Monitoring changes in *Phytophthora* populations in developing countries and the Phytophthora.exe database

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Abstract

Phytophtora infestans is a pathogen that is constantly adapting to the changing environment, increasing its genetic diversity through mutation and because of this, late blight epidemics have become more difficult to manage worldwide. Microsatellites or single sequence repeats (SSR) have proven to be a useful technique to characterize and monitor changes in *P. infestans* populations. This molecular tool is faster and cheaper than previously-used methods, easy to compare across laboratories and therefore could facilitate integration of the various genetic data sets available for this important pathogen. Standardization of microsatellite and other data has recently been facilitated by the development of data-entry software called Phytophthora.exe, which was developed as part of the Eucablight project in Europe. The International Potato Center (CIP) and some partners have embarked on an effort to promote the use of microsatellite markers and Phytophthora.exe in developing countries. To this end, data from Peru and Ecuador have been entered in the global database housed in Denmark and plans are underway to enter data from a number of other locations in South America, Africa and Asia. Potential uses of the microsatellite markers and the global database are discussed.

Keywords: Phytophthora, microsatellites, database.

Introduction

Phytophthora infestans, the oomycete pathogen that causes late blight on potato, tomato and other solanaceous crops occurs around the world. Late blight is considered the most devastating disease of potatoes and one of the most serious of all plant diseases. In central Mexico and South America, *P. infestans* is a pathogen of many different wild solanum species (Chacon, *et al.*, 2006; Garry, *et al.*, 2005; Fry and Smart, 1999). In Canada and the United States, *P. infestans* has been reported to infect hairy nightshade (*Solanum sarachiodes*), bittersweet (*S. dulcamara*) and Petunia (*Petunia hybride*). While in Europe *Solanum nigrum* was found to be a host of the new diverse population of *P. infestans.* (Fry, 2008).

Crop losses and cost of fungicides to control late blight together are estimated in the billions of dollars (US) per year. In most potato-growing areas, frequent fungicide applications are the main method of disease control and this can cause damage to human health and the environment. For this reason, the avoidance of primary inoculum sources and the use of cultivar resistance are increasingly important to minimize costs and environmental impact. However, changes in the population structure of *P. infestans* discovered since the early 1990s make those strategies for late blight management less effective in controlling the disease. These new strains have increased the severity of late blight on potato, because genetic variation, adaptive abilities, aggressiveness and virulence have increased. In addition, in areas where both mating types A1 and A2 are present, oospores as a persistent infectious survival structure constitute another constraint (Turkensteen *et al.,* 2003). To combat these strains it is necessary to use more resistant potato cultivars and to use fungicides more effectively. Potato cultivars with desirable market quality that show high levels of resistance are being developed, particularly in developing countries. New methods of breeding include the use of wild potatoes as resistant sources. The effectiveness of such control strategies will be influenced by changes in the pathogen population and it is thus important to understand population change on local and wider geographical scales. For this reason, accurate and up to date information on fungicide resistance, sources of primary inoculum, and aggressiveness of the pathogen is critical.

To characterize *P. infestans* populations, phenotypic and genotypic markers have been developed, and have contributed to our understanding of the population genetics of *P. infestans*. Mating types (A1 and A2) (Judelson, 1996) provided the first indication of major changes in *P. infestans* populations. Allozyme markers for Glucose-6-

phosphate isomerase (*Gpi*) and Peptidase (*Pep*) loci (Tooley *et al*., 1985) provided the first molecular evidence of diploidy in *P. infestans*. Nuclear DNA fingerprinting has allowed much greater resolution of population structures. A total of 30 markers were identified by the restriction fragment polymorphism (RFLP) (Goodwin *et al*., 1992); mitochondrial DNA haplotypes (Griffith and Shaw, 1998) enable the tracking of specific lineages; neutral nuclear markers (AFLP) (Vos *et al*., 1995) and more recently the approach using simple sequence repeats (SSR) or microsatellites (Knapova and Gisi, 2002; Lees *et al*., 2006) have provided even greater resolution. In a recent review of genetic markers for *P. infestans*, SSR were proposed as a powerful choice because they are highly specific, single locus, codominant, highly polymorphic, highly reproducible and less amount of pathogen DNA is needed (Cooke and Lees, 2004).

One way that researchers and extension workers may enhance their capacity to help farmers deal with the "new" potato late blight is by improved collaboration and exchange of knowledge and information. To this aim, several initiatives were funded. One such initiative was a concerted knowledge sharing effort in Europe called EUCABLIGHT, a Late Blight Network for Europe (www.eucablight.org). An important component of the information sharing of this initiative is a common database of markers (fingerprints) of the pathogen throughout Europe. A program called Phytophthora.exe was developed to ensure standardization of data and facilitate data input. Phytophthora.exe also allows for automatic uploading to a centralized database on the pathogen and thereby makes more informed estimates of risk of introduction of new strains.

In order to standardize methods to get a uniform and accurate data set, microsatellite markers were used at CIP to characterize *Phytophthora* Peruvian populations. Furthermore, to monitor changes of pathogen population, all existing data about Peruvian and Ecuadorian *P. infestans* (and *P. andina*) isolates were uploaded to the EUCABLIGHT database using the program Phytophthora.exe version 2.0 developed for South America.

Materials and methods

Phytophthora isolates

A sample of twenty-two representative isolates of *Phytophthora infestans* from the CIP collection, that previously were characterized using RFLP markers, were examined using SSR. These isolates were collected from potato and wild potatoes from 1998 to 2000 in different potato areas in Peru (Garry, *et al*., 2005; Perez *et al*., 2001). Another fourteen isolates collected in 2005 from tree tomato (*Solanum betaceum*) were also examined. Some Ecuadorian isolates of *P. infestans* and *P. andina* with known SSR loci were used as markers to determine the size of the alleles at different SSR loci (R. Oliva, personal communication).

Genotypic characterization using SSR markers

Isolates were screened with eight microsatellite (SSR) markers developed specifically for *P. infestans* (Knapova *et al*., 2001; Less *et al*., 2006). PCR amplifications were performed in a 10 µl volume containing 5 ng of genomic DNA, 1 µl of 10x reaction buffer B (Promega), 0.01 mM of each dNTP, 0.2 µM each of forward and reverse primers (Table 1), and 0.7 U of *Taq* polymerase (Promega). PCR was performed in a MJ Research cycler under conditions indicated by Knapova *et al*. (2001) and Lees *et al*. (2006) for each marker. Microsatellite alleles were separated by running the reactions on a 6% denaturing acrylamide gel. Before loading, the mixture was denaturated by heating at 94°C for 5 min. A non-radioactive detection method was used and fingerprints were scored visually after silver staining. Cluster analysis was conducted using the unweighted pair-group method with arithmetic mean using the software program NTSYS-pc version 1.70 (Exeter Software, Setauket, NY).

Table 1. The eight microsatellites markers selected for this study

Marker	Repeat	SSR Primer sequence [a]	Size range (bp)	Annealing temp (°C)
4B	$(TC)_{34}$	F:AAAATAAAGCCTTTGGTTCA R:GCAAGCGAGGTTTGTAGATT	205-217	58
G11	$(TC)_{27}$	F: TGCTATTTATCAAGCGTGGG R: ACAATCTGCAGCCGTAAGA	142-166	56
1F	$(TC)_{?}$	F: GAGAGTGAATGAGAGCGAG R:ACAATCTGCAGCCGTAAGAG	94-166	59
Pi63	$(GAG)_{8}$	F:ATGACGAAGATGAAAGTGAGG R: ATTCATTATTGGCAATGTTGG	148-160	58
Pi66	$(GT)_{7}$	F: ACCGACAGCTTCTGAAACC R:AAAATAAGAAGAGATTCGTGCC	153-155	58
Pi89	$(AT)_{9}$	F: GAGAACGCACAATGTAAGGC R: ACATAAATACACGCTGAACGG	179-185	58
2D	$(TC)_{9}$	F: AATTGAGTGAATGCGTCACC R: TTTCCTGCTATCCTCAGCAC	155	58
D13	$(CT)_{27}$	F: TGCCCCCTGCTCACTC R: GCTCGAATTCATTTTACAGA	108-142	50

[a] F, forward primer; R, reverse primer

Data input, storage and management using Phytophthora.exe

A complete Version 2.0 of Phytopthora.exe for South America was installed on a PC in both CIP-Lima and CIP-Quito with instructions from the EUCABLIGHT homepage (http://www.eucablight.org). The user manual can be downloaded from the same webpage. The data entry tool Phytophthora.exe provides a user-friendly interface that facilitates data entry and its submission to the EUCABLIGHT database (Hansen *et al.*, 2006).

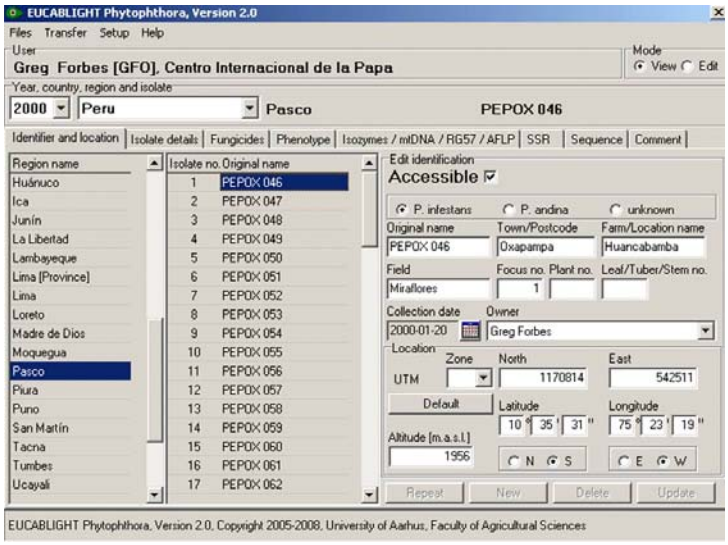


Figure 1. Computer screen showing a data entry page from Phytophthora.exe

All existing data of Peruvian and Ecuadorian *Phytophthora infestans* isolates were entered and stored. Information about country and year of collection for each isolate were required and "regionID" and "isolatedID" were also created (Fig. 1). Further information was filled in the 50 database fields for phenotypic (mating type, fungicide resistant, virulence), genotypic (SSR alleles, RFLP, mt DNA, allozymes) and cropping (cultivar, location, altitude) data. Data were uploaded to the central EUCABLIGHT database in Denmark, where they can be accessed in summarized form via the Internet. All primary data in the Eucablight database is owned by the supplier.

Results and discussion

The use of SSR markers to characterize Phytophthora isolates

From the eight selected microsatellite marker loci (Table 1), three (4B, G11, and 1F) were highly polymorphic and three (Pi63, Pi66 and Pi89) moderately polymorphic for the sample that was evaluated. The other two loci (2D and D13) were not polymorphic for these *P. infestans* isolates but they allow discrimination between *Phytophthora* species. The microsatellite analysis showed that isolates with identical RFLP genotype generally share identical alleles for each SSR marker. An exception was for the RFLP genotype US-1 which had different SSR alleles combinations. All the Peruvian isolates from tree tomato (*S. betaceum*) clustered together and they were genetically more closely related to Ecuadorian isolates from the Anarrichomenum complex (EC-2.1) than to Ecuadorian isolates from *S. betaceum* of *P. andina* (Fig. 2).

The congruence of results from both RFLP and SSR analyses, and the fact that SSR is cheaper and less time-consuming than RFLP, makes SSR markers an ideal tool for efficient analysis of *P. infestans* populations.

Analysis of data using Phytophthora.exe software

The database and Phytophthora.exe are ready to be used by countries outside Europe after minor adaptations. Phytophthora.exe is currently used at CIP, both in Peru and Ecuador. There are also plans to use this software in other countries of South America, Africa and Indonesia.

Phytophthora.exe is a user-friendly tool for the entry of primary data because minimal typing is necessary and uploaded data can be easily exported into common databases for analysis or for the exchange of data among partners. Furthermore, the strict input process in Phytophthora.exe helps avoid errors. Processed data can be seen online in graphs and tables (Fig. 3). Stored data from a number of locations will allow us to monitor changes in the pathogen population and to design effective strategies for late blight management according population structure. For example in Peru *Phytophthora* isolates are resistance to metalaxyl (Fig. 3), therefore, farmers should be aware of the inefficacy of products that include this component. This database has many practical applications like mapping pathogen diversity, in late blight simulation and forecasting. For instance, at the level of South America, the *P. infestans* A1 mating type is present in Ecuador, Peru, Colombia and Chile, while the A2 mating is present in Argentina, Bolivia and Brazil. The risk of presence of both mating types and therefore of sexual recombination, is highest in the area where these two populations meet. Similarly, the tracking of virulence and aggressiveness in *P. infestans* populations is also important. Late blight researchers in developing countries would benefit from greater information sharing and a globalization of the tools developed in EUCABLIGHT, particularly Phytophthora.exe

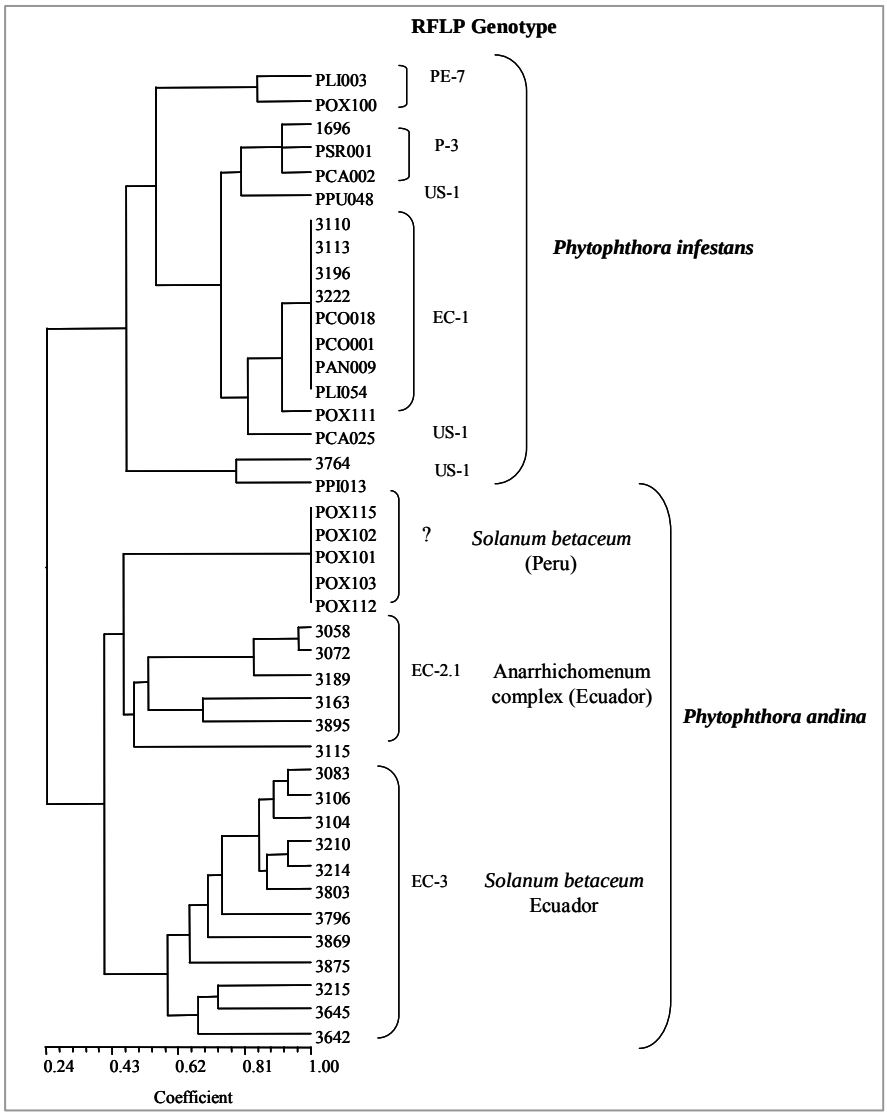
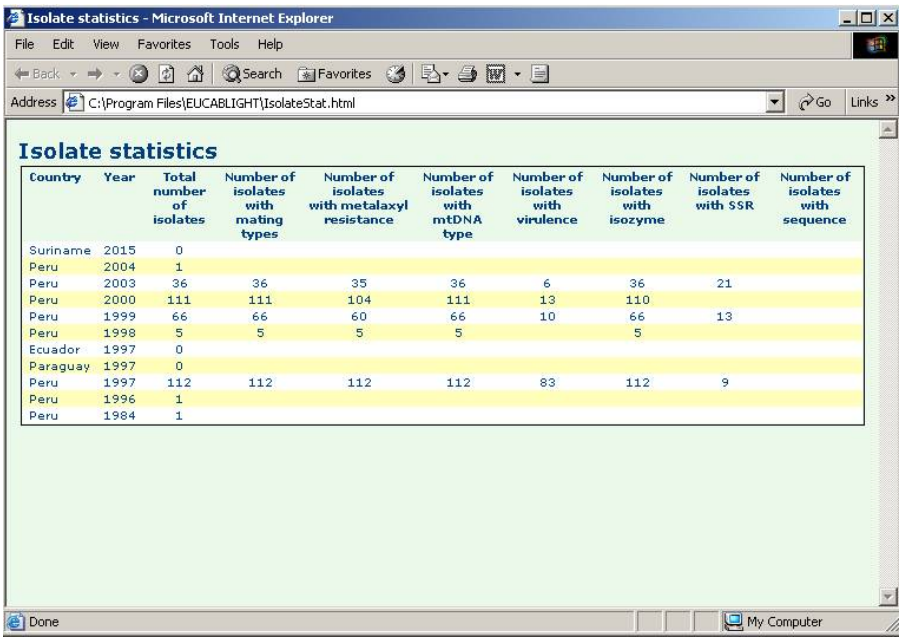


Figure 2. A phenogram derived from microsatellite markers, of isolates of Phytophthora *infestans* and *Phytophthora andina.* Phenogram based on the Dice similarity coefficient



Isolate statistics

Country	Year	Total number of isolates	Number of isolates with mating types	Number of isolates with metalaxyl resistance	Number of isolates with mtDNA type	Number of isolates with virulence	Number of isolates with isozyme	Number of isolates with SSR	Number of isolates with sequence
Suriname	2015	0							
Peru	2004	1							
Peru	2003	36	36	35	36	6	36	21	
Peru	2000	111	111	104	111	13	110		
Peru	1999	66	66	60	66	10	66	13	
Peru	1998	5	5	5	5		5		
Ecuador	1997	0							
Paraguay	1997	0							
Peru	1997	112	112	112	112	83	112	9	
Peru	1996	1							
Peru	1984	1							

Figure 3. Summary table for selected isolates from Ecuador, Peru and Paraguay from the Eucablight central database in Denmark (www.eucablight.org)

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