this needs further confirmation. The molecular and phylogenetic approaches described here could elucidate the taxonomic status of this lineage and the EC-3 clonal lineage (Adler *et al*. 2004).

Flier *et al*. (2002) hypothesized that the central highlands of Mexico, which include the Toluca Valley, were the center of origin of the Waterhouse group IV *Phytophthora* species and a

speciation "hot-spot". The highlands of central Mexico are the presumed center of origin of *P. infestans*, *P. mirabilis*, *P. phaseoli* and the newly described species *P. ipomoeae* (Niederhouser 1991, Goodwin *et al*.1992; Brasier and Hansen, 1992; Flier *et al*. 2003). These species show host specificity in the central highlands of Mexico, with *P. infestans* associated with solanaceous hosts (potato and wild *Solanum*) and *P. mirabilis*, *P. phaseoli*, and *P. ipomoeae* associated with non-solanaceous hosts: *Mirabilis halapa* ("four o'clock"), *Phaseolus*; and *Ipomoea longipedunculata*, respectively. It was suggested that these species might have undergone sympatric speciation in the Toluca Valley (Flier *et al*. 2003; Goodwin *et al*. 1999). The EC-2 group (Ic mtDNA haplotype, *P. andina*) is indigenous to the Andean highlands of South America (Adler *et al*. 2004) thousands of kilometers from the central highlands of Mexico, which raises questions about the center of origin of these closely related species as well as the historical migration route of species in Clade Ic throughout Central and South America (Kroon *et al*. 2004).

Our data suggest that the Andean Highlands of South America are the center of origin of *P. infestans* (Chapter I), supporting the Andean theory, first proposed by Berkeley (1846) and latter supported and documented by Abad *et al*. (1995, 2002) and Abad and Abad (1997). No evidence for host-specificity has been observed in *P. infestans* populations in the Toluca Valley. The same pathogen attacks both cultivated potatoes and wild *Solanum* (Grundwald *et al*. 2001), although only limited gene flow exists between the subpopulations of the pathogen on wild *Solanum* spp. and the cultivated potatoes (Flier *et al*. 2003). In contrast, in the Andean highlands of South America, a high level of host-specificity has been observed in *P. infestans sensu lato* populations. At least four clonal lineages have been described in Ecuador associated with

different hosts in the genus *Solanum* and other hosts (Adler *et al*. 2004). Host specificity seems to be the driving force for maintaining genetic isolation between these subpopulations. Isolates of *P. infestans* obtained from hosts of the *Petota* section (tuber bearing *Solanum*) do not infect hosts in the *Anarrhichomenum* complex (hosts of Ic mtDNA EC-2 group), and vice versa (Adler *et al*. 2004). In addition, postmating mechanisms of reproductive isolation have been documented in subpopulations of *P. infestans sensu lato* in Ecuador (Oliva *et al*. 2002). The evolution of several *Phytophthora* species appears to be associated with host specialization (Cooke *et al*. 2000).

The presence of several lineages closely related to *P. infestans* and associated with solanaceous and non solanaceous hosts suggest the presence of an ancient population of *P. infestans* in the Andean region of South America (Chapter I, Adler *et al*. 2004) and support this region as a speciation "hot-spot". Interestingly, *M. jalapa*, host of *P. mirabilis*, is a native plant from South America and in addition to "four o'clock" is also known as "Marvel of Peru". There are no reports of *Phytophthora* like pathogens on this plant in South America, but few have looked for disease on this host in Latin America. The South American Andes are also the center of diversity for the genera *Ipomoea* (Rajapakse *et al*. 2004) and *Phaseolus* (Gepts, 1998), whose members are hosts of *P. ipomoeae* and *P. phaseoli*, respectively. This opens the possibility that these pathogens also originated in South America and migrated with their hosts to other regions in the past. Flier *et al*. (2003) found that the mitochondrial haplotype associated with *P. ipomoeae* was the Ic mtDNA described previously by Ordonez *et al*. (2000) for the isolates of *P. infestans sensu lato* of the EC-2 group (Ic mtDNA). This suggests a common origin of the *Phytophthora* species from these hosts.

Kroon *et al*. (2004) postulated a hybrid origin of *P*. *andina* (Ic mtDNA EC-2 group) because the presence of several heterozygous sites in the sequences of nuclear genes, and indicated that *P*. *infestans* is one of the parental species. The origin of new *Phytophthora* species through interspecific hybridization has been documented (Brasier *et al*. 1999). The Intron Ras sequence (223-bp) of the *S*. *tetrapetalum* isolates (*P*. *andina*) has nine heterozygous sites (Table 2.4). Other Ecuadorian isolates of *P*. *infestans* from different hosts were homozygous for all sites, suggesting *P*. *infestans* is a putative parent. Interestingly, *P*. *mirabilis* isolates were also homozygous for all sites but polymorphic in the same six sites compared to *P*. *infestans*. Hybridization between *P*. *infestans* and *P*. *mirabilis* might have resulted in the heterozygous sites found in the isolates from *S*. *tetrapetalum* (Table 2.4).

Phylogenetic relationships between *Phytophthora* species are based mainly on sequence data of the rDNA internal transcribed spacers (ITS) (Cooke *et al*. 2000), however, some *Phytophthora* species cannot be resolved based on ITS data, particularly closely related ones. For example *P*. *infestans*, *P*. *mirabilis*, and *P*. *phaseoli* are poorly resolved with ITS data. Mitochondrial genes (*cox 2 and cox 1*) (Martin and Tooley 2003) and multi-gene based phylogenies (Kroon *et al*. 2004) have been published and these and other groups were clearly differentiated. Both phylogenies were largely in congruence with the ITS phylogeny described by Cooke *et al*. (2000). Here we used sequence data from the intron 1 (223-bp) found in the *Ras* gene, a single-copy gene (Chen and Roxby, 1996). Although we covered only a limited subset of *Phytophthora* species, the groupings recovered are congruent with those found using other nuclear and mitochondrial sequences (Cooke *et al*. 2000, Martin and Tooley 2003; Kroon *et al*. 2004). Our data indicate that this intron Ras region might have enough variation to be used as

another tool for reconstructing the evolutionary history in the genus *Phytophthora* and deserves further testing on more *Phytophthora* species. Exons and introns in genes are subjected to different evolutionary selection pressures. Mutations in introns are less likely to impact gene function than mutations in exons. Therefore, introns accumulate more mutations, while the mutation rates of the coding exon sequences are constrained (Rajapakse *et al*. 2004). Most *Phytophthora* genes lack introns, however the *Ras* gene (Chen and Roxby, 1996) and *NiaA* gene, a gene encoding nitrate reductase (Pieterse *et al*. 1995), are notable exceptions (Ospina-Giraldo and Jones, 2003).

Species recognition is traditionally based on morphology (morphological species recognition or MSR); mating or reproductive behavior (biological species recognition or BSR); and DNA sequence divergence (phylogenetic species recognition or PSR) (Taylor *et al*. 2000). Every approach has its limitations and advantages, therefore congruence among them give strong support to the creation of a new species. Ecuadorian isolates of the EC-2 group (Ic mtDNA) fit the morphological description of *P. infestans*. However, Oliva *et al*. (2002) and Adler *et al*. (2004) demonstrated the existence of pre- and post- mating isolating mechanisms between EC-2 isolates and other *P. infestans* isolates. Phylogenetic analyses presented in this study and by others (Kroon *et al*. 2004), based on different nuclear and mitochondrial genes, show that these isolates form an independent lineage closely related to *P. infestans* and other species of the Clade Ic (Cooke *et al*. 2002). Therefore both BSR and PSR strongly support this group as a new species: *Phytophthora andina*, a name given previously by Kroon *et al*. (2004) for relate isolates from *S. muricatum* from Ecuador.

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Table 2.1 Diversity of *P. infestans* sensu lato on *Solanaceous* plants in Ecuador (Adler et al. 2004)

Lineage	mtDNA Haplotype[a]	Mating Type	Allozyme Genotype[b] *Gpi*	Allozyme Genotype[b] *Pep*	Hosts
US-1	Ib	A1	86/100	92/100	Tomato (*S. lycopersicon)*, *S. ochrantum*, *S. caripense* pear melon *(S. muricatum), S. andreanum*[c]
EC-1	IIa	A1	90/100	96/100	*Potatoes (S. tuberosum*, *S. phureja)*, and wild potatoes (*Solanum* spp. in the section Petota, tuber-bearing species)
EC-2	Ic	A2	100/100	76/100	*Anarrhichomenum*-complex[d], pear melon (*S. muricatum)*
EC-2[e]	Ia	A1	100/100	76/100	*Anarrhichomenum*-complex[f], *Brugmansia sanguinea*
EC-3	Ia	A1	86/100	76/100	Tree tomato (*S. betaceum)*

[a] Mitochondrial DNA haplotype determined by methods of Griffith and Shaw (1998)

[b] *Gpi* = Glucose-6-phosphate isomerase, *Pep* = Peptidase

[c] S. *andreanum* is a tuber-bearing species in the section Petota. It is also attacked by isolates of the EC-1.

[d] *Anarrhichomenum-complex* includes *S. brevifolium*, and *S. tetrapetalum*, and other species not clearly identified.

[e] Have the same RFLP fingerprint as EC-2 but differ for other markers (Adler et al. 2004)

[f] *Anarrhichomenum-complex* includes *S. oblongifolium*, and other species not clearly identified

Table 2.2 Isolates of *P. infestans* sensu lato from Ecuador and *P. infestans* used in this study.

Isolate Code	Host	Mating Type	mtDNA Haplotype	Allozyme Genotype *Gpi*	Allozyme Genotype *Pep*	Clonal Lineage	Source
EC3090	*Solanum phureja*	A1	IIa	90/100	96/100	EC-1	CIP-Ecuador
EC3094	*Solanum phureja*	A1	IIa	90/100	96/100	EC-1	CIP-Ecuador
EC3154	*Solanum andreanum*	A1	IIa	90/100	96/100	EC-1	CIP-Ecuador
EC3163	*Solanum tetrapetalum*	A2	Ic	100/100	76/100	EC-2	CIP-Ecuador
EC3164	*Solanum tetrapetalum*	A2	Ic	100/100	76/100	EC-2	CIP-Ecuador
EC3165	*Solanum tetrapetalum*	A2	Ic	100/100	76/100	EC-2	CIP-Ecuador
EC3166	*Solanum tetrapetalum*	A2	Ic	100/100	76/100	EC-2	CIP-Ecuador
EC3167	*Solanum tetrapetalum*	A2	Ic	100/100	76/100	EC-2	CIP-Ecuador
EC3199	*Solanum tuquerense*	A1	IIa	90/100	96/100	EC-1	CIP-Ecuador
EC3253	*Solanum columbianum*	A1	IIa	90/100	96/100	EC-1	CIP-Ecuador
EC3300	*Solanum paucijugum*	A1	IIa	90/100	96/100	EC-1	CIP-Ecuador
94-1	*Solanum tuberosum*	A1	Ib	86/100	92/100	US-1	NCSU

CIP, International Potato Center
NCSU, North Carolina State University

Table 2.3 Isolates of *Phytophthora* spp. used in this study and GenBank accession numbers for *cox1* sequences

Species Name	Group[a]	Isolate Number[b]	GenBank[c]
Phytophthora andina	__	EC3421^{K}	AY564160
Phytophthora botryosa	II	CBS533.92^{K}, P44	AY564166
Phytophthora cactorum	I	NY568^{MT}, P6183^{K}, P7^{G}, P59^{G}	AY129174, AY 564167
Phytophthora capsici	II	302^{MT}, P8^{G}, P30^{G}	AY129166
Phytophthora citricola	III	P1817^{K}, P33^{G}, P53^{G}	AY564170
Phytophthora citrophthora	II	CBS274.33^{K}, P96^{G}, P132^{G}	AY564171
Phytophthora colocasiae	IV	P1696^{MT}	AY129173
Phytophthora gonapodyides	IV	NY353^{MT}	AY129175
Phytophthora hibernalis	IV	P3823^{MT}	AY129170
Phytophthora ilicis	IV	P3939^{MT}	AY129172
Phytophthora inflata	III	IMI 342898^{K}, P122^{G}	AY564187
Phytophthora ipomoeae	__	Pic99129^{K}	AY564158
Phytophthora meadii	II	CBS219.88^{K}, P74^{G}, P75^{G}	AY564192
Phytophthora megakarya	II	IMI 337098^{K}, P42^{G}	AY564193
Phytophthora mirabilis	IV	P3007^{MT}	AY129175
Phytophthora nicotianae	II	332^{MT}, P582^{K}	AY129169, AY564196
Phytophthora palmivora	II	CBS236.30^{K}, P26^{G}, P65^{G}	AY564197
Phytophthora parasitica[d]	II	P21^{G}, P70^{G}	
Phytophthora phaseoli	IV	330^{MT}, CBS556.88^{K}	AY129168, AY564159
Phytophthora pseudotsugae	I	H270^{MT}	AY129167
Phytophthora syringae	III	IMI 1045169^{K}, P35^{G}	AY564203
Phytophthora tropicalis	__	PD97/11132^{K}, P27^{G}, P118^{G}	AY564161
Outgroup species			
Pythium aphanidermatum			AY564163

[a] Waterhouse morphological group (Waterhouse 1963).

[b] Isolate number according to Kroon et al (2004) (K), Martin and Tooley (2003) (MT), and M. Gallegly (M), Virginia Tech University. Isolates provided by M. Gallegly were sequenced for Intron Ras.

[c] Sequeces deposited by Kroon et al. (2004) or Martin and Tooley (2003).

[d] *Phytophthora parasitica* = *Phytophthora nicotianae*, we received them as *P. parasitica*.

Table 2. 4 Heterozygous (two bases same site) and homozygous (one base same site) sites within the 223-bp of Ras Intron 1 region of isolates of *P. infestans*, *P. infestans sensu lato* from *S. tetrapetalum* from Ecuador and *P. mirabilis*.

	Site[a]								
	519	551	589	593	598	603	610	688	706
P. infestans	G	C	C	A	A	A	A	C	G
S. tetrapetalum isolates[b]	G/A	C/T	A/C	A/G	A/C	A/C	A/G	C/T	G/A
P. mirabilis	G	T	A	G	C	C	G	C	G

[a] Numbering is that of GenBank accession No U30474 (Chen and Roxby 1996).
[b] Likely to be *P. andina*.

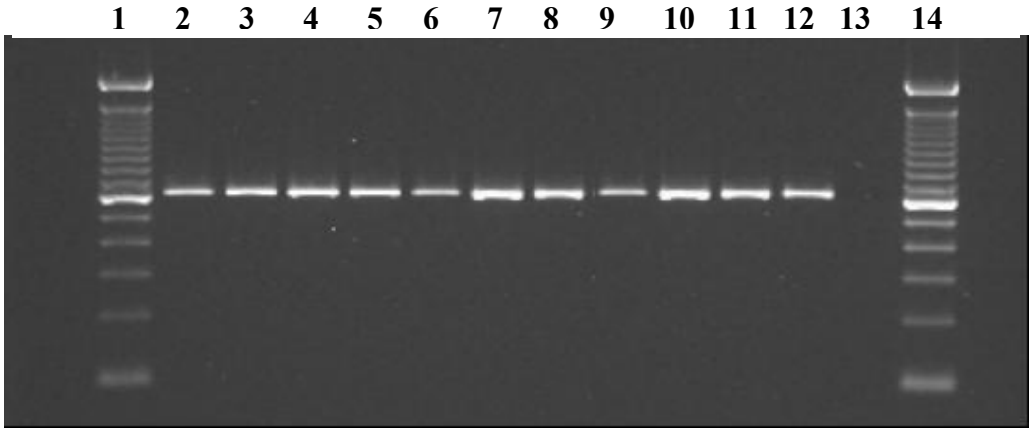
Figure legends

Figure 2. 1 DNA amplified with PINF/ITS5 from isolates of *Phytophthora infestans* sensu lato from Ecuador, and *P. infestans* and *P. mirabilis*. Lanes 2, 3, 4, and 5: PINF amplification products of isolates from *Solanum tetrapetalum* (EC3163, EC3164, EC3165, EC3167). Lane 6: isolate EC3090 from S. *phureja*. Lane 7: Isolate EC3154 from *S. andreanum*. Lane 8: Isolate EC3199 from *S. tuquerene*. Lane 9: Isolate EC3253 from *S. columbianum*. Lane 10: Isolate EC3300 from *S. paucijugum*. Lane 11: isolate 94-1 (US-1) from *S. tuberosum*. Lane 12: *P. mirabilis*. Lane 13: no template DNA control. Lanes 1 and 14 contain a 100-bp DNA ladder. Middle band in the ladder with greater intensity is 600bp fragment.

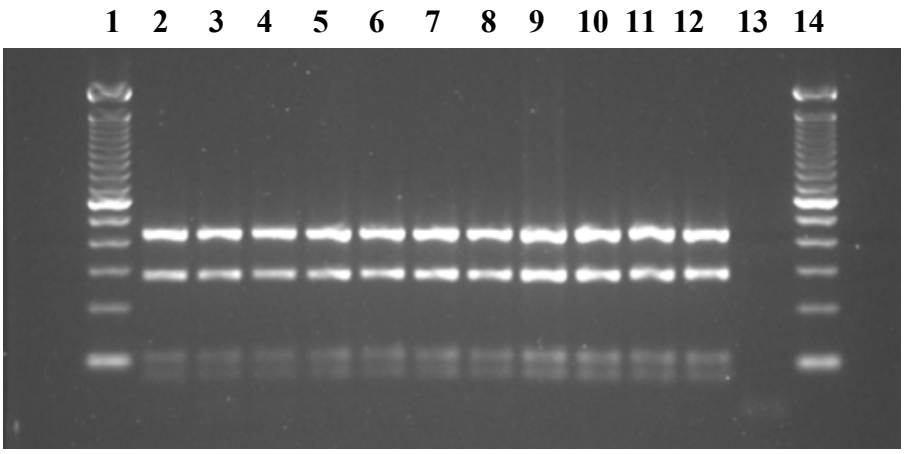
Figure 2. 2 Restriction enzyme analysis with A) *Rsa*I; B) *Msp*I; and C) *Alu*I of DNA amplified with primer pair ITS5 and ITS4 from *P. infestans sensu lato* from Ecuador, *P. infestans*, and *P. mirabilis*. Lanes 2, 3, 4, and 5: Isolates EC3163, EC3164, EC3165, EC3167 from *Solanum tetrapetalum*. Lane 6: isolate EC3090 from S. *phureja*. Lane 7: Isolate EC3154 from *S. andreanum*. Lane 8: Isolate EC3199 from *S. tuquerense*. Lane 9: Isolate EC3253 from *S. columbianum*. Lane 10: Isolate EC3300 from *S. paucijugum*. Lane 11: isolate 94-1 (US-1) from *S. tuberosum*. Lane 12: *P. mirabilis*. Lane 13: no template DNA control. Lanes 1 and 14 contain a 100-bp DNA ladder. Middle band in the ladder with greater intensity is 600bp fragment.

Figure 2. 3 Neighbour-joining tree illustrating phylogenetic relationship among *Phytophthora* species, including the Ecuadorian isolates of *P. infestans* sensu lato from *S. tetrapetalum*, based on the sequence of a fragment of the *cox 1* gene from the mitochondrial genome. The tree was constructed using corrected Kimura 2-parameter distances and 1000 replicates. Gaps were handled using pairwise deletion. Numbers along the branches indicate bootstrap values. Scale bar unit: number of nucleotide substitutions per site.

Figure 2. 4 Neighbour-joining tree illustrating phylogenetic relationship among *Phytophthora* species, including the Ecuadorian isolates of *P. infestans* sensu lato from *S. tetrapetalum*, based on the sequence of an intron of the single-copy Ras gene from the nuclear genome. The tree was constructed using corrected Kimura 2-parameter distances and 1000 replicates. Gaps were handled using pairwise deletion. Numbers along the branches indicate bootstrap values. Scale bar unit: number of nucleotide substitutions per site.


1 2 3 4 5 6 7 8 9 10 11 12 13 14

A)


1 2 3 4 5 6 7 8 9 10 11 12 13 14

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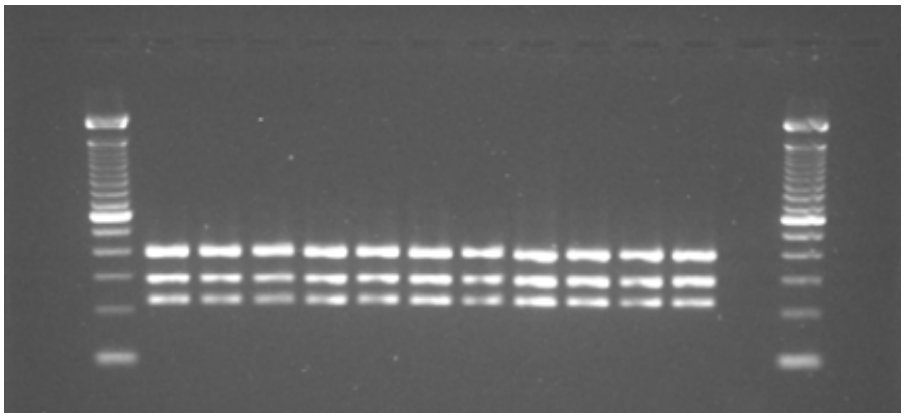
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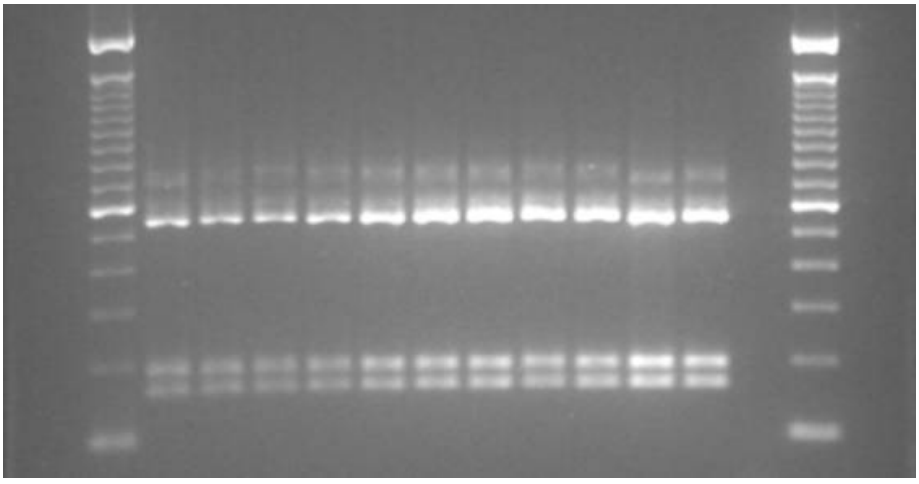
C)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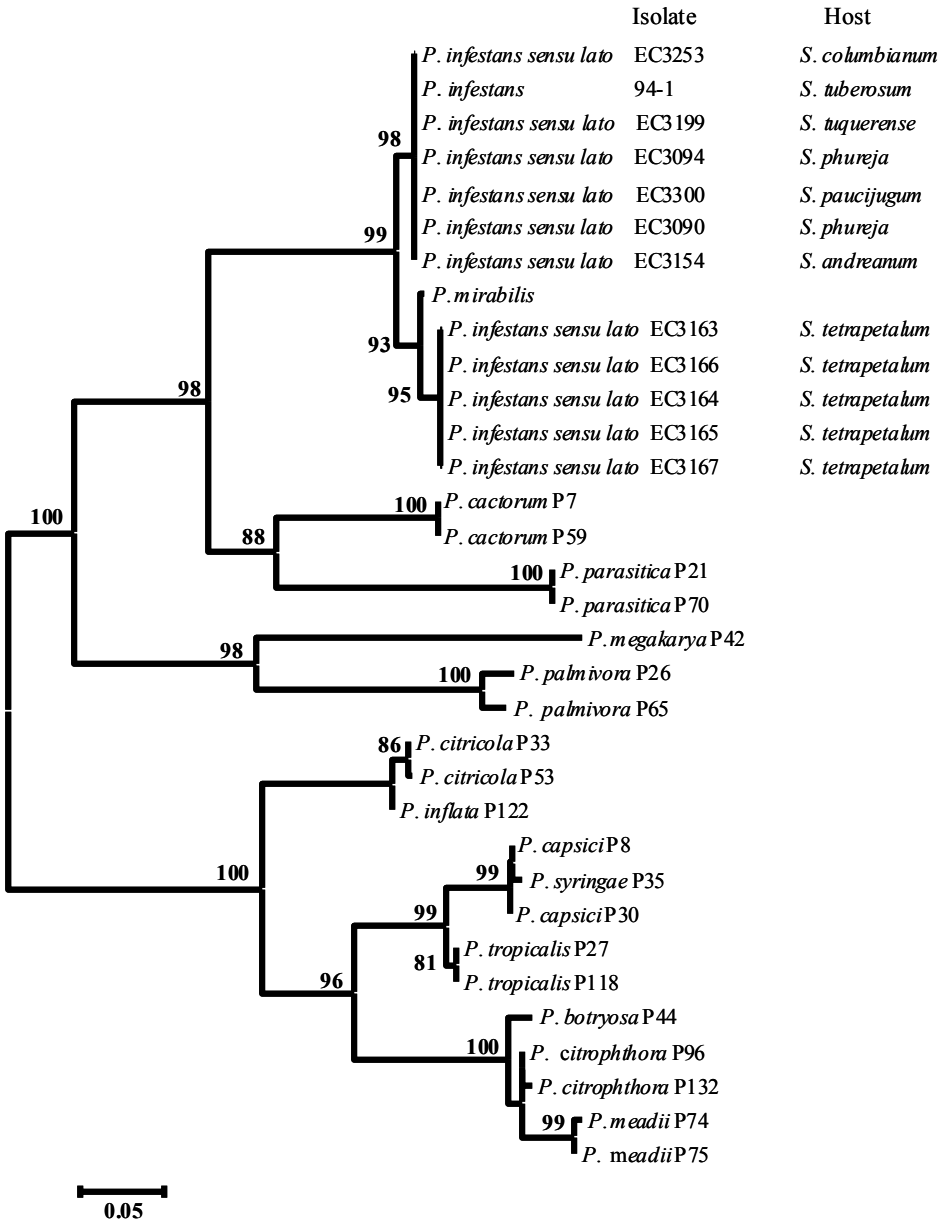
Isolate
Host
P. infestans sensu lato EC3253 S. columbianum
P. infestans 94-1 S. tuberosum
P. infestans sensu lato EC3199 S. tuquerense
P. infestans sensu lato EC3094 S. phureja
P. infestans sensu lato EC3300 S. paucijugum
P. infestans sensu lato EC3090 S. phureja
P. infestans sensu lato EC3154 S. andreanum
P. mirabilis
P. infestans sensu lato EC3163 S. tetrapetalum
P. infestans sensu lato EC3166 S. tetrapetalum
P. infestans sensu lato EC3164 S. tetrapetalum
P. infestans sensu lato EC3165 S. tetrapetalum
P. infestans sensu lato EC3167 S. tetrapetalum
P. cactorum P7
P. cactorum P59
P. parasitica P21
P. parasitica P70
P. megakarya P42
P. palmivora P26
P. palmivora P65
P. citricola P33
P. citricola P53
P. inflata P122
P. capsici P8
P. syringae P35
P. capsici P30
P. tropicalis P27
P. tropicalis P118
P. botryosa P44
P. citrophthora P96
P. citrophthora P132
P. meadii P74
P. meadii P75
98
99
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88
100
98
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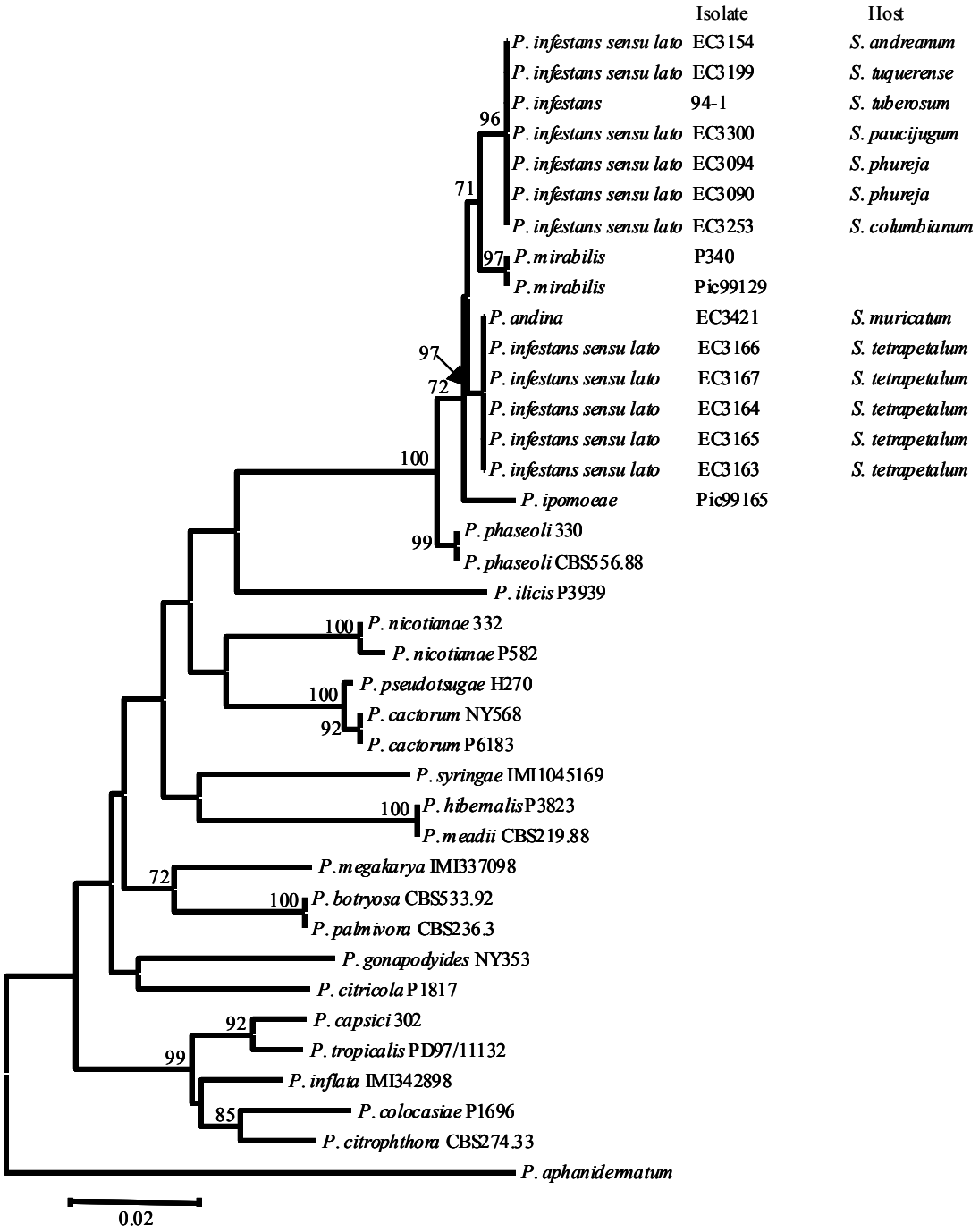
0.05


Isolate
Host
P. infestans sensu lato EC3154 S. andreanum
P. infestans sensu lato EC3199 S. tuquerense
P. infestans 94-1 S. tuberosum
P. infestans sensu lato EC3300 S. paucijugum
P. infestans sensu lato EC3094 S. phureja
P. infestans sensu lato EC3090 S. phureja
P. infestans sensu lato EC3253 S. columbianum
P. mirabilis P340
P. mirabilis Pic99129
P. andina EC3421 S. muricatum
P. infestans sensu lato EC3166 S. tetrapetalum
P. infestans sensu lato EC3167 S. tetrapetalum
P. infestans sensu lato EC3164 S. tetrapetalum
P. infestans sensu lato EC3165 S. tetrapetalum
P. infestans sensu lato EC3163 S. tetrapetalum
P. ipomoeae Pic99165
P. phaseoli 330
P. phaseoli CBS556.88
P. ilicis P3939
P. nicotianae 332
P. nicotianae P582
P. pseudotsugae H270
P. cactorum NY568
P. cactorum P6183
P. syringae IMI1045169
P. hibernalis P3823
P. meadii CBS219.88
P. megakarya IMI337098
P. botryosa CBS533.92
P. palmivora CBS236.3
P. gonapodyides NY353
P. citricola P1817
P. capsici 302
P. tropicalis PD97/11132
P. inflata IMI342898
P. colocasiae P1696
P. citrophthora CBS274.33
P. aphanidermatum
96
71
97
97
72
100
99
100
100
92
72
100
92
99
85
0.02

CHAPTER THREE

GENETIC STRUCTURE OF POPULATIONS OF *PHYTOPHTHORA INFESTANS* FROM COSTA RICA

Abstract

Phytophthora infestans causes late blight and is the most devastating disease of potato worldwide. In Costa Rica, ca. 3000 ha of potato are grown every year mostly under agro-climatic conditions conducive to the disease. In recent years, disease management has become increasingly difficult and could be associated with changes in the population structure of this pathogen. Seventy-five isolates of *P. infestans*, including six isolates from wild *Solanum*, were collected from two geographically distinct potato-growing regions of Costa Rica to determine the genetic diversity and the structure of pathogen populations. All 75 isolates were analyzed for mitochondrial DNA (mtDNA) haplotype, and a subset of isolates was analyzed for mating type, allozyme genotype with *Glucose-6-phosphate isomerase* (*Gpi*), *Peptidase* (*Pep*), restriction fragment length polymorphism fingerprint with probe RG-57, DNA sequence variability from three nuclear gene regions (Ras, Intron Ras, and B-tubulin) and two mitochondrial gene regions (P3 and P4), and metalaxyl resistance. All isolates tested but two were the A1 mating type, *Gpi* 100/100, *Pep* 94/100, and mitochondrial haplotype Ia. No variation was observed for RG57 DNA fingerprint or DNA sequences among these isolates. The remaining two isolates, collected from wild *Solanum* species, were A2 mating type, *Gpi* 100/111/122, and mtDNA haplotype Ia.

The population of *P. infestans* in Costa Rica attacking potatoes is composed of a single clonal linage, whereas a different genotype was found associated with wild *Solanum* species. This is the first report of the presence of A2 mating type in Costa Rica. Minimal importation of seed potatoes and a certified seed program have probably limited genetic diversity of the populations in the country.

Introduction

Phytophthora infestans causes late blight and is the most devastating disease of potato worldwide. Over the past 20 years the population structure of *P. infestans* has undergone major changes in many areas of the world and two major populations known as "old " and "new" have been described (Goodwin *et al*., 1994; Goodwin, 1997, Goodwin *et al*., 1998; Knapova and Gisi, 2002; Cooke *et al*., 2003). It was believed that the "old" population consisted exclusively of A1 mating type, was metalaxyl sensitive, and of the Ib mitochondrial (mtDNA) haplotype (US-1 clonal lineage), whereas the "new" populations consisted of both A1 and A2 mating types, were metalaxyl resistant, and of both Ia and IIa mtDNA haplotypes (Goodwin *et al*., 1994; Goodwin, 1997, Goodwin *et al*., 1998; Knapova and Gisi, 2002). A displacement of the "old " population by the "new" population has been observed in Europe (Goodwin *et al*., 1994; Goodwin, 1997, Goodwin *et al*., 1998; Knapova and Gisi, 2002; Cooke *et al*., 2003). Migration of isolates from Central Mexico, the putative center of origin and diversity of the pathogen, has been proposed as the major force driving the changes (Fry *et al*., 1992; Goodwin *et al*., 1994; Goodwin, 1997).

Others have challenged the theory of Mexico as the putative center of origin of the late blight pathogen and the source of inoculum for 19th century epidemics based on historic and scientific data (Abad and Abad, 1997, Andrivon, 1996; Bourke, 1964; May and Ristaino, 2004; Ristaino *et al*., 2001; Tooley, 1989). DNA sequence analysis of a mitochondrial DNA fragments from Irish famine-era specimens showed that the isolates were not the Ib mtDNA haplotype characteristic of old population (Ristaino *et al*., 2001). The Ia mtDNA haplotype was the most common haplotype found whereas, the Ib haplotype was observed in early 20th century samples from South America (Ristaino *et al*., 2001; May and Ristaino, 2004).

Potato is an important vegetable crop in Costa Rica. Potatoes rank third, after rice and dry bean in the Costa Rican diet, with a per capita consumption of 25 kg/year (Brenes *et al*., 2002). Approximately 3000 ha of potato are grown every year mostly under agro-climatic conditions conducive for the disease. In recent years, late blight management has become increasingly difficult despite the use of large amounts of protectant and systemic fungicides to control the disease. Infection of cultivars released as resistant has been observed as soon as five years after deployment. This increase in disease severity could be associated with changes in the population structure of the pathogen as has occurred elsewhere (Goodwin *et al*., 1994; Goodwin, 1997, Goodwin *et al*., 1998; Knapova and Gisi, 2002; Cooke *et al*., 2003).

The genetic structure of *P*. *infestans* populations in Costa Rica has been studied only to a limited extent. Twenty-two isolates (13 isolates from potato and 9 isolates from tomato) were characterized in three different studies by Hohl and Iselin (1984); Goodwin *et al*. (1994); and Sanchez *et al*. (2000). All isolates were analyzed for mating type, eight for allozyme alleles at

the *Glucose-6-phosphate isomerase* (*Gpi*) and *Peptidase* (*Pep*) loci, seven for DNA fingerprinting with probe RG-57, and six for mitochondrial DNA haplotype (mtDNA haplotype). A single clonal lineage, named CR-1, was identified, and defined as the A1mating type, *Gpi* 100/100, *Pep* 94/100, and mtDNA Ia (Goodwin and Drenth, 1997). The *Pep* 94 allele, however, was not included by Forbes *et al*. (1998) in the Global Marker Database for *Phytophthora infestans* until further confirmation with proper controls was conducted. The RG 57 DNA fingerprinting pattern of the Costa Rican isolates obtained by Sanchez *et al*. (2000) differed from the one obtained by Goodwin *et al*. (1994) in DNA fingerprint band 4 suggesting that several genotypes of *P. infestans* may occur in Costa Rica.

The objective of this study was to further characterize the genetic structure of populations of *P. infestans* in Costa Rica from two geographically distinct commercial potato-growing regions and from wild *Solanum* species adjacent to these areas.

Materials and Methods

Collection of isolates

Seventy-five isolates of *P. infestans* were obtained from naturally infected leaves of potato or wild *Solanum* plants. Samples were collected from commercial fields located in the two main potato-growing regions in Costa Rica: Northern Cartago (Cartago) and Zarcero (Alajuela), and surrounding areas (Figure 3.1). Cartago is located 32 km east of San Jose and Zarcero is 50 km northwest of San Jose. Thirty-seven isolates were collected from 28 different fields in Cartago and 20 isolates were collected from 15 different fields in Zarcero. Twelve isolates were collected from potato differentials grown in a Research Station located in Fraijanes, Alajuela, near

Zarcero. Six isolates from wild *Solanum* species, possibly *S. longiconicum* (Spooner *et al*., 2001) were also collected on the slopes of Irazu and Turrialba Volcanos (Table 3.1).

Isolation of the pathogen

All isolates were taken from single lesions of infected leaves. A small piece (1 cm^2) of infected leaf tissue was placed under potato tuber slices in plastic boxes and incubated at 18°C in the dark. Sporangia were removed from the upper side of the tuber slice and plated on unamended or antibiotic amended Rye B agar media (Caten *et al*., 1968) after 5 to 7 days of incubation. Pea broth (120 g of frozen peas per liter of sterile distilled water) was used for culture of mycelia of *P. infestans* for allozyme analysis, mtDNA haplotyping, restriction fragment length polymorphism analysis, and DNA sequencing. Mycelia was removed from the broth by vacuum filtration on Whatman No. 1 filter paper and kept frozen at –20 °C until use.

Mating type

Mating type was determined on a subset of isolates (n=52) by pairing Costa Rican isolates of *P. infestans* with a known A1 (isolate 94-52, US-6 genotype) or A2 (isolate 97-27, US-8 genotype) tester strain on Rye-V8 medium. A mycelial plug (8 mm in diameter) of a known A1 or A2 isolate was placed on one side of a petri dish (90 mm in diameter), and a mycelial plug of an unknown isolate at the other side. Oospore formation was checked after 10-14 days incubation at 18°C in the dark, using a microscope. Isolates that produced oospores with the known A1 tester isolate were designated as A2 mating type and isolates that produced oospores with the known A2 tester isolate were designated the A1 mating type. Positive controls consisted of pairings

between the opposite mating type of tester isolates, and negative controls consisted of pairings between isolates of the same mating type.

Metalaxyl sensitivity

Sensitivity to metalaxyl was estimated on a subset of isolates (n=32) based on radial growth on metalaxyl-amended Rye-V8 agar, as described previously (Wangsomboondee *et al*., 2002). An 8-mm-diameter mycelial plug from a 7-14 day-old colony was placed on the center of a petri plate containing Rye-V8 agar amended with metalaxyl (Ridomil 2E, Syngenta, Research Triangle Park, NC) at 5 or 100 µg per ml. Control plates contained nonamended Rye-V8 medium (0 µg per ml). Two replications were conducted per treatment for each isolate, and the experiment was repeated at least twice. Plates were maintained at 18°C in the dark. Radial growth of each colony was measured after 10 to 14 days, and the relative growth of each isolate at each metalaxyl concentration was calculated as a percentage of the growth on nonamended Rye-V8 medium (control plate). Isolates with a colony diameter less than 40% of the nonamended control at 5 and 100 µg/ml were classified as metalaxyl sensitive. Isolates with a colony diameter greater than 40% of the nonamended control at 5 µg/ml but less than 40% of the nonamended control at 100 µg/ ml were classified as intermediate. Isolates with a colony diameter greater that 40% of the nonamended control at both 5 and 100 µg/ml were classified as metalaxyl insensitive (resistant) (Deahl *et al*., 1995).

Allozyme analysis

Allozyme genotypes were determined for a subset of isolates at *Glucose-6-phosphate isomerase* (*Gpi*) (n=48) and *Peptidase* (*Pep*) (n=24) loci. Mycelium obtained from pea broth culture as described previously, was cut into small pieces (0.5 to 1 cm^2) and placed in sterile 1.5 ml microcentrifuge tubes. The mycelium was centrifuged at 13,000 rpm for 1 min to remove excess water. Fifty microliters of extraction buffer (20% sucrose, 2% Triton X-100, 0.01% bromophenol blue, and 9.8 ml H_2O) was added to each tube and the mycelia was ground for 30 to 60 sec with a hand drill equipped with sterile Konte pestles. Samples were centrifuged for 2 min at 10,000 rpm, and the supernatant was collected for allozyme analysis.

Allozyme genotypes were determined at the *Gpi* and *Pep* loci by cellulose-acetate electrophoresis (CAE)(Goodwin *et al*., 1995). Isolates of two clonal genotypes US-1 (*Gpi* 86/100, *Pep* 92/100) and US-6 (*Gpi* 100/100, *Pep* 92/100) were used as standards on each acetate plate. Isolates PIC 97310 (92/100), PIC 97605 (92/100), and PIC98366 (100/100), from the PICTIPAPA Program in Mexico were used as comparative standards for *Pep* (Grundwald *et al*., 2001)**.** Allozyme alleles were designated by the numbers representing their percentage of mobility relative to the standards.

Mitochondrial DNA haplotype

All 75 isolates were analyzed for mtDNA haplotype. Four different mitochondrial haplotypes have been described in *P. infestans*: Ia, Ib, IIa, and IIb (Carter *et al*.,1990). DNA extraction, digestion with restriction enzymes, polymerase chain reactions (PCR), and determination of haplotypes were according to the methods of Griffith and Shaw (1998) modified by

Wangsomboondee *et al*. (2002). The mitochondrial haplotypes of tested isolates were determined by comparing their patterns to reference isolates US-8 (Ia), US-1 (Ib), BR-1 (IIa), US-6 (IIb) (Forbes et al. 1998).

DNA fingerprinting

Isolates were grown in pea broth for 14 days at 18 ˚C. The mycelium was vacuum-filtered through Whatman No. 1 filters, lyophilized at -5˚C overnight, and ground in liquid nitrogen. Total DNA was extracted following the mini-prep procedure described by Lee *et al*., (1998) as modified by Jacobson and Gordon (1990). RFLP analysis using the RG-57 probe was carried out using the methodology described by Goodwin *et al*. (1992) on a subset of isolates (n=20). Transfer to Hybond $-N^+$ nylon membrane (Amersham Biosciences), hybridization with non-radioactive RG-57 probe, and autoradiography were all according to the manufacturer's instructions (Renaissance non-radioactive kit NEN^{TM} Life Science Products, Inc, Boston, MA, USA) and methods described previously (Wangsomboondee *et al*., 2002). The DNA fingerprinting of the isolates was determined by comparing their patterns with those of reference isolates US-1, US-8, and US-18 (Forbes *et al*., 1998).

DNA sequencing

Sequences of three nuclear DNA regions and one mitochondrial region were determined for 12 isolates. Nuclear regions included two portions of the Ras gene and one portion of the B-tubulin gene. The mitochondrial regions included a portion of the c*ox 1* gene (P4 region) and the genes *rp114, rp 15,* and *tRNAs* (P3 region). These regions showed variability among a larger set of *P.*

infestans isolates (Gomez *et al*., 2003). Total DNA was extracted from mycelium grown on pea broth using the cetyltrimethylammonium bromide (CTAB) method (Trout *et al*., 1997). PCR conditions for nuclear gene regions were according to Ristaino *et al*. (1998), and for the mitochondrial regions the procedure from Wangsomboondee *et al*. (2002) was followed. The following primer pairs were used to amplify portions of the nuclear genes: RAS F: 5' CGT GTC TGC TTC TCC GTT TCG 3' and RAS R: 5' CCA GGC TTT CGG CAA ATT CC 3'; IntronRASF: 5' TTG CAG CAC AAC CCA GAC G 3' and Intron RASR: 5' CTG CAC GTA CTA TTC GGG GTT C 3'. Primer sequences for TUB are available from C. Andre Levesque, Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Summerland, British Columbia. For the mitochondrial regions the primer pair 3F/3R and 4F/4R from Griffith and Shaw (1998) were used. PCR products were purified with QIAquick Kit (Qiagen, Chatsworth, California). Fragments were sequenced with the Big Dye Terminator reaction kit (ABI PRISM, Applied Biosystems). Sequencing was performed with the same primers used for amplification and in both 5' to 3' and 3' to 5' directions. Sequence alignments were performed using Clustal X (Thompson *et al*., 1997) and adjusted manually by visual inspection using Bioedit (available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Results

Mating type

All isolates of *P. infestans* collected from potatoes and four isolates from wild *Solanum* species were of the A1 mating type (Table 1). They formed abundant oospores with the known A2 isolate 97-27 (US-8), but not with the A1 isolate 94-52 (US-6). Two isolates collected from wild

Solanum were of the A2 mating type (Table 1). They formed abundant oospores with the known A1 isolate 94-52 (US-6), but not with the A2 isolate 97-27 (US-8). Crosses with positive controls of the opposite mating type formed oospores, whereas crosses with negative controls of the same mating type did not form oospores.

Metalaxyl sensitivity

Eight isolates (25%) were sensitive, and the remaining 24 (75%) were resistant to metalaxyl. No isolates with intermediate levels of resistance were found (Table 2).

Allozyme genotypes

Forty-two isolates from potato and four from wild *Solanum* species, 96%, had an allozyme genotype of *Gpi* 100/100 (Table 1, Figure 2a). All isolates tested for *Pep* had an allozyme genotype of *Pep* 94/100 (Table 1, Figure 2b). Presence of the *Pep* 94 allele among Costa Rican populations of *P. infestans* was confirmed using controls with genotypes 92/100 and 100/100 (Figure 2b). Two isolates (4%) from wild *Solanum* had a five-banded pattern for *Gpi*, corresponding to *Gpi* 100/111/122 (Figure 2c).

Mitochondrial DNA haplotype

All isolates, including those from wild *Solanum* species with a different *Gpi* genotype were Type I (Fig. 3). Further PCR amplification using primer pair F2/R2 and digestion with *Msp*I indicated that all isolates tested were the Ia mtDNA haplotype (Fig. 3).

DNA fingerprinting

No variation among potato isolates was observed for DNA fingerprints (Fig. 4). All isolates had a DNA fingerprint identical to DNA fingerprint reported previously by Sanchez *et al*. (2001) and were (1000000001001101000110011). This DNA fingerprint differs from the pattern of CR-1 (Goodwin *et al*., 1998) by the absence of band 4 (Fig. 4).

DNA sequencing

No DNA sequence variation among isolates was found for any of the DNA regions analyzed. One isolate from wild *Solanum* species showing the same *Gpi* genotype, mating type and mtDNA haplotype as the potato isolates (Table 3.1) had the same DNA sequence for the five regions analyzed. No other isolates from wild *Solanum* were sequenced.

Discussion

Two genotypes were found among 75 isolates of *P. infestans* from Costa Rica. One genotype (A1 mating type, *Gpi* 100/100, *Pep* 94/100, mitochondrial DNA haplotype Ia and RG57 fingerprint (1000000001001101000110011)) represented 97% of the total isolates sampled and was found on both wild and cultivated potato regardless of the production area. This genotype is similar to the multilocus genotype CR-1 described by Goodwin *et al*. (1994) from nine isolates collected between 1982 to 1993, except that the RG57 fingerprint band 4 was not present in our isolates. Band number 4 is not reproducible with the RG 57 probe and seems to depend on the

particular batch of probe used (Forbes *et al*., 1998). Forbes *et al*. (1998) and Goodwin *et al*. (1998) indicated that variation at fingerprint locus 4 is difficult to interpret and should be not used for genotype identification. Sanchez *et al*. (2000) were also unable to detect band 4 in other Costa Rican isolates. Thus, the most common Costa Rican genotype described in our study was identical to CR-1.

The second genotype (A2 mating type, *Gpi* 100/111/122, mtDNA haplotype Ia) was found in two isolates collected from wild *Solanum* (Table 3.1). This genotype had the same *Gpi* genotype, mating type and mtDNA haplotype as the US-8 genotype, especially the five-banded pattern observed in its *Gpi* genotype. It is not clear if the same wild *Solanum* species was infected by the two genotypes of *P. infestans* found in Costa Rica. According to Spooner *et al*. (2001) the only member of the section Petota present in Costa Rica is *S. longiconicum*. Morphological variation (flower color, plant size, leaf size and form) in wild *Solanum* exists in Costa Rica indicating that there might be more than one species. In addition other species belonging to other section has been also described in the country.

The most common genotype of *P. infestans* genotype in Costa Rica conforms partially to the reported "new" populations that migrated from Mexico in the mid to late 1970s (Goodwin *et al*., 1994, Goodwin, 1997). However, the Costa Rican genotype differs from both old and new genotypes for the presence of the *Pep* 94 allele, and thus cannot be classified as old or new (Goodwin *et al*., 1994). The *Pep* 94 allele was first described by Goodwin *et al*. (1994) in Costa Rican isolates, but has not been found elsewhere. Forbes *et al*. (1998) did not include the *Pep* 94 allele in the Global Marker Database for *Phytophthora infestans* and suggested that further confirmation with proper controls was needed. In our study, the *Pep* 94 allele was clearly

identified among isolates from Costa Rica (Fig. 2B). If CR-1 originated in Mexico, its introduction into Costa Rica must have involved an unreported migration (Goodwin, 1997). A possible route for the introduction of CR-1 into Costa Rica was the introduction in the decade between 1953-1963 of the Mexican variety Atzimba into Costa Rica for seed stock. Atzimba accounted for 95% of the national production in Costa Rica by 1973 (Rhoades, 1999) and has been a popular variety ever since. Import of infected seed potato tubers is the most common method of long-distance spread of *P. infestans*. Rapid spread of genotypes of *P. infestans* in infected seed has been demonstrated in the United States (Goodwin *et al*., 1998).

Introduction of the CR-1 genotype from the United States or Canada is also possible since Kennebec, a North American variety, was also planted in Costa Rica in the 1970s (Rhoades *et al*., 1999). The genotype CA-4 (previously BC-1) found in British Columbia, Canada, during 1992 and 1993, has the same DNA fingerprint as the Costa Rican genotype, but has a different mating type (A2) and dislocus allozyme genotype (*Gpi* 100/111 and *Pep* 100/100). CA-4 is considered to be a possible sexual recombinant or an immigrant from Mexico (Goodwin *et al*., 1998).

Three Costa Rican isolates of *P. infestans* collected from potato in 1982 by Hohl and Iselin (1984) were also the A1 mating type and Ia mitochondrial DNA haplotype (Knapova and Gisi, 2002). Knapova and Gisi (2002) included these isolates along with several isolates from around the world as reference isolates in the characterization of 134 French and Swiss isolates of *P. infestans* using simple sequence repeats or micro satellites (SSR). Costa Rican isolates showed a unique genotype, B-05. Cluster analysis showed similarity among the Costa Rican isolates and the US-7 (B-02), US-8 (C-01), and MEX 143 (D-07) isolates, indicating a common

ancestor for these genotypes. MEX 143 is a Mexican isolate collected in 1982. US-7 and US-8 are considered to be of Mexican origin and were probably introduced from northwestern Mexico into the United States during or shortly before 1992 (Goodwin *et al*., 1995). Mex 143, US-7 and US-8 all have the Ia mtDNA haplotype, however they are of the A2 mating type. It is interestingly that the second genotype found in Costa Rica associated with wild *Solanum* presents a multilocus genotype similar to US-8. Both Costa Rican genotypes seem to share a common ancestral population according to SSR analysis. No Swiss or French isolate showed the B-05 genotype or was in the same cluster; therefore an introduction via Europe seems unlikely.

May and Ristaino (2004) analyzed the sequences of mtDNA fragments from historical herbarium specimens. Two Costa Rican samples from the 1940s were the Ia mtDNA haplotype, indicating that this mtDNA haplotype was in Costa Rica prior to the 1970s, and could be associated with either CR-1 or the five-banded *Gpi* genotype. The Ia mtDNA haplotype is the predominant haplotype in present day central Mexican populations of *P. infestans* (Gavino and Fry, 2003; Flier *et al*., 2003), but it is also found in South American populations (Perez *et al*., 2001, Gavino and Fry, 2002) which could be another possible source of introduction of the pathogen into Costa Rica (Abad and Abad, 1997, Andrivon, 1996). In a recent study of Peruvian populations of *P. infestans* Perez *et al*. (2001) described the lineage PE-3 from southern Peru. This lineage has the Ia mtDNA, *Gpi* 100/100, and A1 mating type, but differs from CR-1 in the RG-57 fingerprint pattern and *Pep* genotype (100/100).

An intriguing finding from our study was the discovery of a second *P. infestans* genotype (A2 mating type, *Gpi* 100/111/122, and mtDNA haplotype Ia) associated with wild *Solanum* species grown not far from commercial potato fields. The multilocus genotype observed in this

second genotype is similar to the US-8 genotype (Goodwin et al. 1998), especially the five-banded pattern of its *Gpi* genotype. The *Gpi* genotype 100/111/122 was first observed in isolates from northwestern Mexico (Goodwin *et al*., 1992) and subsequently in USA and Canada (Goodwin *et al*., 1998), but has since not been found elsewhere. The presence of the US-8 in USA and Canada is considered a result of migration from northern Mexico in the early 1990s (Goodwin *et al*., 1998). It is likely that the Gpi 100/111/122 genotype was introduced to Costa Rica from Mexico, USA or Canada. However, there is no clear explanation for its concurrent presence in wild *Solanum* species and absence from commercial potatoes growing in the same geographical area. US-8 genotype is especially pathogenic on potatoes and it is sometimes called "super blight" (Shattock, 2002). It is also a very efficient colonizer of potato tubers (Goodwin, 1997) and after its introduction into USA and Canada has become predominant in both places. If this genotype was introduced into Costa Rica, the most likely way was by the movement of potato tubers as has been proposed for other migrations of *P*. *infestans* (Goodwin, 1997). Therefore, one would expect to find this genotype first associated with potatoes, even if its introduction is a recent one, as well as with wild *Solanum* species, but this was not the case. Although, a more intensive survey may uncover the presence of this genotype in potato, at this time it is not the most common genotype on potatoes. We are presently determining the RG-57 pattern for this genotype in order to confirm whether it is US-8 or a variant of it. In Canada, one isolate banding as *Gpi* 100/111/122 proved to be an A1 mating type (Peters *et al*., 1999) and four isolates showing the same bands as US-8, but with different intensities were assigned to a new genotype called UN-4 (Daayf *et al*., 2001). In addition, isolates of US-8 genotype were more diverse in pathogenicity than isolates of UN-4 genotype (Daayf *et al*., 2001).

Host preference among genotypes of *P. infestans* has been observed in different countries and might explain the predominance of one genotype on a particular host (Legard *et al*., 1995; Oyarzún *et al*., 1998; Vega-Sanchez *et al*., 2000; Reis *et al*., 2003; Adler *et al*., 2004). In Ecuador, populations of *P. infestans sensu lato* have been isolated from both cultivated and wild *Solanum* species. So far three clonal lineages (US-1, EC-1, EC-3) and one heterogeneous group (EC-2) have been described (Adler et al. 2004). Each of the four groups attacks one or more hosts in the genus *Solanum* among others, but no two lineages appear to be the primary pathogens of the same host. All lineages have been collected from the same eco-region, but little or no gene flow occurs among them.

This is the first report of A2 mating type in Costa Rica. However, no evidence of sexual reproduction was observed and populations of *P. infestans* in Costa Rica are clonal. A1 mating type was found associated with potatoes and wild *Solanum* species whereas A2 mating type was found associated with wild *Solanum* species. As mentioned before, because of taxonomic uncertainty, it is not clear if the same wild *Solanum* species harbors both mating types. In addition, the absence of the A2 mating type on potatoes could be the result of intensive use of fungicides on commercial potato crops. Recently, it was found that pear melon (*Solanum muricatum*) is a common host for A1 and A2 mating types in Ecuador, although no evidence of sexual reproduction has been yet found there (Adler *et al*., 2002, Adler *et al*., 2004). Both mating types have also occurred in Japan (Mosa *et al*., 1989) and Brazil (Reis *et al*., 2003) for more than a decade, but to date only clonal populations have been found in these countries. Although the coexistence of the A1 and A2 mating types apparently does not always lead to sexual recombination (Oliva *et al*., 2002), a close monitoring of the *P. infestans* populations in Costa

Rica is needed to determine any shift in the genotypes attacking commercial potatoes as well as the uprising of sexual recombinants. Because the two genotypes found have a different *Gpi* genotype, rapid detection of isolates from either clones or the presence of new genotypes will be possible using cellulose acetate electrophoresis (Goodwin *et al*., 1995).

A high level of metalaxyl-resistance (75%) was found among populations of *P. infestans*, despite the fact that the fungicide alone or in mixture with protectants is rarely used in potato production in Costa Rica, especially in Northern Cartago. Following the introduction of metalaxyl in the early 1980s, failure to control the pathogen in the field was quickly observed and growers discontinued its use. Zarcero showed a higher level of metalaxyl insensitivity (87%) than Cartago (68%). Similar results were found by Paez *et al*. (2001) with a larger number of isolates. Metalaxyl-resistance, therefore, seems to be a character associated with Costa Rican isolates before metalaxyl use and not originated by directional selection within a sensitive population. Deahl *et al*. (2003) concluded that clonality and predominance of metalaxyl-resistant isolates in the Uruguayan population of *P. infestans* are consistent with a common origin and migration of resistant genotypes into the country rather than from selection on an existing population. Monitoring for metalaxyl resistance should continue in order to establish whether resistance levels would decrease further.

Based on the results presented here outbreaks of *P. infestans* on potato crops in Costa Rica during the recent past years have likely been caused by a single clonal lineage, CR-1. Therefore, based on the analyzed characters, the population structure of *P. infestans* in Costa Rica attacking potatoes has shown little change in 20 years. No information about the genetic structure of populations of *P. infestans* in Costa Rica is available prior to 1982.

The use of locally produced potato seed tubers and the existence of a national program of certified seed production probably has limited the introduction of new genotypes into the country. In Costa Rica, potato seed tuber production occurs at high altitude (> 2500 m a.s.l) on the slopes of the Irazu Volcano in Northern Cartago. Potato growers have a tradition of unidirectional movement of seed potato from higher to lower altitudes. No officially imported seed has been used in potato production in Costa Rica for more than two decades. New clones for agronomic and disease resistance testing are imported as tissue culture plants or tubers grown in greenhouse from the International Potato Center, Lima, Peru and are further micropropagated and/or greenhouse grown. The practice of using local seed tubers has also limited the introduction of new genotypes and helped to restrict the gene pool of the pathogen in South Africa (McLeod *et al*., 2001), Nepal (Ghimire *et al*., 2003), and Russia (Siberia and Far East regions, Elansky *et al*., 2001). The detection of only one genotype, regardless of the production area in Costa Rica is also consistent with the unidirectional movement of the potato seed in the country. These data demonstrate the importance of local seed certification programs.

The increased severity of the epidemics observed in some recent years is probably due to a combination of conducive weather patterns and incorrect spray programs. The occurrence of complex pathotypes within the Costa Rican *P. infestans* population was recently reported (Paez *et al*., 2002), and could also play a role in the epidemics, if an increased fitness is associated with new races

Costa Rican populations of *P. infestans* present some unusual features: (a) the *Pep 94* allele has not been found elsewhere; (b) the *Gpi* genotype (100/111/122) was found associated with wild *Solanum* and not with commercial potatoes; and (c) wild *Solanum* species harbors two

genotypes whereas commercial potatoes have only one. Although migration from Mexico, North America, or from South America is the most likely hypothesis to explain the presence of both genotypes in Costa Rica, recent evidence from Ecuador (Adler *et al*. 2004) has shown that wild *Solanum* species harbor more genetic variability of *P. infestans sensu lato* than previous realized. As Erselius *et al*. (1999) indicated, very little attention has been paid to the possible role that other less economically important crops and wild species play in the population dynamics of *P. infestans*. In Central and South America, many alternative hosts exist in the same geographical environments as potatoes, but few studies have been done on the pathogen growing on these plants in the wild. Further investigation into the pathogen populations attacking wild *Solanum* and other hosts (e.g. tomatoes) in Costa Rica and Central America is clearly warranted, and may provide important information toward understanding the population history of this important pathogen both locally and globally.

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Table 3. 1 Isolates of *Phytophthora infestans* collected from potato and wild *Solanum* in Costa Rica and tested for metalaxyl sensitivity, mating type, mitochondrial DNA (mtDNA) haplotype and allozyme genotype

Isolate Code	Host	Province	Location	Year	Metalaxyl[a] Sensitivity	Mating Type	mtDNA[b]	Allozyme Genotype[c]	
								Gpi	*Pep*
ZB	Potato	Alajuela	Palmira	2000	R	A1	Ia	100/100	94/100
ZE	Potato	Alajuela	Palmira	2000	R	A1	Ia	100/100	94/100
ZF	Potato	Alajuela	Viveros Rica Flor	2000	R	A1	Ia	-[d]	-
ZG	Potato	Alajuela	Palmira	2000	R	A1	Ia	100/100	94/100
ZN	Potato	Alajuela	Viveros Rica Flor	2000	R	A1	Ia	100/100	94/100
ZO	Potato	Alajuela	Pueblo Nuevo	2000	R	A1	Ia	100/100	94/100
CC	Potato	Cartago	Km 30	2000	R	A1	Ia	100/100	94/100
CD	Potato	Cartago	Inv. F. Calvo	2000	S	A1	Ia	-	-
CE	Potato	Cartago	Km 27	2000	S	A1	Ia	-	-
CG	Potato	Cartago	Inv. F. Calvo	2000	R	A1	Ia	-	-
CI	Potato	Cartago	Km 25	2000	S	A1	Ia	100/100	-
CJ	Potato	Cartago	Pacayas	2000	R	A1	Ia	-	-
CK	Potato	Cartago	Inv. F. Calvo	2000	S	A1	Ia	-	-
CL	Potato	Cartago	Sanatorio	2000	R	A1	Ia	100/100	94/100
CM	Potato	Cartago	Calle Acenjos	2000	R	A1	Ia	-	-
CP	Potato	Cartago	Calle Acenjos	2000	S	A1	Ia	-	-
CQ	Potato	Cartago	Pacayas	2000	R	A1	Ia	-	-
TR5	Potato	Cartago	Tres Rios	2000	-	A1	Ia	100/100	94/100
CS	Potato	Cartago	Tres Rios	2000	S	A1	Ia	-	-
CT	Potato	Cartago	Tres Rios	2000	R	A1	Ia	100/100	94/100
CW	Potato	Cartago	Quebrador	2000	R	A1	Ia	100/100	94/100
CY	Potato	Cartago	Tierra Blanca	2000	R	A1	Ia	100/100	94/100
F1	Potato	Alajuela	Fraijanes	2000	R	A1	Ia	-	-
F4	Potato	Alajuela	Fraijanes	2000	S	A1	Ia	-	-
H1	Potato	Heredia	San Joaquin	2000	S	A1	Ia	-	-
H2	Potato	Heredia	San Joaquin	2000	R	A1	Ia	-	-
H5	Potato	Heredia	San Joaquin	2000	R	A1	Ia	-	-
H6	Potato	Heredia	San Joaquin	2000	R	A1	Ia	-	-
C1	Potato	Cartago	Tierra Blanca	2001	R	A1	Ia	100/100	94/100
C2	Potato	Cartago	Llano Grande	2001	R	A1	Ia	-	-
C3	Potato	Cartago	Tierra Blanca	2001	R	A1	Ia	100/100	94/100
C5	Potato	Cartago	Tierra Blanca	2001	R	A1	Ia	100/100	94/100
F6	Potato	Alajuela	Fraijanes	2001	-	A1	Ia	-	-
F7	Potato	Alajuela	Fraijanes	2001	-	A1	Ia	100/100	94/100
F8	Potato	Alajuela	Fraijanes	2001	-	A1	Ia	-	-
F10	Potato	Alajuela	Fraijanes	2001	-	A1	Ia	-	-
Dr	Potato	Alajuela	Fraijanes	2001	-	A1	Ia	-	-
DR1	Potato	Alajuela	Fraijanes	2001	-	A1	Ia	100/100	94/100
DR4	Potato	Alajuela	Fraijanes	2001	R	A1	Ia	100/100	94/100
DR6	Potato	Alajuela	Fraijanes	2001	-	A1	Ia	100/100	94/100
DR7	Potato	Alajuela	Fraijanes	2001	-	A1	Ia	-	-
DR10	Potato	Alajuela	Fraijanes	2001	-	A1	Ia	-	-
Z1	Potato	Alajuela	Zarcero	2001	-	A1	Ia	-	-
Z2	Potato	Alajuela	Zarcero	2001	-	A1	Ia	-	-
Z14	Potato	Alajuela	Zarcero	2001	-	A1	Ia	-	-
Z12	Potato	Alajuela	Zarcero	2001	-	A1	Ia	-	-
Z17	Potato	Alajuela	Zarcero	2001	-	A1	Ia	100/100	94/100
GB-1	Potato	Alajuela	Zarcero	2002	-	A1	Ia	100/100	94/100
GB-2	Wild	Cartago	San Gerardo Dota	2002	-	A1	Ia	100/100	94/100
GB-4	Potato	Cartago	Guarumos	2002	-	A1	Ia	100/100	94/100

Table 3.1 continued

GB-10	Potato	Alajuela	Palmira	2002	-	A1	Ia	100/100	94/100
GB-10	Potato	Alajuela	Palmira	2002	-	A1	Ia	100/100	94/100
41	Potato	Cartago	Volcan Turrialba	2003	-	-	Ia	100/100	-
51	Wild	Cartago	El Convenio	2003	-	-	Ia	100/100	-
52	Wild	Cartago	El Convenio	2003	-	-	Ia	100/100	-
61	Potato	Cartago	Guarumos	2003	-	-	Ia	100/100	-
71	Potato	Cartago	San Martin de Irazu	2003	-	-	Ia	100/100	-
81	Potato	Alajuela	Zarcero	2003	-	-	Ia	100/100	-
91	Potato	Alajuela	Zarcero	2003	-	-	Ia	100/100	-
92	Potato	Alajuela	Palmira	2003	-	-	Ia	100/100	-
111	Potato	Alajuela	Zarcero	2003	-	-	Ia	100/100	-
141	Wild	Cartago	Cordillera Talamanca	2003	-	-	Ia	100/100	-
151	Potato	Cartago	Cañon del Guarco	2003	-	-	Ia	100/100	-
152	Potato	Cartago	Cañon del Guarco	2003	-	-	Ia	100/100	-
201	Potato	Cartago	Hacienda Las Yolgas	2003	-	-	Ia	100/100	-
211	Potato	Cartago	Las Parcelas	2003	-	-	Ia	100/100	-
221	Potato	Cartago	Las Parcelas	2003	-	-	Ia	100/100	-
120	Wild	Cartago	Volcan Irazu	2004	-	A2	Ia	100/111/122	-
154	Wild	Cartago	Near V. Turrialba	2004	-	A2	Ia	100/111/122	-
TB-1	Potato	Cartago	La Laguna	2004	-	A1	Ia	100/100	-
TB-2	Potato	Cartago	El Porton-La Laguna	2004	-	A1	Ia	100/100	-
CG-1	Potato	Cartago	Llano Grande	2004	-	A1	Ia	100/100	-
RR	Potato	San Jose	Rancho Redondo	2004	-	A1	Ia	100/100	-
RM	Potato	Cartago	Rancho Macho	2004	-	A1	Ia	100/100	-
BA	Potato	Cartago	Pacayas	2004	-	A1	Ia	100/100	-

a Metalxyl sensitivity was determined according to Wangsomboondee et al. (2002)
b mtDNA haplotype was determined by methods of Griffith and Shaw (1998)
c Allozyme Genotype: *Gpi* (*glucose-6-phosphate isomerase*) and *Pep* (*peptidase*) were determined by cellulose-acetate gel electrophoresis (Goodwin, Schneider, and Fry, 1995)
d Not determined.

Table 3. 2 Sensitivity to metalaxyl of isolates of *Phytophthora infestans* collected from fields in the two main potato-growing regions in Costa Rica.

	Response to Metalaxyl			
Region	Sensitive[a]	Intermediate	Resistant	Total
Cartago	6	0	13	19
Zarcero	2	0	11	13
Total	8	0	24	32

[a] Isolates with a colony diameter less than 40% of the nonamended control at 5 and 100 μg/ml were classified as metalaxyl sensitive. Isolates with a colony diameter greater than 40% of the nonamended control at 5 μg/ml but less than 40% of the nonamended control at 100 μg/ ml were classified as intermediate. Isolates with a colony diameter greater that 40% of the nonamended control at both 5 and 100 μg/ml were classified as metalaxyl resistant

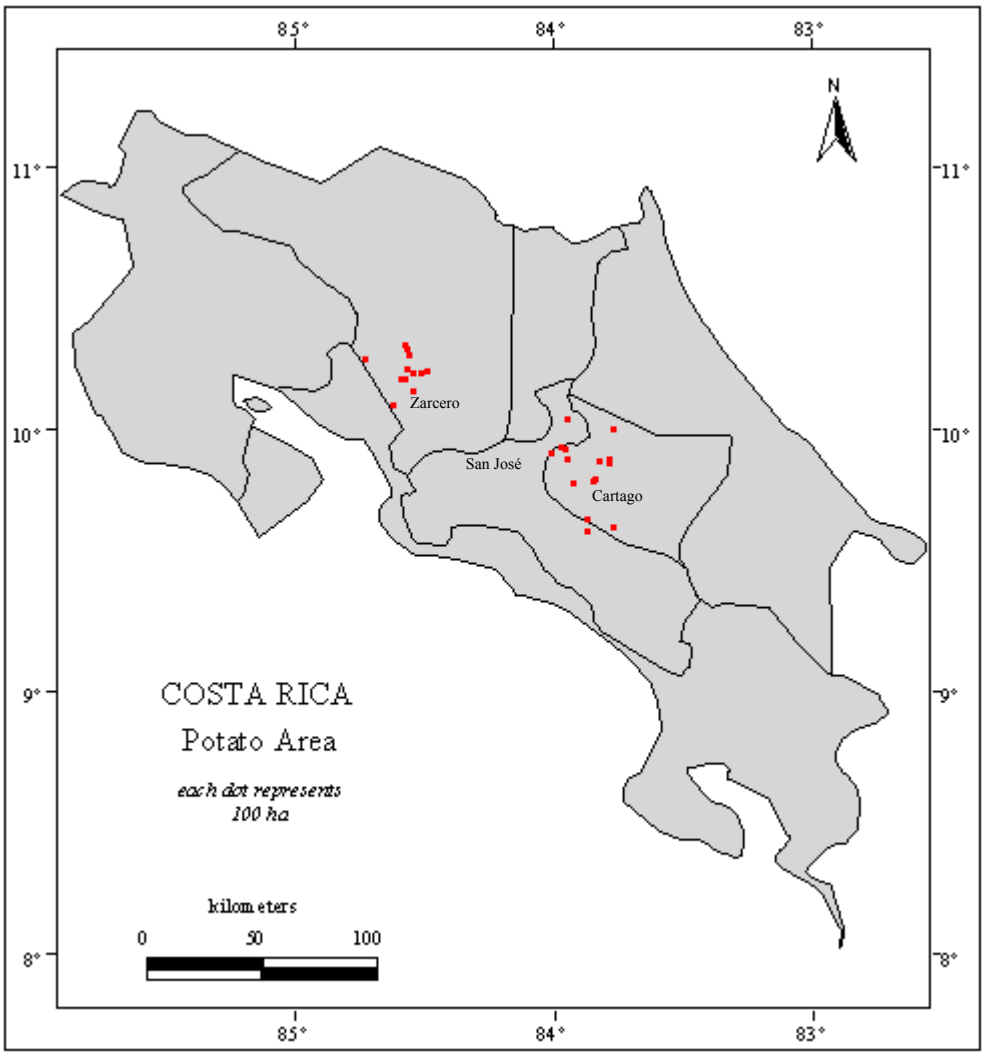
Figure legends

Figure 3. 1 Locations of the two major sampling areas in Costa Rica

Figure 3. 2 Cellulose-acetate gel stained for *Glucose-6-phosphate isomerase* (*Gpi*) (A and B), and *Peptidase* (*Pep*) (C) genotypes of *Phytophthora infestans*. A) *Gpi* genotypes of *P. infestans* isolates are in lanes from left to right: lanes 1-9) Costa Rican isolates (100/100); lane 10) US-6 (100/100); lane 11) US-1 (86/100). B) *Gpi* genotypes of *P. infestans* isolates are in lanes from left to right: lane 1) US-6 (100/100); lanes 2, 8 and 9) US-1 (86/100); lane 3) US-7 (100/111); lane 4) US-8 (100/111/122); lanes 5 and 7) Costa Rican isolate 120 (100/111/122), lane 6) Costa Rican isolate 154 (100/111/122); lanes 10, 11 and 12) Costa Rican isolates TB-1, TB-2 and RM (100/100). C) *Pep* genotypes of *P. infestans* isolates are in lanes from left to right: lane 1) Mexican isolate Pic97605 (92/100), lanes 2 and 4) Costa Rican isolates ZE and CT (94/100), lane 3) Mexicann isolate Pic97310 (92/100), lane 5) Mexican isolate Pic97605 (92/100); lane 6) Mexican isolate Pic98366 (100/100).

Figure 3. 3 Mitochondrial DNA polymorphisms revealed by PCR-RFLP of Costa Rican isolates of *Phytophthora infestans*. A) PCR amplification with primer pair F4/R4 and digestion with EcoRI. B) PCR amplification with primer pair F2/R2 and digestion with MspI. Lanes 2-5 are control isolates of the Ia, Ib, IIa, and IIb haplotypes digested with *EcoR*1 and *Msp*I respectively. M = molecular marker, 100 bp.

Figure 3. 4 DNA fingerprint patterns with RG57 probe of *Phytophthora infestans*. Lane 1, US-1 genotype; Lane 2, US-8 genotype; Lanes 3 and 4, US-18 genotype, Lanes 5 to 9 Costa Rican isolates of the most common allozyme genotype found. RG57 fingerprint band numbers are indicated in the right.



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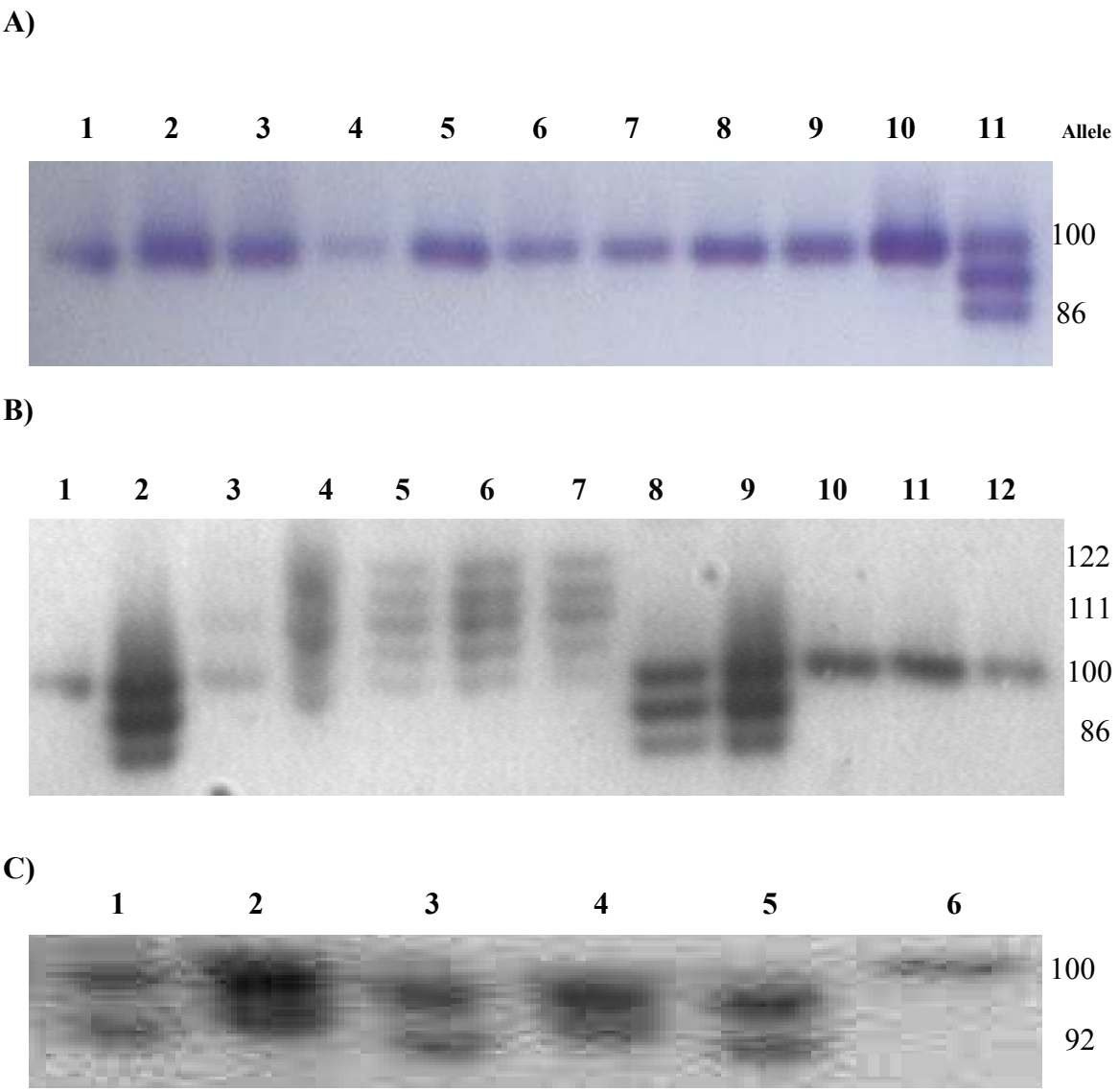

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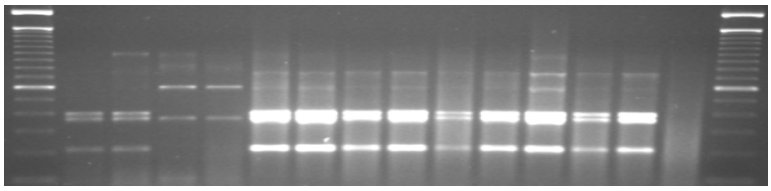
A)

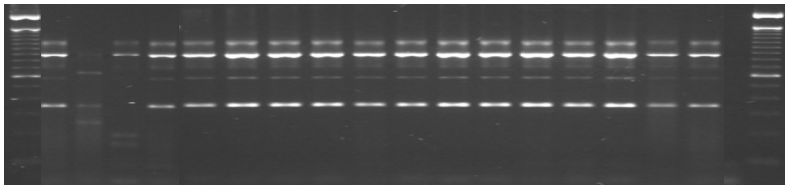
P4
*Eco*R1
digest



M Ia Ib IIa IIb

B)

P2
*Msp*I
digest



MIa Ib IIa IIb

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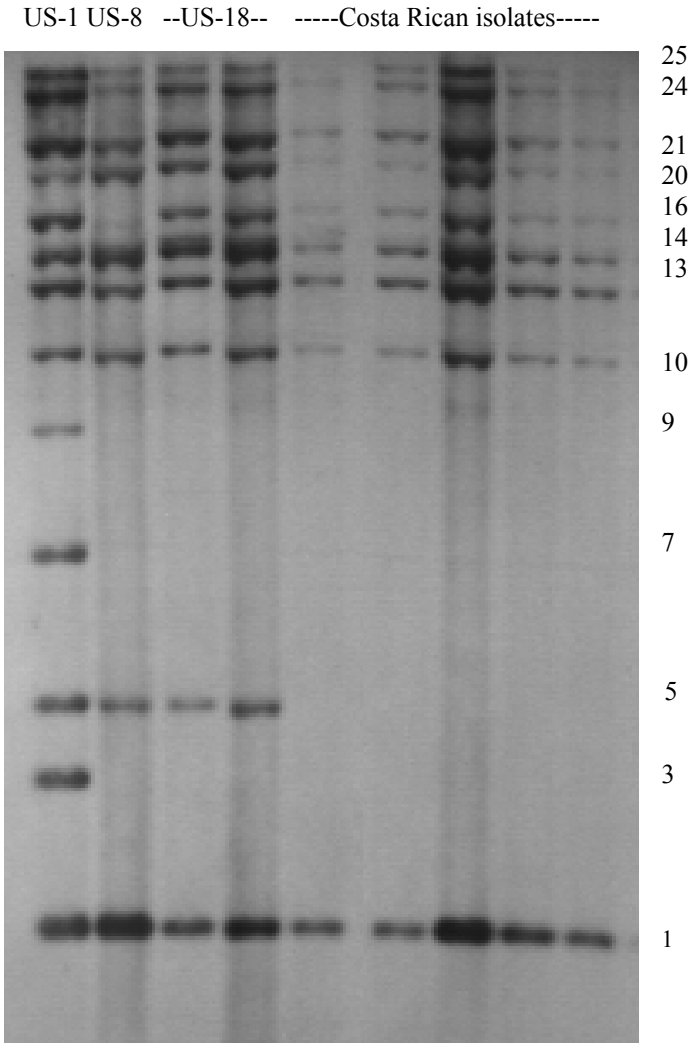

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