

8-2008

# Generating Genetic Resources for *Phytophthora capsici* (L.) and Studying *P. capsici* and *Phytophthora* Hybrids in Peru

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To the Graduate Council:

I am submitting herewith a dissertation written by Oscar Pietro Hurtado-Gonzales entitled "Generating Genetic Resources for *Phytophthora capsici* (L.) and Studying *P. capsici* and *Phytophthora* Hybrids in Peru." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

Kurt H. Lamour, Major Professor

We have read this dissertation and recommend its acceptance:

Feng Chen, John K. Moulton, Beth Mullin

Accepted for the Council:
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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Bonnie H. Ownley

Accepted for the Council:

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Carolyn R. Hodges, Vice Provost
and Dean of the Graduate School

(Original signatures are on file with official student records.)

# Generating genetic resources for *Phytophthora capsici* (L.) and studying *P. capsici* and *Phytophthora* hybrids in Peru

A Dissertation
Presented for the Degree Of
Doctor Of Philosophy

University Of Tennessee, Knoxville

**Oscar Pietro Hurtado-Gonzales**
**August 2008**

# Acknowledgments

I would like to thank God for the gift of Life.

I would like to thank to my family (far and near) because they are a source of inspiration and constant motivation to move forward. I want to thanks my wife Sarah Hendricks for her moral support and proof reading of this document.

I would like to express my gratitude to my advisor Dr. Kurt Lamour for giving me the great opportunity to work under his mentoring and for all the good lessons to become a better researcher and a better human being. I would like to thank to the members of my committee Dr. Feng Chen, Dr. John Moulton, Dr. Bonnie Ownley, and Dr. Beth Mullin for the availability and support throughout my years at UT. I would like to extend my acknowledgments to all faculty, students, and departmental staff from the Department of Entomology and Plant Pathology who have made my days in the school more enjoyable.

In memory of my beloved Mother

Cristina

# Abstract

The genus *Phytophthora* includes more than 90 described species infecting over 1000 plant species. Population studies were conducted to investigate the survival and spread of *P. capsici* in the Peruvian coastal region. A total of 227 *P. capsici* isolates, recovered at widely distant localities from 2005-2007, were fingerprinted with AFLPs and SNP genotyping. A clonal population (PcPE-1) represented by 221 isolates was found to be distributed throughout the country. Atypical isolates of *P. nicotianae* were isolated from loquat trees in Peru and nuclear (internal transcribed spacer [ITS], the phenol acid carboxylase gene, and AFLPs) and mitochondrial genotyping (cytochrome oxidase gene [*cox*I]) identified this species as a hybrid between *P. nicotianae* and *P. cactorum*. A comparison of five *Phytophthora* hybrid isolates from Peru and Taiwan (also infecting loquat trees) suggested that isolates from Peru likely originated from a single hybridization event and that the two isolates from Taiwan originated through different hybridization events.

The generation of genetic resources for the study of complex genetic traits in *P. capsici* was initiated by studying its inbreeding up to the sixth generation. A total of 692 oospore-derived isolates were fingerprinted and a subset was characterized for pathogenicity in cucumber and jalapeno fruits and for segregation of the mating type. The traits tested revealed no-Mendelian segregation, and apomixis were observed to be more prevalent (100%) in deep (fifth generation) inbreeding crosses. Inbreeding was measured by studying

the segregation of 20 AFLP markers, which indicated a loss of heterozygosity of ~75% by the sixth generation. The seminal cross from this study was used as a mapping population ($F_1$) for generating a genetic linkage framework with 189 AFLP and 18 SNP markers. A total of 18 linkage groups were produced for each parental isolate using 65 and 42 markers for CBS121657 and CBS121656 isolates respectively covering 409 cM. SNP markers FL5 and FL6 were used for estimating the genome size of *P. capsici* and precision of the genome assembly.

In order to conduct functional studies in *P. capsici*, we tested the efficacy of the polyethylene glycol mediated transformation. We regenerated up to 30 antibiotic resistant isolates and 53% of them were stable after three months of subculturing.

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# Chapter One Introduction

Currently the genus *Phytophthora* includes more than 90 described species infecting over 1000 plant species, arguably making *Phytophthora* the most devastating pathogens of dicotyledonous plants (1, 8, 11, 16, 18). The genus *Phytophthora* is classified in a unique group of organisms known as oomycetes (1). Due to their fungal-like morphology, *Phytophthora* were historically considered fungi. Detailed physiological, biochemical and phylogenetic studies placed oomycetes in a unique lineage of eukaryotic organisms more related to brown algae and greatly distant from true fungi. Among these differences between fungi and oomycetes we can mention that: (i) oomycete cell walls are primarily composed of β-1,3-glucans and lack or contain small only proportions of chitin; (ii) oomycetes are sterol and thiamine auxotrophs and their membranes are composed of unusual lipids, (iii) the major part of an oomycete's life cycle is diploid, unlike filamentous fungi which are haploid, (iv) oomycete hyphae is not septate, and (v) oomycetes produce biflagellate swimming spores (1). Due to these differences, most strategies to control fungi are not effective against oomycetes (6, 17, 21).

*Phytophthora infestans*, the causal agent of potato late blight and the Irish famine in the 19$^{th}$ century, is the most thoroughly studied oomycete (2, 3). Research conducted in this pathosystem has greatly contributed to our current understanding of the different biological processes in this group of organisms. At the present time there are three oomycete draft genomes (*P. sojae*, *P. ramorum*, and *P. infestans*) and two more are underway (*P. capsici* and *Hyaloperonospora parasitica*). The current genomic era, with improved technological capabilities, offers oomycete researchers the opportunity to pursue more comprehensive and detailed studies (4, 7, 22).

Although oomycete and filamentous fungi are phylogenetically distant, they share common strategies of invasion (17). Both the fungal rice blast pathogen *Magnaporthe grisea* and *P. infestans* develop specialized pre-penetration structures or terminal swellings called appresoria (5, 20). The appresorium allows the pathogen to penetrate the hydrophobic cuticle of the host. A combination of secreted lytic enzymes and external force (turgor pressure) permits the pathogen to enter, forming a penetration peg. The penetration peg develops into an infection vesicle that subsequently ramifies inter- and intracellularly throughout the plant cells (10). *Phytophthora* usually adopts a hemibiotrophic lifestyle. During the early stages of infection, *Phytophthora* keeps the host cells alive, but the pathogen subsequently secretes specific proteins into the host (known as necrosis-induced proteins, NIPs) that induce cell death (19). *Phytophthora* then spreads rapidly through the host and then specialized tissue (also known as sporangium) emerges from natural openings in the host. Sporangia contain non-differentiated cytoplasm that undergoes cleavage after perceiving certain environmental cues. Cytoplasmatic cleavage results in the formation of small uninucleate cells known as zoospores that are released from the sporangium and swim by means of two flagella. Zoospores perceive specific exudates from the root of the host (chemotropism) and encyst once they contact the host surface. Zoospores can then start a new cycle of infection.

During host colonization *Phytophthora* also secrets a battery of proteins (known as effector proteins) that are believed to evade the plant immune system and to modulate the transcriptional host machinery (9). Data mining of *Phytophthora* genome sequences

complemented with ectopic expression assays have determined that a group of these effector proteins contain an aminoacidic motif known as RxLR (7).

*Phytophthora capsici* (L.) is a soilborne plant pathogen distributed worldwide. It is capable of infecting a wide variety of hosts including members of the Solanaceae, Leguminoseae and Cucurbitaceae plant families (13). Consequently, losses due to *P. capsici* outbreaks have serious economic implications. *Phytophthora capsici* is an outcrossing species, requiring both mating types (A1 and A2) to complete the sexual cycle. In the United States, fields naturally infected with *P. capsici* have both mating types. The sexual stage is under strong selection pressure because *P. capsici* does not produce thick-walled asexual chlamydospores and the primary hosts (cucurbits, tomatoes, and peppers) are unavailable during the winter months (15). For this reason, U.S. populations carry a large amount of genetic variation, and in many cases the isolates are highly fecund (12-14). When studying *P. capsici* under laboratory conditions, the life stages of this pathogen easily generated. All these characteristics point to *P. capsici* as an attractive organism for studying oomycetes.

In chapter two, the genetic diversity of *P. capsici* populations in pepper fields from Peru is presented. Peru is located in the west side of South America and is considered a center of diversity for *Capsicum* species, the main hosts for *P. capsici*. Genetic diversity was studied using amplified fragment length polymorphic (AFLPs) and single nucleotide polymorphism (SNPs) markers. Our studies concluded that *Phytophthora capsici* has the profile of an introduced pathogen in the coastal fields in Peru, and that isolates with exactly the same AFLP profile survived for at least three years. We only identified one mating type in all sampled fields (A2). *Phytophthora*

*capsici* propagates clonally in coastal Peru and shows a very low genetic variability, in contrast to the high genetic diversity found in naturally infested fields in the U.S. This finding suggests that Peruvian pepper farmers would only need to deploy a small set of resistant pepper varieties combined with good agricultural practices to control *P. capsici* outbreaks.

Our interest in studying different species of *Phytophthora* led us to focus on an interesting group of isolates collected from dying loquat trees in Peru. Studies of these isolates are presented in the third chapter. Our collaborator in Peru, Professor Liliana Aragon, brought to our attention this *Phytophthora* species that was difficult to identify using classical morphological keys. We employed molecular tools (AFLP fingerprinting, nuclear gene sequencing, and mitochondrial inheritance using hi-resolution DNA melting analysis) to determine the identity of this pathogen. Reports of a similar pathogen infecting loquat trees in Taiwan also directed us to investigate and compare the isolates from Taiwan and compare results from both locations.

Our analysis shows that these isolates were natural hybrids between the species *P. nicotianae* and *P. cactorum*. Moreover, our results suggest that these *Phytophthora* hybrids from Peru likely originated from a single hybridization event, that the two isolates from Taiwan originated through different hybridization events, and that the hybrids in Peru have persisted over at least three years at three separate field locations as a clonal pathogen.

In a separate line of study, homozygous lines for *P. capsici* were developed with an aim toward studying complex genetic traits, such as sporangiogenesis, oosporogenesis, and pathogenicity that are believed to be ruled by an orchestrated expression of several

genes. The current models used for the study of biology in oomycetes, such as *P. sojae*-soybean and *P. infestans*-potato, focus their attention on a particular gene, or gene family, under the genetic background of one or very few isolates. Studies that exploit naturally occurring variations (e.g., SNPs) found in *Phytophthora* isolates have not been possible because of the limited ability of generating laboratory crosses with measurable phenotypes.

As mentioned above, *P. capsici* is highly fecund, the generation of laboratory crosses is routine, and natural US populations carry large amount of genetic variation. Thus, *P. capsici* is an attractive model for conducting experiments in quantitative genetic analysis of complex traits in oomycetes by employing recombinant inbred lines, near isogenic lines, and other genetic resources. These resources have already proved to be useful in the determination of complex traits and in the study of genetic networks in other organisms, such as maize, fruit fly, potato, etc.

In the fourth chapter, the possibility of generating recombinant inbred lines in *P. capsici* is explored. Laboratory crosses were conducted up to the sixth generation of inbreeding. Loss of heterozygosity was performed by tracking the segregation of 20 AFLP markers through five generations, resulting in the generation of oospore-derived isolates with more than 60% of their alleles fixed to homozygosity. Subsets of these laboratory populations were tested for their pathogenicity on cucumber and jalapeno fruits, and mating type. SNP genotyping was required for an accurate identification of recombinant progeny by the fourth generation. We observed that either oospore-derived isolates recovered from the different crosses with exact AFLP profiling or SNP genotyping profile was prevalent during our crosses. The entire progeny of two crosses

from the fourth generation of inbreeding yielded this type of clonality with the same genotype as one of the parents, phenomena that we called apomixes. An oospore-derived isolate from the second backcross generation was selected for the genome sequencing project for its reduced heterozygosity to facilitate the genome assembly.

The development of genetic resources for studying quantitative traits required the association of loci to the trait under study in a segregating population. Genetic linkage maps are the first step in building these resources. In the fifth chapter, DNA markers (AFLP and SNP genotyping) were employed to build genetic linkage maps in *P. capsici*. Oospore progeny derived from a cross generated in the third chapter were used as a mapping population. One hundred eighty-nine clearly resolvable AFLP markers were generated with 16 primer combinations plus 18 SNPs with 18 sets of primer pairs.

Our analysis generated 18 linkage groups at LOD (Logarithm of Odd) threshold values between 4 and 6 for parent CBS121656 and 18 linkage groups for CBS121657. We also mapped 10 SNP markers. SNP markers were useful for comparison between their physical location in the genome assembly and their location in the genetic linkage groups.

After the identification of candidate gene(s) associated with the trait of interest using the resources described in chapter three and four, functional analyses were required for further validation. One method that can provide further validation is through activation of intrinsic gene silencing machinery by overexpression of a particular gene.

In chapter six, a transformation technique currently used with *P. sojae* was adapted for use with *P. capsici*. The technique was based on the transformation of protoplast via polyethylene glycol and $CaCl_2$. We generated protoplast from isolate

CBS121656 and then transformed the protoplasts with three different constructs (pNC-GFP, pHA, and pUBIN) using the selectable antibiotic G418 (geneticin). We recovered a total of 30 antibiotic resistance isolates (50 µg/ml) and confirmed their identity with PCR. Isolates were subcultured at least three months under selective and non-selective media and observations were made for their stability. Although transformation using the PEG protocols remains the favorite among different oomycete laboratories, this method still generates a high percentage (65%) of non-stable transformants that can lose their ability to grow on selective media.

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# Chapter Two Survival and spread of *Phytophthora capsici* in coastal Peru

**ABSTRACT**

*Phytophthora capsici* Leonian is a soilborne pathogen that causes significant losses to pepper production in Peru. Our objective was to investigate the mechanisms by which *P. capsici* is able to survive and spread. During 2005-2007, 227 isolates of *P. capsici* were collected from four species of pepper (*Capsicum annum*, *C. baccatum*, *C. chinense*, and *C. pubescens*) and tomato (*Solanum lycopersicum*) at 33 field sites in 13 provinces across coastal Peru. All 227 isolates were of the A2 mating type and AFLP (amplified fragment length polymorphism) analysis indicated that 221 of the isolates had the same genotype. Analyses of six polymorphic SNP (single nucleotide polymorphism) loci showed fixed heterozygosity suggesting a single clonal lineage is widely dispersed. Members of the same clonal lineage were recovered during 2005-2007 from geographically separate locations from each of the host types sampled. Our results indicate that clonal reproduction drives the population structure of *P. capsici* in Peru. The impacts of continuous cropping and irrigation from common river sources on the population structure in Barranca valley are discussed.

**INTRODUCTION**

*Phytophthora capsici* Leonian is a soilborne plant pathogen that causes fruit, crown and root rot in a wide range of vegetable hosts including peppers, tomato, squash, melons, and most recently green and lima beans (5, 9, 29, 35, 60). *Phytophthora capsici* was first described as a pathogen of pepper in New Mexico (42). Since the initial species description, *P. capsici* has been reported on additional hosts (18, 28, 30, 32-34, 44, 46,

50, 58, 68, 72). *Phytophthora capsici* can produce massive amounts of asexual deciduous sporangia on infected host and large-scale epidemics can be initiated from a limited number of infected plants (62). Unlike many other *Phytophthora* diseases that are favored by cooler temperatures, disease is favored by warm (25°C to 28°C) and wet conditions (9). *Phytophthora capsici* is heterothallic requiring the interaction of two mating types (A1 and A2) to complete the sexual stage and produce thick-walled sexual oospores. Oospores are capable of surviving in the soil for several years, germinating in favorable environmental conditions (9, 16, 49, 61). In the U.S., both mating types are found at most locations and oospores are thought to play an important role in the epidemiology (36-39, 58). In addition, *P. capsici* has been isolated from creeks used for irrigation of vegetables in Michigan, and it has been shown to infect diverse weeds (15, 19, 56). In many cases crop rotation has not reduced the residual inoculum sufficiently to provide effective protection (40).

In Peru, *Phytophthora capsici* was first reported as the causal agent for wilt and root rot infecting several *Capsicum* spp. in 1971 (10). Since then, no other investigation has been conducted despite increasing losses due to *P. capsici* for pepper growers in Peru. Various practices are applied to control the epidemics, such as moderate irrigation, the use of well-drained fields, crop rotation, and fungicide treatments (3, 48). The Andean region of Peru-Bolivia is considered one of the centers of origin for species within the genus *Capsicum*. Archeological studies in coastal Peru have revealed evidence for the presence of *Capsicum* spp. dating back 4000 years (54). It is not known how long *P. capsici* has been present in and around the *Capsicum* center of origin, nor is it known whether *P. capsici* infected other ancient local crops, such as cucurbits, prior to *Capsicum*

(6). Several varieties of *C. annuum*, as well as other species of *Capsicum* such as *C. baccatum*, *C. chinense*, and *C. pubescens*, are cultivated across the coastal area of the country and several other *Capsicum* spp. are grown in the Amazon (55). Thus, a high diversity of the host may impact the pathogen population structure and evolution (73). A better understanding of the mechanisms by which *P. capsici* is surviving and spreading will assist in developing effective management and breeding strategies.

Our primary objective was to characterize the survival and spread of *P. capsici* in pepper production areas of coastal Peru. We report on the absence of diversity in *P. capsici* recovered from peppers and tomatoes from across coastal Peru for mating type, AFLP and SNP markers, and we present a fine scale genotypic analysis of *P. capsici* recovered from pepper in the Barranca valley.

## MATERIALS AND METHODS

### Isolate recovery and sampling scheme

Samples collected in 2005 (n=23) were received as standard diagnostic specimens and processed by the diagnostic clinic at the National Agricultural University La Molina (Lima, Peru). Isolates were recovered from seven different hosts at 23 field sites (Fig. 2.1 and Table 2.1; tables and figures are located in appendices). During 2006-2007, more intensive sampling was performed at ten pepper fields located in Barranca, a coastal province 120 miles north of the city of Lima. The fields spanned four districts: Barranca, Supe, Caral and Minas (see Table 2.2 and Fig. 2.2). Fields were previously cultivated with the following hosts: tomato, potato, paprika, and/or artichoke. Fields varied in size from 3 to 10 hectares and isolates were recovered from the following *Capsicum* species:

*C. annum*, *C. baccatum*, *C. chinense*, and *C. pubescens*. Plants varied in age from 3 weeks-old to 3 months-old and wilting was the primary symptom of infected plants. Adjacent plants within a field were avoided. All of the isolates were recovered from the crown region of infected plants. A minimum of 12 symptomatic plants were collected per field and transported to Lima for pathogen isolation at the diagnostic clinic (National Agricultural University, La Molina). *Phytophthora capsici* culture collection isolates CBS121656 (isolated from infected cucumbers in Michigan, U.S. in 1997) and CBS121657 (isolated from infected pumpkins in Tennessee, U.S. 2004) were also included in the study.

For pathogen isolation, infected plants were thoroughly washed with tap and sterile distilled water. Small sections of tissue were excised from the edge of an expanding lesion at the crown level and transferred to potato dextrose agar plates amended with PARP (100 ppm of pimaricin, 100 ppm of ampicillin, 30 ppm of rifampicin, and 100 ppm of pentachloronitrobezene). Plates were incubated for 2 days at room temperature. Hyphal tips were sub-cultured from actively expanding mycelium and transferred to V8 juice agar amended with PARP. For long term storage, agar plugs of mycelium were stored in 2 ml screw cap tubes with 1 ml of sterile distilled water and three sterile hemp seeds.

To determine mating type of *P. capsici* isolates, plugs of actively expanding mycelium were placed at the center of V8 juice agar plates approximately 2 cm distant from the "tester" isolate CBS121656 (mating type A1) or CBS121657 (mating type A2). Plates were wrapped with Parafilm® and incubated in the dark at room temperature for at least one week, after which observations were made for the production of oospores at the

interface using a light microscope. Isolates able to produce oospores when crossed against CBS121656 were determined as A2 mating type. Conversely, isolates that were able to form oospores when crossed against CBS121657 were determined as A1 mating type.

**DNA isolation and AFLP analyses**

To determine genetic similarity among *P. capsici* isolates, AFLP profiles were generated for all 227 isolates. Isolates were grown and treated as previously described for DNA isolation (41). AFLP genotyping was done using *Eco*RI and *Mse*I restriction endonucleases, adapters, and primers as described by Vos et al. (70). Pre-selective amplification was done using no selective nucleotides (Eco+0/Mse+0) and selective amplification was conducted with selective primer pair (Eco+CG/Mse+CG). Selective amplifications were diluted and labeled in a separate reaction according to Habera et al. (25). AFLP fragments were resolved and analyzed on a CEQ$^{TM}$ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) following the manufacturer's protocols. A 600 bp DNA size standard was used to resolve AFLP fragments (Beckman Coulter, Fullerton, CA). AFLP marker sizes ranged from 70 to 500 bp. AFLP profiles were generated twice for each isolate using independent DNA extractions. A binary matrix was constructed using only clearly resolved, replicated markers. Using the program NTSYSpc 2.11a (Exeter Software, Setauket, NY), the combined data matrix was used to construct a genetic similarity matrix of all possible pairwise comparisons of individuals using Jaccard's similarity coefficient: $GS(ij) = a/(a + b + c)$. $GS(ij)$ is the measure of genetic similarity between individuals $i$ and $j$, where $a$ is the number of polymorphic bands

shared by *i* and *j*, *b* is the number of bands present in *i* and absent in *j*, and *c* is the number of bands present in *j* but absent in *i*. A dendrogram was constructed by employing an unweighted pair-group method with arithmetic average (UPGMA) cluster analysis. Bootstraps values were generated in PAUP version 4.0b10 with 1000 replicates (66).

**SNP genotyping**

Twenty isolates from the Barranca valley (2006-2007) and 23 isolates from the diagnostic clinic in 2005 were selected for subsequent analyses using six nuclear SNP markers (Table 2.3). The SNP markers were heterozygous in at least one of the samples tested. Custom TaqMan® SNP Genotyping Assays (Foster City, CA) were designed according to the manufacturer's instructions (Table 2.3). SNP assays were performed using 7.5 µl of iQ™ Supermix (Bio-Rad), 0.325 µl of TaqMan® probe/primers allelic discrimination cocktail (40X), 2 µl of DNA (~15-20 ηg) and 3.2 µl of molecular biology grade $H_2O$. PCR reactions were carried out in 96-well plates (Bio-Rad) in triplicates for each isolate on an iQ5 Real-Time thermal cycler (Bio-Rad) using the following parameters: 95°C for 10 minutes, 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. Each assay was standardized for their optimal cycling PCR conditions. Results were analyzed with the accompanying iQ5 optical system software 1.0 (Bio-Rad) using the allelic discrimination option, adjusting parameters for Ct values and RFU (relative fluorescent units) according to the manufacturer's instructions. Results were scored as: Allele 1 = 11 (FAM), Allele 2 = 22 (VIC) and Heterozygous = 12. A concatenated similarity tree with bootstraps (AFLP and SNP) was built as described above.

## RESULTS

### AFLP genotyping and mating type

AFLP using the Eco+CG/Mse+CG primer pair generated 50 reproducible markers. Four of the 50 alleles identified were only found in the U.S. isolates (CBS121656 and CBS1211657). Isolates recovered from the Barranca valley in 2005 (n=2) and 2006-2007 (n=204, represented by isolate LT3738) were found to exhibit a single AFLP genotype with 35 AFLP markers. Seventeen of the twenty-three isolates submitted to the clinic in 2005 had the LT3738 genotype. Of the remaining clinic isolates all except one had a different AFLP genotype composed of 45 AFLP markers (represented by isolate LT2137). Representative isolates LT3738 and LT2137 had 34 AFLP markers in common. The last isolate (LT2145) had a unique AFLP genotype consisting of 37 AFLP markers. AFLP genotypes LT3738 and LT2137 were identified as clonal types and are referred to as PcPE-1 and PcPE-2.

Isolates belonging to the clonal lineage PcPE-1 were recovered from widely dispersed geographic fields (e.g. Ica and Trujillo, Fig. 2.1) whereas isolates from PcPE-2 were confined to Lima and Ica (Fig. 2.1). Mating type analysis revealed that all 227 isolates were the A2 mating type.

### SNP and genotyping

Analysis of six SNP markers showed a strong correlation with the AFLP analysis. Thirty-four of the 37 isolates identified as the PcPE-1 type using AFLP had SNP profiles identical to PcPE-1, five isolates with the PcPE-2 AFLP profile had SNP profiles identical to PcPE-2, and LT2145, which had a unique AFLP profile, also had a unique

multi-locus SNP profile (Table 2.4). Two isolates identified as the PcPE-1 type using AFLP (LT3726 and LT3762) had a single SNP marker, SNP 15, changed from AG to AA (Table 2.4). Additionally, LT3753 identified as PcPE-1 with AFLP had two SNP markers changed from hetero- to homozygosity; URA3 changed from AG to GG, and SNP 15 changed from AG to AA (Table 2.4). Clone corrections of the 227 isolates reduced the total number of unique multi-locus genotypes to five and for all six SNP markers only two of the three possible genotypes were observed. Due to the small size of the clone corrected data set, Hardy-Weinberg and other population genetic metrics that are based on allele frequencies were not calculated. A UPGMA cluster analysis combining AFLP and SNP markers revealed that the two distinct clusters (PcPE-1 and PcPE-2) shared ~70% of the markers (Fig. 2.3).

## DISCUSSION

This is the first large-scale spatiotemporal population study of *P. capsici* infecting peppers and tomatoes in Peru using molecular tools. *Phytophthora capsici* is a major biological threat to peppers in Peru and our objective was to better understand how *P. capsici* is surviving and spreading. There are few population studies characterizing the genotypic diversity of *P. capsici* outside of the U. S. and a limited number of studies indicating the frequency of the A1 or A2 mating types at specific sites. In Northwest Spain, 16 isolates from 11 locations had a low level of genetic variation based on RAPD analysis and all were a single (A1) mating type (63). Additional reports of only a single mating type include reports from Bulgaria (45 isolates, all A1), southern Italy (60

isolates, all A2) and southeastern Spain (3 isolates, all A1) (27, 31, 52). Both mating types have been reported from Brazil, Canada, Mexico and northern Italy (1, 11, 45, 67).

AFLP and SNP analysis of 227 *P. capsici* isolates recovered from 2005 to 2007 from four different *Capsicum* spp. and tomato at 33 locations throughout Peru revealed a surprisingly homogenous population with much lower genotypic and overall genetic diversity compared to populations analyzed in the U.S. (26, 38, 39). A single clonal lineage (PcPE-1) accounts for 221 of the isolates and this clonal type was recovered from all of the locations sampled except for three isolates from the Ica Valley (Fig. 2.1, Fig. 2.3). Also surprising, but consistent with the molecular data, was the finding that all of the isolates had the A2 mating type. For the U. S. populations that have been analyzed in detail, the overall picture is significantly different. In the U. S., both the A1 and A2 mating types have been recovered from many different locations and the genetic structure clearly reflects the impact of sexual recombination on the overall population biology (4, 12, 26, 30, 33, 36, 38, 40, 51, 58). In Michigan, the expansion of clonal lineages is common within single fields during a single year; but spread of clonal lineages over long distances or survival over the winter has not been detected (39, 40).

Although AFLP data showed no new AFLP genotypes in the Barranca valley from 2005 to 2007, SNP genotyping of a subset of isolates revealed changes from heterozygosity to homozygosity at two loci; SNP15 and URA3 (Table 2.4). Since the overall AFLP profile is identical to the PcPE-1 clonal type, there is no evidence that sex has occurred and most likely these changes are due to some kind of mitotic effect (e.g. gene conversion) (20). Mitotic recombination as well as mutation and gene conversion

are thought to be sources of variation for *Phytophthora* spp. that spread as large clonal lineages such as *P. infestans*, *P. cinnamomi*, and *P. ramorum* (7, 21, 57).

Currently it is unclear how a single clonal lineage has spread throughout coastal Peru. In the U.S., *P. capsici* does not appear to be spread long distances aerially similar to *P. infestans* and movement is most likely via infested water, soil, or plant parts (59). The finding of a limited number of widely dispersed clonal lineages parallels the situation with *P. infestans* in Peru where clonal lineages define the population structure. For both *P. capsici* and *P. infestans* migration of the opposite mating type (A1 for *P. capsici* and A2 for *P. infestans*) could significantly impact the overall diversity of populations and may contribute to increased crop losses and pathogen survival (2, 8, 13, 14, 17, 21-24, 53, 64, 65, 69).

Coastal Peru is a very dry agricultural system with very little annual rainfall (less than 50 mm/year) and above-ground infection and sporulation is generally not observed. The geography at many of the sites sampled precludes infested irrigation water accounting for the observed population structure as they are separate valleys and rely on separate water sources. Further investigations of movement on seedlings and/or seed are warranted. For the isolates collected from the ten fields in the Barranca valley, the situation may be easier to explain. The pepper cropping season in the Barranca coastal areas starts in October and plants are mature in April. Further up the valley, at a higher elevation (Caral and Minas districts), the climate allows and farmers practice year-round pepper cultivation. Irrigation water in the Barranca valley comes from the Supe River, which runs west from the slope Andean mountains to the Pacific Ocean (Fig. 2.2) and it is possible that *P. capsici* outbreaks occurring in higher areas of the valley contribute to

the incidence of pepper root rot and crown rot in lower parts of the valley. In addition, alternative explanations such as movement of plant seedling, tools, and survival of latent mycelium or sporangia in plant debris, should not be excluded. Current methods to control *P. capsici* include avoiding excess water in the plant rhizosphere through conservative irrigation and planting on raised beds. Drip irrigation is employed in some cases, but not all farmers have access to this technology. In light of our findings it will be important to test the above-ground irrigation water for the presence of *P. capsici*.

Peppers, originally cultivated in South America, now have more than five cultivated species and over twenty wild species (27). Archeological studies revealed deposits of cultivated types of *Capsicum baccatum* dating back 4000 years in coastal areas in Peru (54) and a major portion of *Capsicum* evolution appears to have occurred in the south-central Peruvian and Bolivian Andes (43). Our studies suggest that *P. capsici* in Peru has the profile of an introduced pathogen. Additional investigation into the population structure of *P. capsici* at other locations in Peru (e.g. *C. pubescences* cultivated in the Amazon, in the eastern side of the Andes) and other areas in South America may provide some clues to the current situation and may help identify current and past possible routes of dissemination.

Breeding for resistance against *P. capsici* has been challenging due to the high diversity of pathogen populations in the U.S. (47, 71). If the genotypic clonality of *P. capsici* in Peru is reflective of fewer race types of *P. capsici*, then this could offer the advantage for pepper breeders to generate resistant pepper lines that protect against one or a few clonal lineages. In light of the mitotic genetic changes that may be occurring

within the context of a clonal population it will be important to include a panel of clonal isolates from different geographic locations in the screening program.

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## APPENDIX 2

### Tables

Table 2.1. *Phytophthora capsici* isolates collected during 2005 from pepper and tomato in Peru.

| Isolate | Isolation date | MT[1] | Origin (Province, State) | Host |
|---|---|---|---|---|
| LT2135 | 01/24/05 | A2 | Viru, La Libertad | *Capsicum annum* |
| LT2136 | 01/25/05 | A2 | Huaral, Lima | *Solanum lycopersicum* |
| LT2137 | 01/25/05 | A2 | Carabayllo, Lima | *Solanum lycopersicum* |
| LT2138 | 01/27/05 | A2 | Huaral, Lima | *Solanum lycopersicum* |
| LT2139 | 01/27/05 | A2 | Santa Rosa Irrigation, Lima | *Capsicum annum* |
| LT2140 | 02/02/05 | A2 | Santiago Valley, Ica | *Solanum lycopersicum* |
| LT2141 | 02/14/05 | A2 | Santa Rosa Irrigation, Lima | *Capsicum annum* |
| LT2142 | 03/08/05 | A2 | Pampas de Villacuri , Ica | *Capsicum annum* |
| LT2143 | 04/27/05 | A2 | Nepeña, Ancash | *Capsicum annum* |
| LT2144 | 05/04/05 | A2 | Barranca, Lima | *Capsicum chinense* |
| LT2145 | 05/10/05 | A2 | Viru, La Libertad | *Capsicum annum* |
| LT2146 | 05/12/05 | A2 | Mala, Lima | *Capsicum baccatum* |
| LT2147 | 05/12/05 | A2 | Mala, Lima | *Capsicum baccatum* |
| LT2148 | 05/31/05 | A2 | Chincha, Ica | *Capsicum annum* |
| LT2149 | 05/31/05 | A2 | Chincha, Ica | *Capsicum annum* |
| LT2150 | 08/17/05 | A2 | Ica Valley, Ica | *Capsicum annum* |
| LT2151 | 08/17/05 | A2 | Ica Valley, Ica | *Capsicum annum* |
| LT2152 | 09/07/05 | A2 | Viru, La Libertad | *Capsicum annum* |
| LT2153 | 10/23/05 | A2 | Ica Valley, Ica | *Solanum lycopersicum* |
| LT2847 | 06/27/05 | A2 | Curahuasi, Abancay | *Capsicum annuum* |
| LT2848 | 06/27/05 | A2 | Curahuasi, Abancay | *Capsicum annuum* |
| LT2849 | 06/27/05 | A2 | Curahuasi, Abancay | *Capsicum annuum* |
| LT2850 | 09/19/05 | A2 | Supe, Lima | *Capsicum annuum* |

[1] MT=mating type

Table 2.2. *Phytophthora capsici* isolates collected from peppers at ten locations in Lima, Barranca province, in 2006-2007.

| Field | No isolates | Year | Origin | Host |
|---|---|---|---|---|
| 1 | 19 | 2006 | Barranca, Lima | *Capsicum pubescens* |
| 2 | 18 | 2006 | Supe, Lima | *Capsicum chinense* |
| 3 | 12 | 2006 | Supe, Lima | *C. baccatum* var. *pendulum* |
| 4 | 13 | 2006 | Supe, Lima | *C. baccatum* var. *pendulum* |
| 5 | 18 | 2006 | Barranca, Lima | *Capsicum annum* |
| 6 | 36 | 2006 | Barranca, Lima | *Capsicum annum* |
| 7 | 36 | 2007 | Caral, Lima | *Capsicum annum* |
| 8 | 18 | 2007 | Minas, Lima | *Capsicum annum* |
| 9 | 14 | 2007 | Minas, Lima | *Capsicum annum* |
| 10 | 20 | 2007 | Minas, Lima | *Capsicum annum* |

Table 2.3. Primers and probe sequences used for SNP genotyping with Taqman assays. Underlined bold nucleotide refers to the assessed SNP. F=forward and R=reverse.

| Locus name | Primer sequence | Internal reporter oligonucleotide (probe) VIC | Internal reporter oligonucleotide (probe) FAM |
|---|---|---|---|
| SNP11 | F_5'-agattgagaagaaggaacttggtcatg-3'<br>R_5'-ccttctcacctgtaaccttgctaat-3' | 5'-acgcacagt**g**ccgtag-3' | 5'-acgcacagt**a**ccgtag-3' |
| SNP14 | F_5'-cgctgtgtcgatagtgggaatg-3'<br>R_5'-tcttctctctgtgttcgactacca-3' | 5'-cttgagct**a**attctct-3' | 5'-ttgagct**c**attctct-3' |
| SNP15 | F_5'-ttccgacgagcaacgaaca-3'<br>R_5'-gtaccgtgccacgcagat-3' | 5'-tcttgcatac**a**ttctgg-3' | 5'-tcttgcatac**g**ttctgg-3' |
| SNP16 | F_5'-gccagcatgctcgatattgg-3'<br>R_5'-gcagtcagggccaagca-3' | 5'-caattgcaac**a**aggcaa-3' | 5'-ccaattgcaac**t**aggcaa-3' |
| SNP20 | F_5'-ccactcggccgacaactc-3'<br>R_5'-gatgatgctaagatcgtaccaaagc-3' | 5'-tactct**g**gtggaaatg-3' | 5'-ctactct**a**gtggaaatg-3' |
| URA3 | F_5'-cgaaggacaacgcgaacttg-3'<br>R_5'- ctgcgtgacgccatcaac-3' | 5'-tgctgc**g**tcgactg-3' | 5'-tgctgc**a**tcgactg-3' |

Table 2.4. Summary of SNP multi-locus genotypes for 43 representative isolates of the PcPE-1 and PcPE-2 clonal lineages and the unique isolate LT2145. N represents the number of isolates identified with a particular SNP genotype configuration.

| | **Clonal lineage** | | | | |
|---|---|---|---|---|---|
| | | **PcPE-1** | | **PcPE-2** | **Unique isolate** |
| **Locus name/ Isolate ID** | **LT3738** | **LT3726** | **LT3753** | **LT2137** | **LT2145** |
| SNP11 | A/A | A/A | A/A | G/A | G/A |
| SNP14 | A/A | A/A | A/A | A/C | A/C |
| SNP15 | A/G | A/A | A/A | A/A | A/G |
| SNP16 | A/T | A/T | A/T | A/T | T/T |
| SNP20 | A/G | A/G | A/G | A/G | A/A |
| URA3 | A/G | A/G | G/G | A/G | A/G |
| **Genotype frequency** | 0.79<br>N = 34 | 0.05<br>N = 2 | 0.02<br>N = 1 | 0.12<br>N = 5 | 0.02<br>N = 1 |

**Figures**

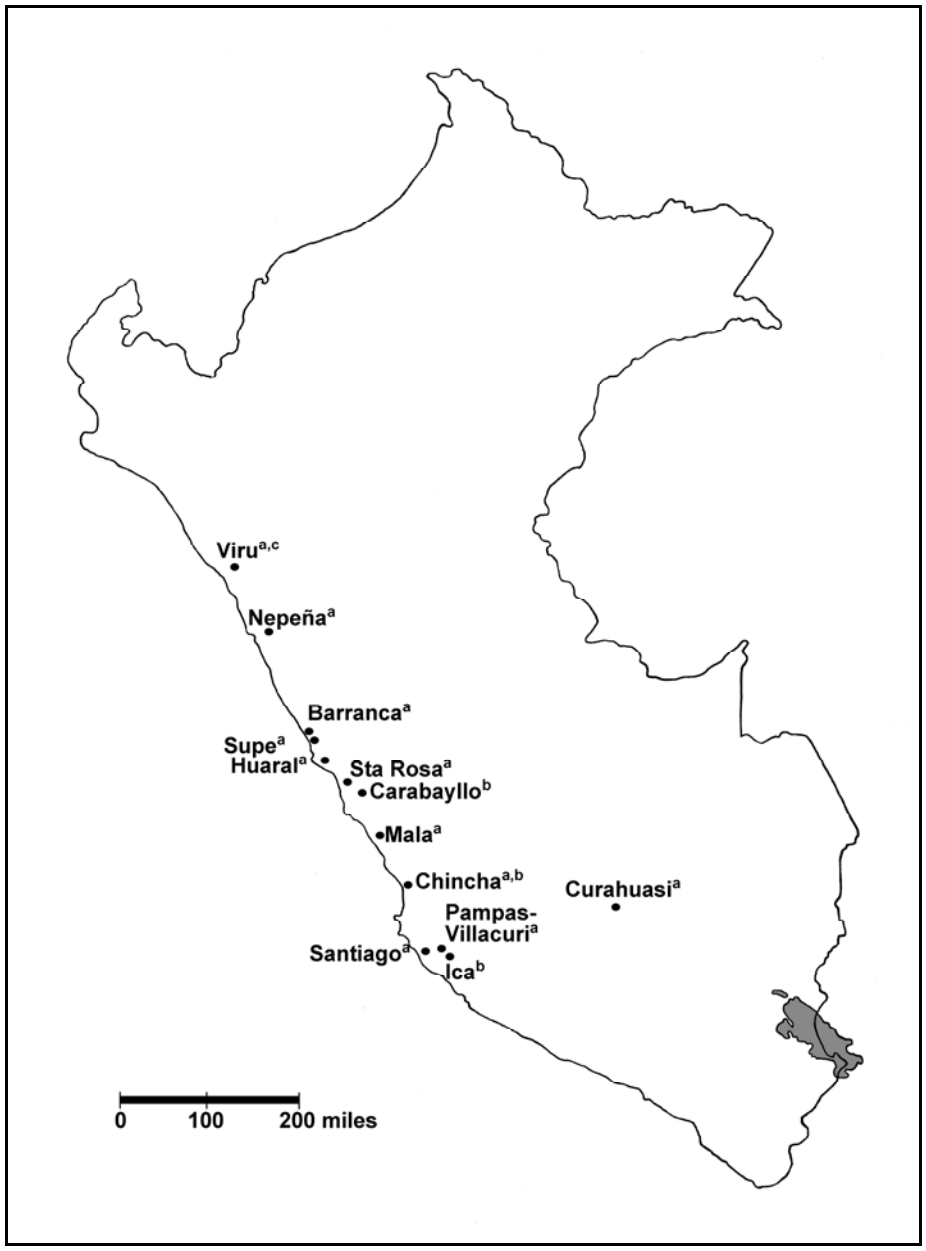


Figure 2.1. Geographic locations for *Phytophthora capsici* isolates collected in Peru. Superscript **a** indicates that isolates from clonal lineage PcPE-1 were present; **b** indicates isolates from PcPE-2 were present and **c** indicates the location of LT2145.

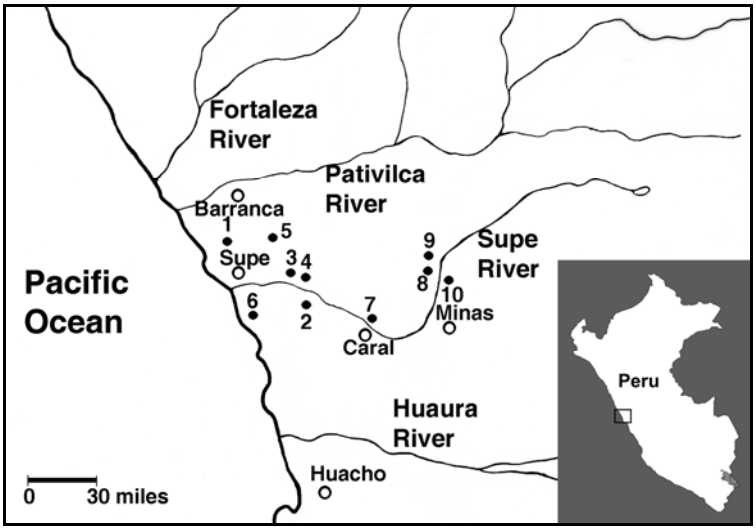


Figure 2.2. Close-up depicting the location of ten fields sampled in the Barranca valley. Black circles indicate specific fields.

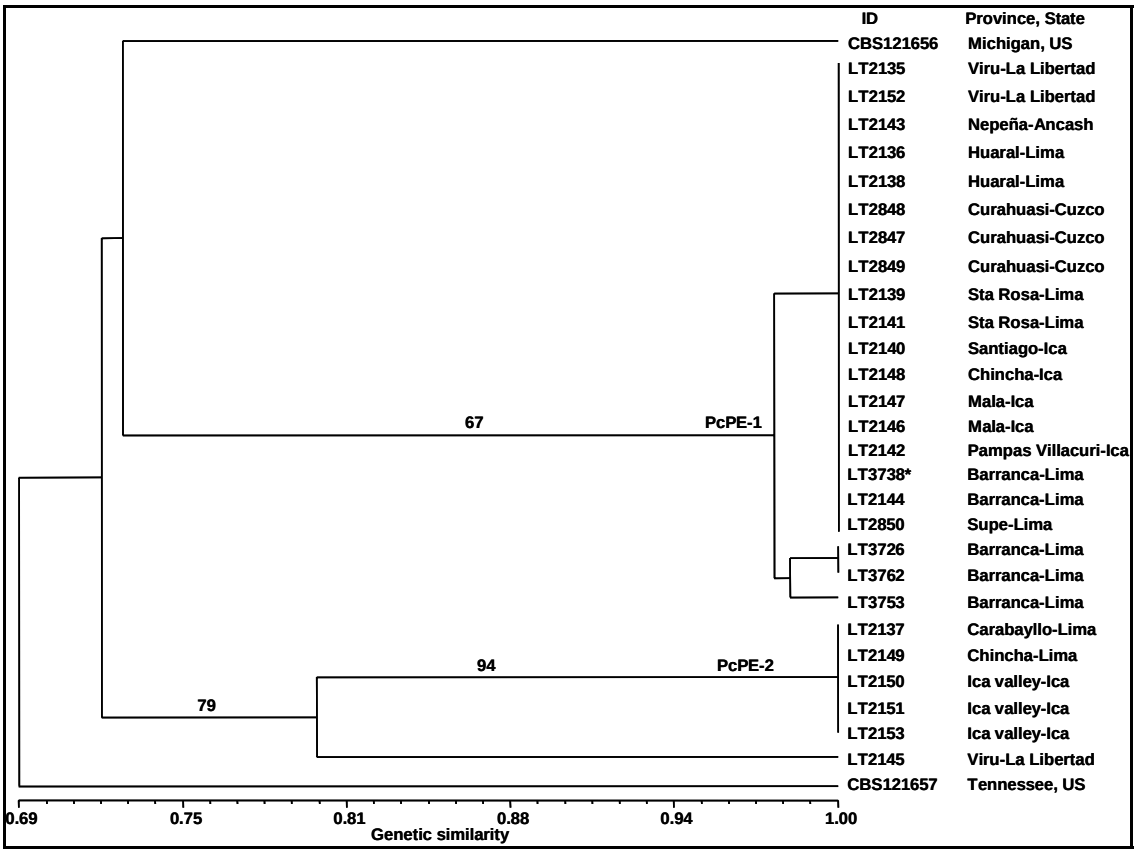


Figure 2.3. Unweighted pair-group method with arithmetic average (UPGMA) cluster analysis of *Phytophthora capsici* isolates from 13 provinces across Peru during 2005-2007 using 50 amplified fragment length polymorphism markers (AFLP) and six SNP loci. LT3738* represents 200 *P. capsici* isolates with identical AFLP profiles from Barranca province in Lima from 2006-2007. LT # refers to isolate identification for isolates that have not been deposited into a collection. Bootstrap values are based on 1000 replicates and the major clonal lineages are indicated by the designators PcPE-1 and PcPE-2.

# Chapter Three Molecular comparison of natural hybrids of *Phytophthora nicotianae* and *P. cactorum* infecting loquat trees in Peru and Taiwan

**ABSTRACT**

Natural *Phytophthora* hybrids (*P. nicotianae* x *P. cactorum*) infecting loquat in Peru and Taiwan were characterized using AFLP (amplified fragment length polymorphism) markers, sequencing of the internal transcribed spacer (ITS) region and the phenol acid carboxylase gene (*Pheca*), and inheritance of the mitochondrial cytochrome oxidase I gene (*cox*I). AFLP profiles of two Taiwanese isolates, recovered in 1995, were polymorphic in ~50% of the fragments whereas five Peruvian isolates, recovered in 2002-2003 and 2007, showed no genotypic variation. Sequencing analysis of the cloned ITS region indicated the presence of *P. nicotianae* and *P. cactorum*, containing double bases at those positions where the sequences of *P. nicotianae* and *P. cactorum* differ. Direct sequence analysis of the *Pheca* gene revealed sequences matching both *P. nicotianae* and *P. cactorum*. Melting analyses of *cox*I revealed that all seven *Phytophthora* hybrids inherited the mitochondrial DNA from *P. nicotianae*. Our results suggest that *Phytophthora* hybrids from Peru likely originated from a single hybridization event and that the two isolates from Taiwan originated through different hybridization events. The hybrid in Peru appears to have persisted over at least three years at three separate locations. Possible factors influencing the population structure of *Phytophthora* hybrids infecting loquat are discussed.

**INTRODUCTION**

The genus *Phytophthora* includes more than 90 described species that infect over 1000 plant species and cause large economic losses in food and ornamental crops every year (12, 17). Methods for identification of *Phytophthora* species have traditionally relied

on observations of phenotypic characteristics (12). More recently, genomic and mitochondrial sequence data have been used to support and expand the definition of *Phytophthora* species (10, 17, 22).

There have been recent reports of atypical isolates of *Phytophthora* that do not fit any described species. An unknown *Phytophthora* was reported to be responsible for the death of alder trees in Britain (6). Later, this pathogen was formally described as an allopolyploid *Phytophthora* hybrid named *P. alni* with several subspecies (8). The possible progenitors of *P. alni* are thought to be *Phytophthora cambivora* and *P. fragariae*, none of which are pathogenic in alder trees (7). Thus, the possibility of hybrid *Phytophthora* with an increased or new host range may be an important mechanism of speciation in oomycetes (5, 23).

In 1998, an unknown *Phytophthora* was isolated from diseased *Spathiphyllum* and *Primula* growing in hydroponic cultures in the Netherlands (21). Using molecular approaches, these isolates were described as natural hybrids with *P. nicotianae* and *P. cactorum* as possible progenitors (21). In 2000, atypical *Phytophthora* isolates were recovered in hydroponic cultures on different hosts. DNA fingerprinting demonstrated that these isolates were also *P. nicotianae* x *P. cactorum* hybrids and that they had likely emerged through different hybridization events (3).

Reports of *Phytophthora* infecting loquat trees date back to the early 1930s (25). However, a detailed description of the *Phytophthora* species infecting loquat was not recorded until 1967 when Weltzien and Schwinn described *Phytophthora nicotianae* as the causal agent of outbreaks in commercial loquat orchards in Lebanon (27). More recently, Chern et al. (9) reported two different types of *Phytophthora* species infecting

loquat trees in Taiwan. Isolates were described as typical and atypical *P. nicotianae* (9). Atypical isolates of *P. nicotianae* were further characterized using isozyme patterns, demonstrating their similarity to isozyme patterns of previously identified natural hybrids of *P. nicotianae* and *P. cactorum* (20, 21). During 2006, atypical *P. nicotianae* isolates were identified infecting loquat trees in Peru. Preliminary studies of the ITS region from a monosporic isolate revealed ITS sequences from *P. nicotianae* and *P. cactorum*, which suggested a possible hybrid nature of the atypical *Phytophthora* (1).

Our goal was to characterize *Phytophthora* hybrids infecting loquat in two distant geographic locations (Peru and Taiwan). Using AFLP and sequence analysis of nuclear genes, we determined that a single hybridization event may account for the isolates recovered in Peru whereas separate hybridization events (distinct from the event in Peru) may have led to the isolates in Taiwan. Mitochondrial inheritance indicates only one mitotype (*P. nicotianae*) in both hybrid populations.

## MATERIALS AND METHODS

### Isolate recovery and maintenance

*Phytophthora* spp. were isolated in October 2002, March 2003 and April 2007 from loquat trees (*Eriobotrya japonica* [Thunb.] Lindl.) with die-back symptoms at Coayllo (Lima, Peru). Sampled trees (N=5) varied in age from one to 15 years and were located in three different fields at least 400 feet apart. Root systems were processed for pathogen isolation in the Diagnostic Clinic at the National Agricultural University La Molina, Peru. Small sections of symptomatic root tissue were thoroughly washed with tap and sterile distilled water. Root tissue was transferred to corn meal agar plates (CMA)

and incubated for three to five days at 24ºC. Single hyphal tips were sub-cultured to amended PARP (100 ppm of pimaricin, 100 ppm of ampicillin, 30 ppm of rifampicin, and 100 ppm of pentachloronitrobezene) V8 juice agar plates (840 ml of distilled water, 163 ml of unclarified V8 juice, 3 g of $CaCO_3$, and 15 g of agar). For long-term storage, a 7-mm plug of expanding mycelium from each culture was placed in 2 ml screw-cap tubes with three sterilized hemp seeds and 1 ml of sterile distilled water. *Phytophthora nicotianae* isolate LT215, isolated from tobacco in Tennessee (2004), and *P. cactorum* isolate LT198, isolated from *Rhododendron* in Tennessee (2004), were included in the study for comparative analysis and crossing experiments (see below).

### *Phytophthora cactorum* x *P. nicotianae* crosses

Laboratory crosses were conducted using the *P. nicotianae* and *P. cactorum* isolates mentioned above. Actively expanding mycelal plugs of *P. cactorum* isolate LT198 were placed at the center of V8 juice agar plates approximately 2 cm away from the *P. nicotianae* isolate LT215. Plates were wrapped with Parafilm and incubated in darkness at room temperature for at least eight weeks. Oospores were separated from the surrounding mycelium by blending with a TissueTearor (Fisher Scientific Inc, Hampton, NH) and treated overnight with 0.05 mg/ml crude lysing enzyme from *Trichoderma harzianum* (Sigma, St Louis, MO). Solution was then diluted with sterile distilled water and incubated at room temperature under constant white-fluorescent light for at least seven days at room temperature (19). Microscope observations were made daily to identify germinated oospores.

**DNA isolation and AFLP fingerprinting**

Isolates from Peru were grown in antibiotic-amended V8 broth and DNA isolation was performed as previously described in Lamour and Finley (18). DNA of two previously identified *Phytophthora* hybrid isolates 95023 and 95034 from Taiwan, were also included in the study (9). AFLP genotyping was performed using *Eco*RI and *Mse*I restriction endonucleases from Invitrogen (Carlsbad, CA). Sequence adapters and primers as described by Vos et al. (26) were acquired from Integrated DNA Technologies (Coralville, IA). Restriction, ligation, pre-amplification and amplification were performed according to Habera et al (14). Pre-selective amplification was performed using non-selective primers matching the adaptor sequences and selective amplification was conducted with the selective primer pair (Eco+CG/Mse+CG). Selective amplifications were diluted and re-amplified in a separate reaction using FAM labeled primers from Proligo (Boulder, CO). AFLP fragments were resolved and analyzed on a CEQ$^{TM}$ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) following the manufacturer's protocols. AFLP profiles were generated twice for each isolate using independent DNA isolations and only AFLP markers consistent among replicates were considered for further analyses.

**Hi-resolution DNA melting analysis of mitochondrial DNA**

Mitochondrial inheritance in *Phytophthora* hybrids was done using hi-resolution DNA melting analysis (15, 28). Primers were designed to amplify a 221 bp region (from bases 793 through 1013) of the cytochrome oxidase mitochondrial gene I (*cox*I). This region contained nine single nucleotide polymorphic (SNP) sites previously identified

between *P. nicotianae* and *P. cactorum* (22). Primer sequences were Forward 5'-ccaccccataaagtagctaacc-3' and Reverse 5'-caagtttctgcagcttttgct-3' and PCR parameters were: 95°C for 5 min, 45 cycles of denaturing at 95°C for 30 s, annealing at 74°C for 30 s, synthesis at 72°C for 30 s with a final extension at 72°C for 5 min. After PCR amplification and heteroduplex formation, PCR products were subjected to melting analysis using a light scanner instrument (Idaho Technologies, Salt Lake City, UT). Parameters were adjusted accordingly and melting curve profiles from each *P. hybrid* were compared with those from *P. nicotianae* and *P. cactorum*. PCR amplifications and melting analyses were performed twice using DNA from two biological replicates.

**ITS and nuclear gene sequencing**

Sequencing of the ribosomal internal transcribed spacer (ITS) and a single-copy nuclear gene (phenol acid decarboxylase, *Pheca*) were performed in order to determine the genomic contribution of each parental *Phytophthora* (*P. nicotianae* and *P. cactorum*) in the hybrid isolates. PCR amplification of the ITS region for hybrid isolates (95023, 95034, and LT2852) was done as previously described by Cooke et al. (10) using primers ITS4 5'-tcctccgcttattgatatgc-3' and ITS6 5'-gaaggtgaagtcgtaacaaagg-3'. The amplified region contains the ITS1, 5.8S gene and ITS2. PCR products were cloned using the TOPO® TA Cloning™ kit (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Clone PCR products were inserted into One Shot® TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. At least six *E. coli* recombinant colonies per isolate were randomly selected for sequencing. Plasmid isolation was performed using the PureLink™ Quick Plasmid miniprep kit (Invitrogen,

Carlsbad, CA). Plasmids were submitted for sequencing to the Sequencing Facilities of the University of Tennessee. Both strands of the cloned ITS region were sequenced. Sequencing files were manipulated with CodonCode aligner software v2.0.2 (CodonCode Corp. Dedham, MA).

Phenol acid carboxylase (*Pheca*) gene amplification was performed using degenerate primers that were designed from a multiple nucleotide sequence alignment (Clustal W) of *P. sojae*, *P. infestans*, and *P. ramorum* (24). Primer design was performed manually and primers were flanking ~ 750 bp of the open reading frame region of the gene. Primer sequences were Forward 5'- gtbccygghtwycacaccaacac-3' and Reverse 5'- ctcgasgatrkyrgcctgtcgc-3'. PCR reactions were performed using Platinum Taq® from Invitrogen (Carlsbad, CA, US) following the manufacturer's instructions. Cycling parameters were as follows: 95°C for 5 min, 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extending at 72°C for 30 s. PCR products were cleaned using the Qiagen PCR purification kit following manufacturer's instructions (Valencia, CA) and submitted for sequencing to the Molecular Biology Resource Facility of the University of Tennessee. Sequencing files were manipulated as described above.

## RESULTS

### AFLP fingerprinting

AFLP profiling of the five *Phytophthora* hybrid isolates from Peru (2002-2003, and 2007) revealed identical AFLP genotype. Isolate LT2852 was used for further comparative analysis. AFLP profiles from Taiwan isolates (95023 and 95034) showed that out of 29 clearly resolvable markers, 15 were polymorphic between both isolates

(Table 3.1 and Fig. 3.1). The AFLP profile of isolate LT 2852 was similar to that of isolate 95023 from Taiwan, sharing 19 out of 29 identified markers (Fig. 3.1). No unique markers were detected for isolate LT2852 that were not present in the Taiwanese isolates (Table 3.1 and Fig. 3.1). Isolate 95023 had the highest number of AFLP markers (26 markers), followed by isolate LT2852 (20 markers) and isolate 95034 (17 markers) (Table 3.1).

**Mitochondrial inheritance**

The inheritance of mitochondrial genome in hybrids was studied by comparing the hi-resolution DNA melting curve profile of each *Phytophthora* hybrid isolate to that of each parental *Phytophthora* (*P. nicotianae* and *P. cactorum*). The *Phytophthora* hybrids from Peru and Taiwan inherited the mitochondrial genome from *P. nicotianae*. *Phytophthora* hybrids had similar melting curve profiles to that of *P. nicotianae* isolate LT215 (Fig. 3.2).

**Nuclear content of *Phytophthora* hybrids**

**ITS analysis.** Eighteen *E. coli* recombinant colonies containing the ITS PCR product cloned from three *Phytophthora* hybrid isolates were sequenced: six clones from isolate LT2852, six clones from isolate 95023 and six clones from isolate 95034. Blast searches in a public database (National Center for Biotechnology Information, NCBI) revealed the presence of distinct ITS sequences within each of the isolates: ITS sequences with high homology (99%) to previously deposited *P. nicotianae*=AF266776, and ITS sequences with high homology to previously deposited *P. cactorum*=AF266772).

***Pheca* analysis**. Blast searches in *Phytophthora* databases (Virginia Bioinformatics Institute, Broad Institute, and the National Center for Genome Research) suggest that *Pheca* is a single copy gene. Sequencing analysis of the phenol acid carboxylase gene (*Pheca*) using degenerate primers in both parental *Phytophthora* (*P. nicotianae* and *P. cactorum*) indicated the presence of thirteen nucleotide differences across 354 bp of the gene at positions 84, 99, 105, 111, 114, 156, 165, 201, 243, 285, 294, 300, and 359 bp (Table 3.2). Direct sequencing of the PCR product from the same region in the *Phytophthora* hybrid isolates revealed the presence of the same nucleotide differences but in the heterozygous stage. These results indicate that the *Phytophthora* hybrids from Peru and Taiwan carry the phenol acid carboxylase alleles from both *P. nicotianae* and *P. cactorum* (Table 3.2 and Fig. 3.3).

## DISCUSSION

Several studies have described the presence of strains of *Phytophthora* as the causal agent of root rot in loquat. *Phytophthora nicotianae* was identified as the causal agent of outbreaks in commercial loquat orchards in Lebanon. The description of some isolates from Lebanon agreed significantly with those observations made by Chern et al. during 1995 in Taiwan and Aragon-Caballero et al. during 1998 in Peru (1, 9). Isolates from Taiwan and Peru were initially described as atypical *P. nicotianae* isolates based on taxonomic keys. However, based on molecular approaches and comparison to previously described hybrids of *P. nicotianae* and *P. cactorum* (3) the atypical *P. nicotianae* isolates from Peru and Taiwan were identified as the same *Phytophthora* hybrids (1, 20).

Previously identified interspecific hybrids of *Phytophthora* species (*P. nicotianae* x *P. cactorum*) were recovered from hydroponic systems in the Netherlands (3, 21). Continuous hydroponic systems harboring multiple crops provide an ideal environment to different *Phytophthora* species for evolutionary experimentation and possibly the generation of new interspecific hybrids (3). Generation of interspecific *Phytophthora* hybrids has been shown to be possible through zoospore fusion or laboratory crosses and in some cases with modified host range (2, 11, 13). Our attempts to germinate oospores from a laboratory cross of *P. nicotianae* and *P. cactorum* failed. This was primarily due to the finding that very few oospores were formed and it was also difficult to visualize germinating oospores due to abundant germination of chlamydospores that survived after enzyme treatment.

Fingerprinting from previously reported interspecific hybrids of *P. nicotianae* x *P. cactorum* showed that isolates arose from independent hybridization events between the parental *Phytophthora* species (3). Highly polymorphic AFLP profiles of *Phytophthora* hybrids recovered in Taiwan also suggests that the isolates have arisen from independent hybridization events (Table 3.1 and Fig. 3.1). In contrast, AFLP profiles of Peruvian isolates indicate the presence of a clonal population maintained at separate locations across the three years that samples were collected. Genetic characterization of natural hybrid species of *Phytophthora alni* showed that hybrids may have been generated on several occasions through the hybridization of its potential progenitors (16).

Mitochondrial inheritance studies demonstrated that *P. nicotianae* participates as a permanent donor of the mitochondria in the *Phytophthora* hybrids, which contrasts with

the *Phytophthora* hybrids infecting alder trees where two mitotypes have been identified from the two possible parental progenitors (16).

It is possible that irrigation practices in Peruvian loquat orchards are maintaining the environmental conditions for the propagation of a clonal population of the pathogen. Loquat orchards in Peru are normally flooded for irrigation by the Omas River in Coayllo, Peru, whereas Taiwanese orchards are irrigated through drip systems. Die-back of loquat trees in Peru was observed after El Niño-Southern Oscillation occurred in 1998. During El Niño 1998, the Omas River overflowed its banks and flooded loquat orchards in Coayllo for several weeks. Similarly, die-back of loquat trees in Taiwan was reported after heavy rainfalls in 1995 (9). Flooding rivers could bring a pathogen into new geographic areas (such as loquat orchards). Permanent flooding or heavy rainfalls (which simulated the conditions present in hydroponic systems) allows the resident species (i.e., *P. nicotianae*) and the immigrant species (*P. cactorum*) to come together for hybridization (4). Among isolates infecting loquat trees, researchers have also identified strains that fit well with the taxonomic characteristics of *P. nicotianae* (1, 9, 25, 27). In addition, phylogenetic studies have shown that *P. nicotianae* and *P. cactorum* are closely related species (10). The occurrence of *P.* hybrids suggests that their divergence is not completed to the extent to which the genomes are no longer compatible (21).

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## APPENDIX 3

### Tables

Table 3.1. Summary of AFLP markers identified in *Phytophthora* hybrids from Peru (LT2852) and Taiwan (95023 and 95034) isolates using primer combination Eco+CG/Mse+CG. One [1] = presence of the marker and cero [0] = absence of the marker.

| Marker (bp) | Isolate | | |
|---|---|---|---|
| | LT2852 | 95023 | 95034 |
| 62 | 0 | 1 | 1 |
| 109 | 1 | 1 | 0 |
| 113 | 1 | 1 | 1 |
| 121 | 1 | 1 | 0 |
| 131 | 1 | 1 | 0 |
| 143 | 1 | 0 | 1 |
| 149 | 0 | 0 | 1 |
| 167 | 0 | 1 | 0 |
| 172 | 1 | 1 | 1 |
| 182 | 1 | 1 | 1 |
| 239 | 0 | 1 | 0 |
| 248 | 1 | 1 | 1 |
| 252 | 1 | 1 | 0 |
| 262 | 1 | 1 | 1 |
| 340 | 1 | 1 | 0 |
| 363 | 1 | 1 | 1 |
| 382 | 1 | 1 | 0 |
| 385 | 1 | 1 | 1 |
| 400 | 1 | 1 | 0 |
| 423 | 1 | 1 | 0 |
| 429 | 1 | 1 | 1 |
| 440 | 0 | 1 | 0 |
| 450 | 0 | 1 | 0 |
| 460 | 1 | 1 | 1 |
| 500 | 1 | 1 | 1 |
| 516 | 0 | 1 | 1 |
| 543 | 0 | 0 | 1 |
| 556 | 0 | 1 | 1 |
| 587 | 1 | 1 | 1 |

Table 3.2. Summary of nucleotides differences between parental and hybrids of *Phytophthora* in a 359 bp fragment of the Phenol acid carboxylase gene. *Phytophthora nicotianae*= LT215; *P. cactorum*=LT198, LT2852=*Phytophthora* hybrid from Peru, 95023=*Phytophthora* hybrid from Taiwan.

| **Isolate** | **Base** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **84** | **99** | **105** | **111** | **114** | **156** | **165** | **201** | **243** | **285** | **294** | **300** | **359** |
| *P. nicotianae* | G | G | A | G | C | C | C | A | A | A | A | G | G |
| *P. cactorum* | A | A | G | A | G | G | G | G | G | G | G | A | T |
| LT2852 | R | R | R | R | S | S | S | R | R | R | R | R | K |
| 95023 | R | R | R | R | S | S | S | R | R | R | R | R | K |

## Figures

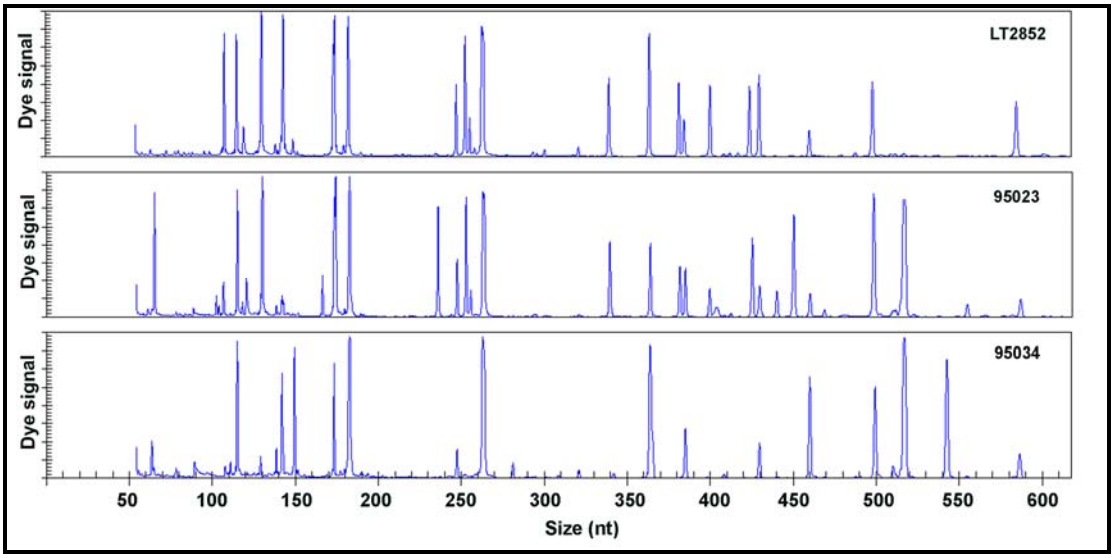


Figure 3.1. AFLP profiles for *Phytophthora* hybrids recovered from loquat in Peru and Taiwan. LT2852 = Peru; 95023 and 95034 = Taiwan.

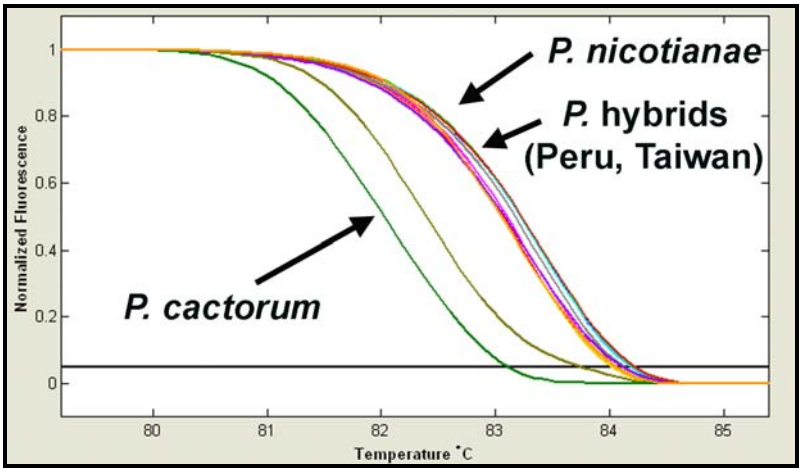


Figure 3.2. DNA melting analysis curves of the *cox*I gene for *Phytophthora cactorum*, *P. nicotianae*, and *Phytophthora* hybrids.

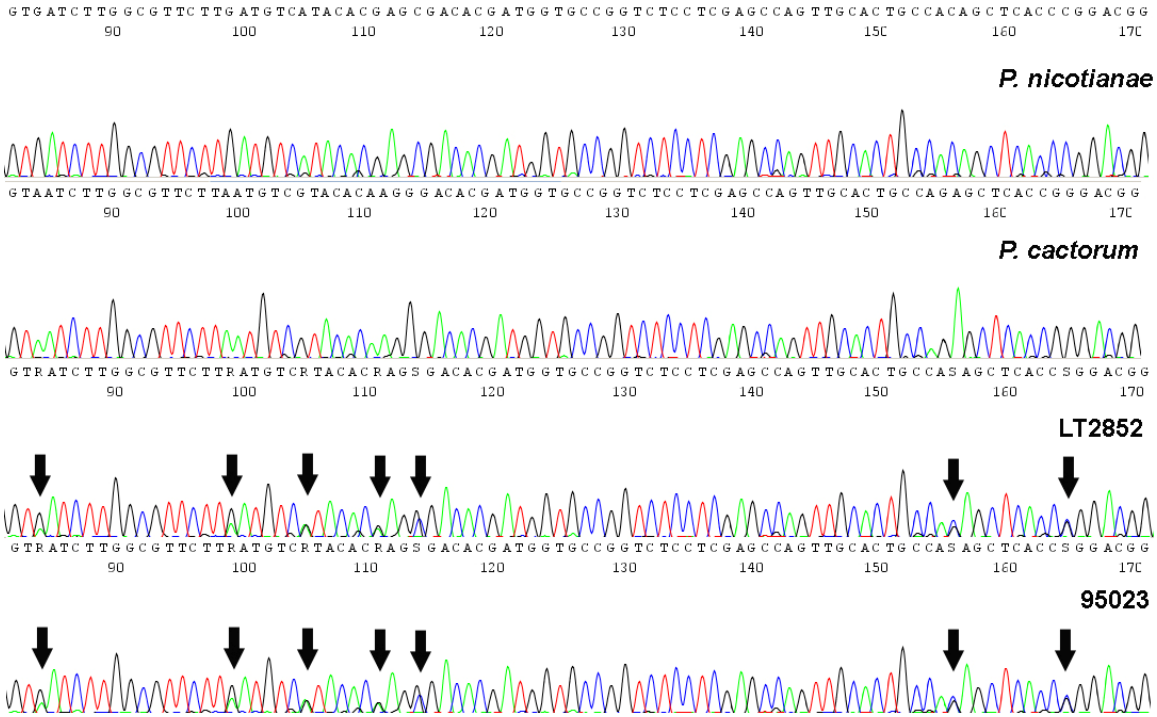


Figure 3.3. Trace electropherograms for a portion of the phenolcarboxylase gene in *Phytophthora nicotianae*, *P. cactorum*, and hybrid isolates from Peru (LT2852) and Taiwan (95023). Black arrows indicate the position of the heterozygous sites in *P.* hybrids.

# Chapter Four Inbreeding, apomixis, and the genetics of *Phytophthora capsici*

**ABSTRACT**

A series of inbreeding crosses, recurrent backcrosses, and successive sibling crosses were completed up to the sixth generation in the plant pathogen *Phytophthora capsici* generating a total of 692 oospore-derived isolates. All of the crosses stem from an initial cross between two wild type *P. capsici* isolates and the resulting progeny varied throughout the different crosses for mating type, colony morphology, and pathogenicity on cucumber and pepper fruits. The heterozygosity level, as measured through the inheritance of 20 amplified fragment length polymorphism (AFLP) markers, decreased incrementally with continued inbreeding and was reduced ~ 60 to 75% by the second consecutive sibling cross. Of the eight crosses analyzed, all but one produced oospore-derived progeny that were identical to one or the other parent indicating that apomixis can play a role in *P. capsici* intraspecific crosses and that mature (two- to five- months-old) oospores may serve as resistant, asexual spores. There was no evidence of isolates emerging through selfing or generation of homothallic isolates. Overall our results suggest that the large reservoirs of naturally occurring genetic variation in *P. capsici* can be exploited to develop inbred lines useful for characterizing complex genetic traits in *Phytophthora*.

**INTRODUCTION**

The genus *Phytophthora* includes 90+ species and infects more than 1000 plant species worldwide (5, 19). Draft genome sequences of two oomycetes are now available (*P. sojae* and *P. ramorum*) (41) and the genomes for the oomycetes *Hyaloperonospora parasitica*, *P. capsici* and *P. infestans* are currently being sequenced. Functional genetic

analyses in *Phytophthora* are conducted by means of transformation, heterologous expression systems, gene silencing, and gene disruption via mutagenesis (17, 24, 43-45, 47). In general, these approaches focus on the effects of single genes in a few "wild type" background genotypes. There are few studies of phenotypic variation that is inherited as complex genetic traits or quantitative trait loci (QTLs). Most likely the majority of the key developmental processes such sporangiogenesis, zoosporogenesis, oosporogenesis and host colonization (pre- and postinvasion) are complex traits and these processes are likely regulated by the orchestrated expression of multiple genes (30, 40).

In other organisms, such as mouse, fruit fly, roundworm, *Arabidopsis* and crops such as tomato, maize and beans, elucidating complex traits is greatly facilitated by the availability of mapping populations, recombinant inbred lines (RILs), and near isogenic lines (NILs) (11, 25-27, 29, 32, 42). Researchers working with hosts such as *Capsicum annuum, Cucurbita moschata*, and *Solanum lycopersicum* have developed recombinant inbred lines toward the dissection of the resistance against *P. capsici* (3, 15, 31). A few of these economically important hosts are also being sequenced. The development of similar inbred line resources for the oomycete community is highly desirable. However there are a number of factors that have slowed the progress of strategies that rely on extensive crossing including low oospore germination rates for some species (e.g., *P. infestans*), aberrant segregation ratios, difficulties in separating the sexual oospores from the surrounding parental asexual spores and mycelium, and a limited number of well characterized nuclear markers (14, 16).

A primary goal in our research program is the development of genetic and genomic resources for the vegetable pathogen *Phytophthora capsici* (23). *Phytophthora*

*capsici* is unique because it regularly completes the sexual stage in natural populations in the U.S. (21). Populations are composed of genotypically diverse individuals of both mating types (20). The sexual stage appears to be under strong selection pressure in the U.S. because *P. capsici* does not produce thick-walled asexual chlamydospores and the primary hosts (cucurbits, tomatoes, and peppers) are unavailable during the winter months. Consequently, U.S. populations carry a large amount of genetic variation and laboratory crosses are in many cases highly fecund (20, 21, 33). In addition, *P. capsici* has a wide host range including members of the Solanaceae, Cucurbitaceae and Leguminoseae (5, 10). The ability to easily complete crosses provides a unique opportunity for genetic investigation.

Our objective was to investigate the impact of inbreeding on *P. capsici* and to determine if the segregating populations may be useful for studying complex traits. We present genetic data from segregating AFLP and/or single nucleotide polymorphism (SNP) markers as well as phenotypic information (mating type, pathogenicity on pepper and/or cucumber fruits, and colony morphology) for 692 oospore-derived isolates from eight inbreeding crosses. We also discuss the implications and difficulties for the development of *P. capsici* genetic resources.

## MATERIALS AND METHODS

### Isolates and crossing scheme

All of the crosses stem from an initial cross of *P. capsici* between two field isolates: LT51 (mating type A1) isolated from a cucumber fruit in Michigan in 1997 and LT263 (mating type A2), isolated from a pumpkin fruit in Tennessee during 2004 (cross

named: LT51xLT263). To generate oospores the parent isolates were plated approximately 2 cm apart on UCV8 juice agar amended with PARP (100 ppm of pimaricin, 100 ppm of ampicillin, 30 ppm of rifampicin, and 100 ppm of pentachloronitrobezene). Plates were wrapped with Parafilm® and kept in the dark at room temperature and incubated for at least 2 months before the oospores were separated from the parental material and stimulated to germinate (described below). The first backcross ($BC_1F_1$) was between isolates LT1021 (A1, $F_1$ progeny) and LT263 (A2) (cross named LT1021xLT263). A second recurrent backcross ($BC_2F_1$) was then set up between isolates LT1422 ($BC_1F_1$ derived progeny, A1) and LT263 (A2) (cross named LT1422xLT263). A first full sibling-cross ($SC_1F_1$) was initiated with offspring from cross LT1422xLT263 using isolates LT1503 (A1) and LT1530 (A2) (cross named: LT1503xLT1530). Two different full-sibling crosses ($SC_{2\text{-}1}F_1$ and $SC_{2\text{-}2}F_1$) were set up using progeny isolates from cross LT1503xLT1530. The second sibling crosses, cross name LT2209xLT2222 and LT2211xLT2222, were germinated at two, three and five months following mating. Recombinant progeny derived from cross LT2209xLT2222 was used for generating a third sib-cross: LT3382xLT3394. A diagram of the crosses is presented in Figure 4.1.

**Oospore germination and mating type**

Mycelium was scraped from the surface area between the two parental inoculum plugs using a sterile spatula and the material transferred into 20 ml of sterile distilled water in a 50-ml falcon tube. The mycelium was then thoroughly disrupted by blending with a Tissue Tearor (Fisher Scientific Inc, Hampton, NH) for at least 1 min at 30,000

rpm. The wand was continuously moved up and down until no visible chunks remained. The homogenized solution was then passively filtered through a single layer of sterile Kimwipe (Kimberly Clark, Dallas, TX) into a clean 50 ml Falcon tube. The volume was adjusted to 18 ml with sterile water and amended with filter-sterilized crude lysing enzyme from *Trichoderma harziarum* (Sigma, St Louis, MO) to a final concentration of 1 mg/ml and a final volume of 20 ml. The 50 ml tubes were incubated overnight (16-20 h) at room temperature under laboratory lighting with gentle agitation using a Minilab Roller shaker (Labnet Int., Edison, NJ) at 25 rpm. Following the overnight incubation, an aliquot of the oospore preparation was transferred to 60-mm plates and observed under a light microscope for viable mycelium, sporangia, or zoospore contamination. Oospores were quantified (number of oospores per µL) and the oospore preparation was diluted with amended filtered V8 broth to a final concentration of one oospore per 50 µL and arrayed into 384-well plates using an Apricot 96-channel pipette model PP-550DS (Apricot Designs, Inc., Monrovia, CA) to a volume of 50 µL per well. Plates were incubated at room temperature for 3-7 days and colonies were picked and transferred to PARP amended water agar media (15 g of agar in 980 ml of distilled water) in 60 mm plates. A single hyphal-tip subculture was then transferred after two days at room temperature and each oospore-derived isolate was assigned a unique identifier (LT number). For long-term storage, 7-mm plugs of agar with expanding mycelium were placed into 2-ml screw-top tubes containing 1 ml of sterile distilled water and three hemp seeds and stored at room temperature.

To determine mating type of *P. capsici* isolates, plugs of actively expanding mycelium were placed at the center of V8 juice agar plates approximately 2 cm away

from “tester” isolates CBS121656 (mating type A1) or CBS121657 (mating type A2). Plates were wrapped with Parafilm and incubated in the dark at room temperature for at least one week after which observations were made for the production of oospores at the interface using a light microscope. Isolates able to produce oospores when crossed against CBS121656 were determined as A2 mating type. Conversely, isolates that were able to form oospores when crossed against CBS121657 were determined as A1 mating type. Isolates unable to produce oospores with either parent were considered sterile.

**DNA isolation and AFLP profiles**

Isolates were grown in V8 broth, the mycelium lyophilized, genomic DNA extracted, and amplified fragment length polymorphism (AFLP) profiles generated as described previously (9, 22). AFLP genotyping was done using *Eco*RI and *Mse*I restriction endonucleases, adapters, and primers as described by Vos et al. (46). Pre-selective amplification was done using no selective nucleotides (Eco+0/Mse+0) and selective amplification was conducted with selective primer pair (Eco+CC/Mse+CA). Selective amplifications were diluted and labeled in a separate reaction according to Habera et al. (2004). AFLP fragments were resolved and analyzed on a CEQ$^{TM}$ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) following the manufacturer’s protocols. AFLP profiles were generated twice for each isolate using independent DNA extractions. AFLP markers were scored for each isolate from the different crosses as one [1] for presence of the marker and zero [0] for absence. A binary matrix was constructed using only clearly resolved, replicated markers. A similarity analysis was carried out using NTSYSpc 2.11a (Exeter Software, Setauket, NY) and a dendrogram constructed by

employing an unweighted pair-group method with arithmetic average (UPGMA) cluster analysis.

**Loss of heterozygosity**

Due to the limitations of AFLP markers (dominant markers) only a specific group of markers were considered for calculating the loss of heterozygosity. The markers used segregated consistent with the following schemes: (1) polymorphic but homozygous in each parent and not segregating in the offspring (case: AA x aa); (2) polymorphic and heterozygous in one parent with segregation 1:1 ratio in the offspring (Aa x aa); (3) heterozygous in both parents and segregation 3:1 in the offspring (Aa x Aa). No attempts were made to determine if alleles were hetero- or homozygous based on the intensity of the fluorescent markers. Loss of heterozygosity was calculated directly by dividing the number of heterozygous alleles that had switched to a homozygous conformation in each of the inbreeding crosses by the total number of heterozygous alleles observed in the seminal cross LT51 x LT263.

**Pathogenicity**

Infection assays were conducted as previously described (8) with slight modifications. The pathogenicity tests were conducted on both wounded and unwounded fruit. For the wounded assays, a sterile scalpel was used to make small punctures at the site of inoculation. For cucumber fruits (*Cucumis sativus*), plugs of agar containing expanding mycelium were placed onto the surface of the fruit, covered with 1.5 ml Eppendorf tubes without caps and sealed with petroleum jelly to avoid desiccation. For

jalapeño fruits (*Capsicum annum*), the plug of agar was placed on the surface of the fruit and the fruits were laid on a moist paper towel in a plastic covered container. Inoculated fruits were incubated at room temperature under laboratory lighting and observations were made daily for 3 to 6 days following inoculation and an isolate was considered pathogenic if a visible lesion was produced by day six.

**SNP genotyping**

Five nuclear SNP markers were identified *in silico* from EST sequences generated from *P. capsici* mRNA from isolate LT1534, a second backcross derived isolate, as part of the *P. capsici* genome sequencing project (unpublished). Custom TaqMan® SNP Genotyping Assays (ABI, Foster City, CA) were designed according to the manufacturer's instructions. SNP assays were performed using 7.5 µL of iQ™ Supermix (Bio-Rad Inc, Hercules, CA), 0.325 µL of Taqman® probe/primers allelic discrimination cocktail (40X), 2 µL of DNA (~15-20 ηg) and 3.2 µL of molecular biology grade $H_2O$. PCR reactions were carried out in 96-well plates (Bio-Rad) in triplicates for each isolate on an iQ5 Real-Time thermal cycler (Bio-Rad) using the following parameters: 95°C for 10 minutes, 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. Each assay was standardized for optimal cycling PCR conditions and results were analyzed with the accompanying iQ5 optical system software 1.0 (Bio-Rad) using the allelic discrimination option, adjusting parameters for Ct values and RFU (relative fluorescent units) according to the manufacturer's instructions. Table 4.1 shows SNP markers used throughout the different crosses.

## RESULTS

### Inbreeding reduces heterozygous alleles

A total of 692 oospore-derived isolates were germinated from eight different crosses (Fig. 4.1). The number of recombinant isolates identified through AFLP fingerprinting and/or SNP genotyping was 201, 71, 79, 88, 11, and 42 (N=492) from the $F_1$, $BC_1F_1$, $BC_2F_1$, $SC_1F_1$, $SC_{2\text{-}1}F_1$, and $SC_3F_1$ crosses respectively (Fig. 4.1). AFLP fingerprinting with the primer combination Eco+CC/Mse+CA revealed 40 clearly resolvable markers on the $F_1$ population. These markers were tracked for their presence/absence across the different offspring ($BC_1F_1$, $BC_2F_1$, $SC_1F_1$, and $SC_{2\text{-}1}F_1$ and $SC_3F_1$). Twenty of the 40 markers were determined to be heterozygous in the wild type parents (LT51 and LT263) based on observations of their segregation in each of the recombinant progeny sets (Table 4.2). Based on these observations it was determined that within the progeny sets the original heterozygosity was reduced by 10% at $BC_1F_1$, 35% at $BC_2F_1$, 50% to 52% at $SC_1F_1$, and 60% to 75% by the $SC_{2\text{-}1}F_1$ (Table 4.2).

AFLP markers were useful for identifying sexual progeny in the first four inbred generations ($F_1$, $BC_1F_1$, $BC_2F_1$ and $SC_1F_1$) as there were between 16 and four segregating markers in the $F_1$ and $SC_1F_1$, respectively (Table 4.2). However, AFLP profiles from advanced inbred crosses ($SC_{2\text{-}1}F_1$ and $SC_3F_1$) revealed that most of the markers were fixed to one of the parents, making it impossible to accurately differentiate sexual progeny from parental genotypes. An UPGMA similarity tree illustrates the low level of genetic variability present at the higher levels of inbreeding as there were ~90% of the identified markers shared by the majority of isolates derived from $SC_{2\text{-}1}F_1$ cross (Fig. 4.2).

Co-dominant SNP markers fixed for different alleles were employed to overcome the AFLP limitations (Table 4.1). SNP14 marker was fixed in the parents from cross LT2209xLT2222 (LT2209=G/G and LT2222=T/T) and recombinant progeny carried both alleles (G/T) (Table 4.3). Additionally, 60 and 10 oospore-derived isolates had identical SNP14 conformation to LT2209 and LT2222, respectively. Three additional SNP markers (SNP18, SNP19 and URA3), were also tested for their inheritance in this recombinant progeny and all except one followed Mendelian segregation (Table 4.3). In the same way, identification of recombinant isolates from cross LT2211xLT2222 was done using the SNP12 marker (LT2211=G/G and LT2222=A/A) but non-recombinant progeny were recovered. Instead, all 46 oospore-derived isolates recovered had SNP12 allelic conformation identical to one of parents (two isolates were identical to LT2222 and 44 isolates identical to LT2211).

Assessment of URA3 SNP marker on $F_1$, $BC_1F_1$, $BC_2F_1$, $SC_1F_1$, and $SC_{2\text{-}1}F_1$, revealed significant correlation with Mendelian ratios ($P<0.05$) except for cross $BC_1F_1$ (Table 4.4).

**Inbreeding reveals a wide range of segregating phenotypes**

All of the $F_1$ isolates produced oospores when mated with the opposite mating type (tester isolate). The number of sterile isolates throughout all of the inbreeding crosses ranged between 0.5 and 12% (Table 4.5). The ratio for mating type (A1:A2), varied from a 1:1 ($F_1$, $BC_1F_1$, $SC_{2\text{-}1}F_1$ and $SC_3F_1$) to approximately 1:2 for the $BC_2F_1$ progeny and ~1:3 for $SC_1F_1$ (Table 4.5). No self-fertile isolates were recovered from any of the crosses.

The proportion of non-pathogenic isolates varied throughout the entire inbreeding process. Eighty-five of the 86 oospore progeny isolates tested from the initial cross LT51xLT263 were able to cause vigorous infection on unwounded jalapeno fruits (Table 4.5). All tested isolates from cross LT51xLT263 were pathogenic on cucumber fruits. Sixty-five isolates from the first backcross population (LT1021xLT263) were tested and 14 isolates were unable to cause infection on wounded or unwounded cucumber fruits. No water-soaking lesions were observed even 10 days after inoculation and therefore were considered non-pathogenic. The remaining isolates (51) were pathogenic on cucumber fruits but eight isolates were only pathogenic on wounded fruit (Table 4.5). Of the 69 progeny tested from the second backcross LT1422xLT263, 59 (85%) and 40 (57%) isolates were able to cause infection on unwounded cucumbers and jalapenos fruits respectively (Table 4.5). Nonpathogenic isolates were detected in this cross. Interestingly, a high percentage (~60%) of the nonpathogenic isolates had either a predominantly submerged or an appressed type of colony morphology when grown on V8 juice agar media (Fig. 4.3). All isolates from cross LT1503xLT1530 (88 isolates) were pathogenic on cucumber but eight were only able to cause infection on wounded cucumber fruits. Infection results on jalapeno fruits were similar: 86 isolates were pathogenic on unwounded jalapenos and only two isolates were non-pathogenic. A large proportion of progeny isolates from cross LT3382xLT3394 (76% of the total tested progeny) were only pathogenic on wounded jalapeno fruits, and four isolates were completely nonpathogenic (Table 4.5).

**Mature oospores can germinate as parental clones (Apomicts)**

Oospore-derived isolates with AFLP or SNP profiles identical to one or the other parent were recovered from all but one cross (LT1021xLT263) (Fig. 4.1). The seminal cross LT51xLT263 produced 20 out of 221 oospore progeny with identical AFLP genotype to the A2 mating type parent LT263. The first backcross LT1021xLT263 did not produce any parental clones whereas the second backcross LT1422xLT263 had 10% of the isolates (nine out of the 88 oospore progeny) with AFLP genotypes identical to LT263 parent isolate (A2 mating type). The first sibling cross LT1503xLT1530 had six oospore-derived isolates identical to LT1503 (A2 mating type). The majority of the isolates recovered from the second sibling crosses (LT2209xLT2222 and LT2211xLT2222) were identical to the A2 compatibility type parent with 73% of isolates recovered from LT2209xLT2222 and 95% of the isolates recovered from LT2211xLT2222 identical to the A2 parent type. No recombinant isolates were recovered from LT2211xLT2222 (two-month old oospores). Three-month old oospores from cross LT2209xLT2222 generated seventeen oospore progeny. SNP genotyping using marker SNP14 revealed a single recombinant progeny, ten isolates (59%) identical to the A2 compatibility type parent (LT2209), and six isolates identical to LT2222 (data not shown). Likewise, three-month old oospores germinated from cross LT2211xLT2222 did not produce any recombinant isolates and the same pattern was observed: a large number of isolates (28 out of 34 oospore progeny recovered) were identical to the A2 compatibility type parent (LT2211) and only 6 isolates were identical to LT2222 (A1) (data not shown).

In addition, of the 46 oospore-derived isolates recovered from a cross between LT2198xLT51, none appeared to be products of sexual recombination; 42 isolates had identical fingerprint to LT51 (A1 mating type) and four isolates had identical AFLP genotypes to the LT2198 parent (A2 mating type).

## DISCUSSION

The wild type isolates used as parents in this study are typical of *P. capsici* found on vegetables in the U.S. Both isolates readily infect common cucurbit and solanaceous vegetable hosts, grow rapidly at room temperature on V8 medium, produce sexual and asexual spores abundantly, and in many parental pairings are highly fecund (21, 33). Despite their apparent morphological similarities growing under laboratory conditions, population studies indicate that individual *P. capsici* isolates carry significant genetic variation (21). Close crosses (inbreeding) can produce genotypes that may not survive under natural conditions due to deficiencies in survival, pathogenicity and virulence which is consistent with our findings. The progeny from the initial outcross ($F_1$) showed very little change in the overall characteristics of the parent isolates; they were consistently vigorous, pathogenic, virulent, and all of the isolates produced sexual spores. It was only at the first backcross and subsequent backcross and sibling cross generations that the effects of recombination on the previously masked store of recessive variation came to light (Table 4.5). These findings are similar to reports for *P. infestans* where $F_2$ isolates were less pathogenic on tomato and potato than their initial parental isolates (18).

Inbred isolates varied in colony morphology, ability to produce sexual spores, and the ability to infect common hosts. Not surprisingly, none of these traits appear to be

controlled by a single gene with simple Mendelian inheritance (Table 4.5). This likely reflects a complex polygenic inheritance for these diverse functions. For pathogens such as *P. infestans* and *P. sojae,* different races have been identified carrying specific avirulence genes that interact with host resistance genes defined in specific differential lines (1, 2, 7, 34). In the *P. capsici-Capsicum* pathosystem similar discrete gene for gene interactions have yet to be established and investigations have rather focused on polygenic resistance to *P. capsici* (28, 39).

An interesting finding that should be considered when generating more advanced inbreeding crosses is the recovery of apomictic, or clonally derived, progeny from mature, germinating oospores. Neither of the parent isolates produces thick-walled asexual chlamydospores and it is unlikely that our results are due to asexual material contaminating the oospore preparations. Meticulous visual observations of the overnight enzyme-treated oospore solution never revealed any visible hyphal fragments, sporangia, or germinating zoospores. Also, there was no evidence that any of the oospore progeny were produced via selfing, as has been previously reported for *P. infestans* (35-37). For selfing, we expect to see some of heterozygous AFLP markers in the parental fingerprint type lost due to recombination and production of the absence allele. In addition, the products of selfing should show a wide variety of different phenotypes due to inbreeding. In all of the crosses presented here the apomictic oospore progeny were phenotypically and genotypically identical to the parent type. Aberrant segregation ratios have been reported as a limiting factor in *P. infestans* genetic analyses (6, 14). Previous investigations in crosses of *P. infestans* (backcrosses and sibcrosses) yielded distorted

ratios for allozyme markers (38). It is possible that non-recombinant oospores (apomicts) may have contributed to the distorted ratios (4, 13).

Although both parental *P. capsici* isolates contributed apomictic oospore progeny, it is interesting to note that the A2 mating type parent contributed the majority. *Phytophthora* isolates are thought to vary for "maleness" and "femaleness" based on whether they are contributing more to the production of antheridia or oogonia formation (5, 12) and this may play a role in our observed results. It is possible that the A2 type was preferentially male or female and that the underlying mechanism for the observed apomixis occurs primarily in the oogonium or antheridium. At this point it is difficult to speculate on the underlying mechanism(s). Similar results were observed in a series of interspecific crosses between *P. capsici* and isolates of the closely related *P. tropicalis*, as well as crosses between *P. capsici* and *P. nicotianae* (Donahoo, unpublished).

Finally, our results suggest that developing inbred lines in the oomycete *Phytophthora capsici* is possible and may help identify genetic factors underlying complex traits. Currently, the main limiting factor is the lack of co-dominant, low-cost markers. AFLP analysis provided sufficient segregating markers early in the inbreeding process but by the second sibling cross the number of segregating AFLP markers among the siblings was very low and it became difficult to accurately genotype individual progeny. One of the goals of the current *P. capsici* genome project is to catalogue single nucleotide polymorphism (SNP) markers and develop a large-scale marker resource. We expect that the availability of a large database of co-dominant markers will provide the necessary tools to fully develop and exploit inbreeding resources for *P. capsici*.

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## APPENDIX 4

## Tables

Table 4.1. Primers and probe sequences used for SNP genotyping with Taqman assays. Underlined bold nucleotide refers to the assessed SNP. F=forward and R=reverse.

| Locus name | Accession Number | Primer sequence | Internal reporter oligonucleotide (probe) VIC | Internal reporter oligonucleotide (probe) FAM |
|---|---|---|---|---|
| SNP12 | EF566463 | F_5'-cccgctagagattcattcatcca-3'<br>R_5'-gcggaccgtaattctgtgcataa-3' | 5'-cagcca**g**cagacgc-3' | 5'-acagcca**a**cagacgc-3' |
| SNP14 | EF566464 | F_5'-cgctgtgtcgatagtgggaatg-3'<br>R_5'-tcttctctctgtgttcgactacca-3' | 5'-cttgagct**a**attctct-3' | 5'-ttgagct**c**attctct-3' |
| SNP18 | EF566465 | F_5'-gatgcgccccacaatgg-3'<br>R_5'-ttggcccactgcgatgt-3' | 5'-cagcgct**c**caccacg-3' | 5'-cagcgct**t**caccacg-3' |
| SNP19 | EF566466 | F_5'-agggaagcaccgcattagg-3'<br>R_5'-cgtgtcaatctccgtcactaataga-3' | 5'-ctcccgaa**c**cggatga-3' | 5'-cccgaa**g**cggatga-3' |
| URA3 | EF151190 | F_5'-cgaaggacaacgcgaacttg-3'<br>R_5'-ctgcgtgacgccatcaac-3' | 5'-tgctgc**g**tcgactg-3' | 5'-tgctgc**a**tcgactg-3' |

Table 4.2. Summary table of AFLP alleles followed throughout the inbreeding process. Alleles were named by their size (bp). Percentage of fixed alleles was calculated based on the total number of described alleles.

| Allele (bp) | Cross | | | | |
|---|---|---|---|---|---|
| | **$F_1$** | **$BC_1F_1$** | **$BC_2F_1$** | **$SC_1F_1$** | **$SC_{2\text{-}1}F_1$** |
| | **LT51xLT263** | **LT1021xLT263** | **LT1422xLT263** | **LT1503xLT1530** | **LT2209xLT222** |
| 119 | Aa x Aa | Aa x Aa | aa x Aa | aa x aa | aa x aa |
| 125 | aa x Aa | Aa x Aa | AA x Aa | Aa x Aa | A* x A* |
| 202 | aa x Aa | aa x Aa | aa x Aa | Aa x aa | Aa x aa |
| 215 | Aa x aa | Aa x aa | aa x aa | aa x aa | aa x aa |
| 217 | Aa x aa | Aa x aa | aa x aa | aa x aa | aa x aa |
| 226 | Aa x aa | aa x aa | aa x aa | aa x aa | aa x aa |
| 233 | Aa x Aa | aa x Aa | aa x Aa | Aa x Aa | aa x Aa |
| 267 | Aa x aa | Aa x aa | aa x aa | aa x aa | aa x aa |
| 290 | aa x Aa | Aa x Aa | AA x Aa | Aa x Aa | A* x A* |
| 322 | aa x Aa | aa x Aa | Aa x Aa | aa x Aa | aa x aa |
| 363 | Aa x aa | aa x aa | aa x aa | aa x aa | aa x aa |
| 378 | AA x Aa | Aa x Aa | AA x Aa | A* x A* | A* x A* |
| 387 | aa x Aa | Aa x Aa | aa x Aa | aa x Aa | aa x Aa |
| 427 | aa x Aa | Aa x Aa | AA x Aa | Aa x Aa | A* x A* |
| 453 | aa x Aa | aa x Aa | aa x Aa | Aa x aa | aa x aa |
| 469 | AA x aa | Aa x aa | aa x aa | aa x aa | aa x aa |
| 492 | aa x Aa | Aa x Aa | Aa x Aa | Aa x Aa | aa x Aa |
| 595 | Aa x aa | Aa x aa | aa x aa | aa x aa | aa x aa |
| 614 | aa x Aa | aa x Aa | aa x Aa | aa x aa | aa x aa |
| 622 | aa x Aa | aa x Aa | Aa x Aa | aa x aa | aa x aa |
| Fixed alleles (%) | not calculated | 10 | 35 | 50 – 52** | 60- 75** |

* Allelic conformation was ambiguous. ** Calculated excluding ambiguous alleles.

Table 4.3. SNP genotypes for cross LT2209 x LT2222.

| **Marker** | **SNP14** | **SNP18** | **SNP19** | **URA3** |
|---|---|---|---|---|
| **Allelic conformation** | G/G[A] x T/T[B] | T/C x T/C | G/G x G/C | C/T x C/C |
| **Expected conformation** | GT | TT:2TC:CC | GG:GC | CC:CT |
| **No. isolates** | 11 | 1:8:2* | 8:3** | 6:5* |

[A] An additional 60 isolates had identical SNP genotype. [B] An additional 10 isolates had identical SNP genotype. * Chi Square value significant at *P*=0.05. ** not significant at *P*=0.05.

Table 4.4 Summary data for inheritance of the URA3 single nucleotide polymorphism in a series of inbreeding crosses of *P. capsici*

| | **Cross** | | | | |
|---|---|---|---|---|---|
| | **$F_1$** | **$BC_1F_1$** | **$BC_2F_1$** | **$SC_1F_1$** | **$SC_{2\text{-}1}F_1$** |
| **SNP marker** | **LT51xLT263** | **LT1021xLT263** | **LT1422xLT263** | **LT1503xLT1530** | **LT2209xLT222** |
| **URA3** | CC x CT | CC x CT | CC x CT | CT x CC | CT x CC |
| **No. isolates** | 44:48 | 20:51 | 32:48 | 47:41 | 5:6 |
| **Chi-square** | 0.17* | 13.53** | 3.2* | 0.41* | 0.09* |

* Chi Square value significant at *P*=0.05. ** not significant at *P*=0.05.

Table 4.5. Summary data for compatibility type and pathogenicity for the progeny of *Phytophthora capsici* inbreeding crosses

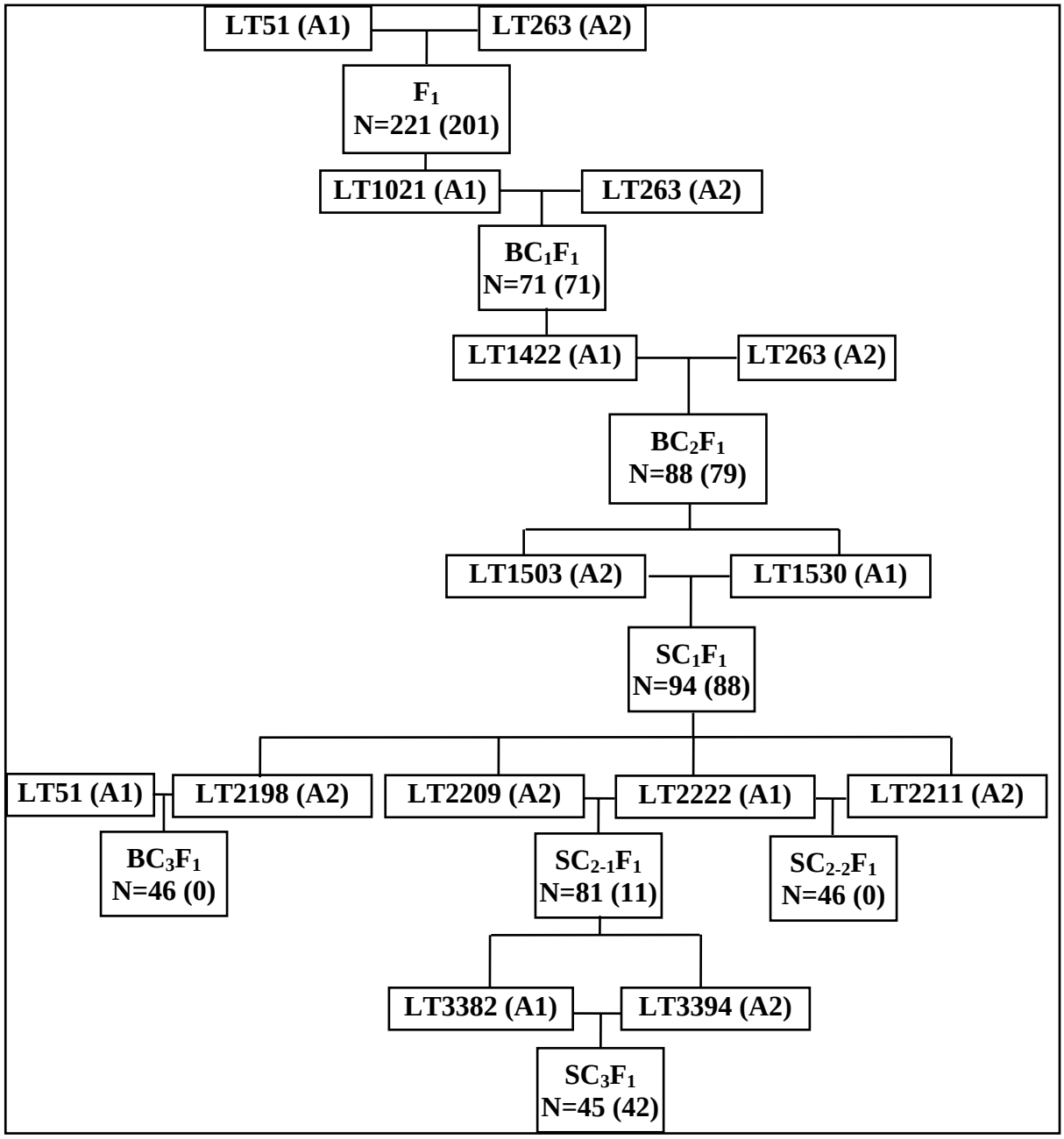
| [1]Population | Cross name | A1 | A2 | Sterile | [2]Ratio A1:A2 | [3]Pathogenicity | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cucumber | | Jalapeno | |
| | | | | | | P | NP | P | NP |
| $F_1$ | LT51xLT263 | 36 | 50 | 0 | 1:1 | 86 | 0 | 85 | 1 |
| $BC_1F_1$ | LT1021xLT263 | 31 | 38 | 2 | 1:1 | 51 (8) | 14 | N/T | N/T |
| $BC_2F_1$ | LT1422xLT263 | 26 | 42 | 2 | 1:2 | 59 | 10 | 40 | 29 |
| $SC_1F_1$ | LT1503xLT1530 | 22 | 65 | 1 | 1:3 | 80 (8) | 0 | 86 | 2 |
| $SC_{2\text{-}1}F_1$ | LT2209xLT2222 | 5 | 6 | 0 | 1:1 | 11 | 0 | 11 (1) | 0 |
| $SC_3F_1$ | LT3382xLT3394 | 18 | 19 | 5 | 1:1 | N/T | N/T | 38 (32) | 4 |

[1] $F_1$=filial 1, $BC_1F_1$=first backcross, $BC_2F_1$=second backcross, $SC_1F_1$=first sib-cross $F_1$, $SC_2F_{1\text{-}1}$ =second sib-cross $F_1$. $SC_3F_{1\text{-}1}$=third sib-cross. [2] Chi Square value significant at P=0.05

[3] Isolates were tested on both wounded and non-wounded fruit. Values in parentheses are the number of isolates pathogenic only on wounded fruit; P=pathogenic, NP=non pathogenic, N/T= non tested

## Figures

Figure 4.1. Overview of *Phytophthora capsici* crosses. N = the total number of oospore-derived isolates with the number of genetically recombined progeny in parenthesis. Tennessee (LT263) and Michigan (LT51) wild type isolates were initially crossed (cross name LT51xLT263) rendering 201 recombinant isolates (F1 population). Isolate LT1021 (F1 - derived) was mated with LT263, generating 71 recombinant isolates (cross name LT1021xLT263). Isolate LT1422 (BC1F1- derived) was mated with LT263 (cross LT1422xLT263) producing 79 recombinant isolates. First full sibling cross (cross name LT1503xLT1530) was done using isolates derived from cross LT1422xLT263, generating 88 recombinant isolates (F1 population). Second full sibling crosses (LT2209xLT2222 and LT2211xLT2222) generated 11 and 0 recombinant populations respectively. A third consecutive sibling cross (LT3382xLT3394) generated 42 recombinant isolates.


LT51 (A1)
LT263 (A2)
F1
N=221 (201)
LT1021 (A1)
LT263 (A2)
BC1F1
N=71 (71)
LT1422 (A1)
LT263 (A2)
BC2F1
N=88 (79)
LT1503 (A2)
LT1530 (A1)
SC1F1
N=94 (88)
LT51 (A1)
LT2198 (A2)
LT2209 (A2)
LT2222 (A1)
LT2211 (A2)
BC3F1
N=46 (0)
SC2-1F1
N=81 (11)
SC2-2F1
N=46 (0)
LT3382 (A1)
LT3394 (A2)
SC3F1
N=45 (42)

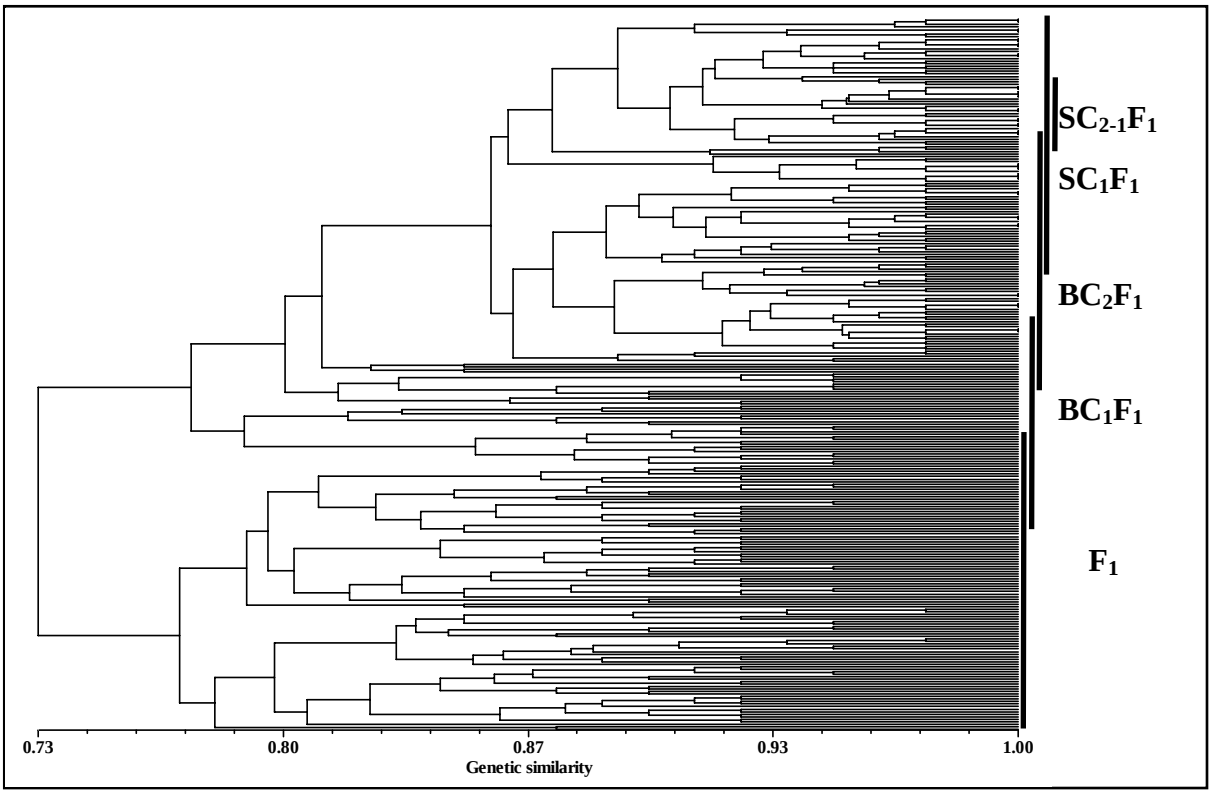



Figure 4.2. Unweighted pair-group method with arithmetic average (UPGMA) cluster analysis of *Phytophthora capsici* isolates from five crosses using 40 amplified fragment length polymorphic (AFLP) markers. Bars at the right represent the position of at least 85% of the isolates from each population. $F_1$=filial one; $BC_1F_1$=first backcross; $BC_2F_1$=second backcross; $SC_1F_1$=first sib-cross $F_1$; $SC_{2\text{-}1}F_1$=second sib-cross $F_1$.

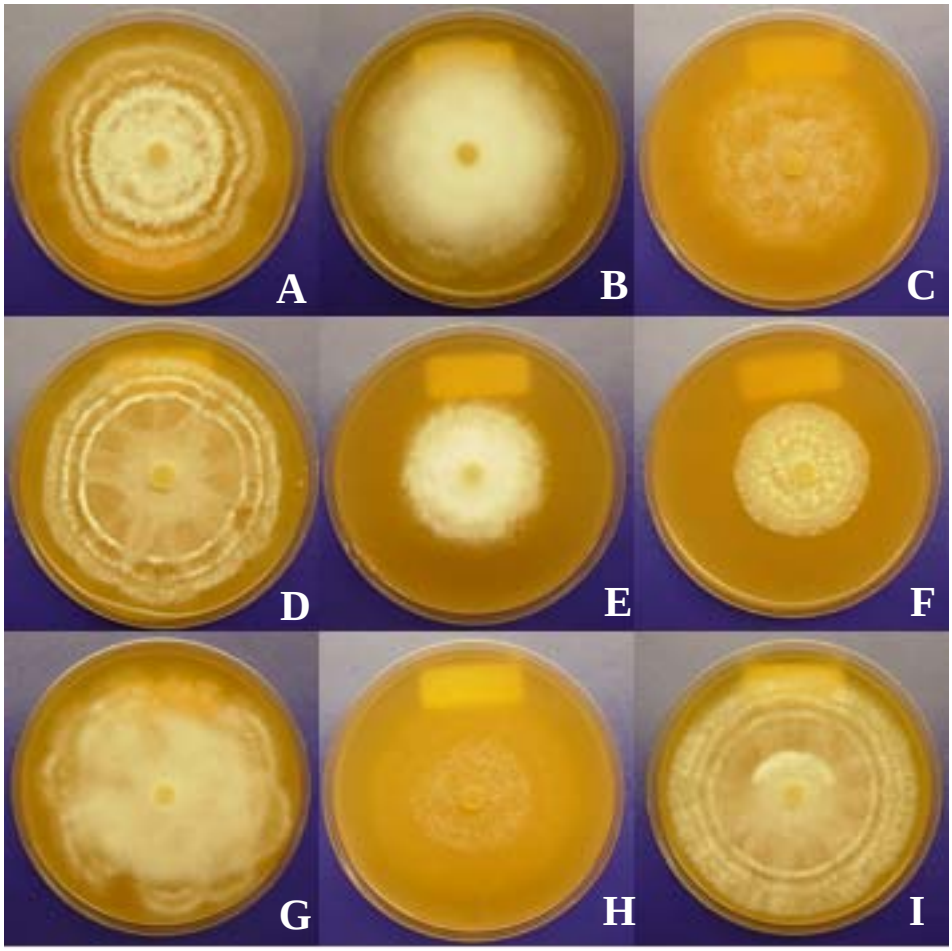



Figure 4.3. Representative colony morphologies for *Phytophthora capsici* isolates derived from sexual reproduction. A and B are LT263 and LT51 wild type parent isolates respectively. C is LT1021 (F1 isolate). D through I are single-oospore isolates derived from cross LT1021xLT263 (first backcross).

# Chapter Five Construction of genetic linkage framework for the oomycete *Phytophthora capsici* (L.)

**ABSTRACT**

We mated two wild type isolates, CBS121656 (mating type A1) and CBS121657 (mating type A2) to construct partial genetic linkage maps in the outcrossing species of *Phytophthora capsici* (L.). We analyzed a set of 46 $F_1$ progeny isolates with 16 primer combinations, generating 189 AFLP (amplified fragment length polymorphism) markers. Additionally 18 SNP (single nucleotide polymorphism) markers were generated for the $F_1$ population. Only 7% (18 AFLP markers) demonstrated significant segregation distortion ($P$<0.01). At a minimum LOD score of 3.0 and a maximum recombination frequency of 0.3, the framework map for each parental isolate was composed of 15 linkage groups. The genetic linkage groups for CBS121657 and CBS121656 were comprised of 65 and 42 markers respectively. Six SNP markers were mapped into CBS121657 with two SNP markers (FL5 and FL6) located in the same linkage group (LG9) separated by 7.7 cM, and allows assembly and genetic comparisons. The highest marker density was identified in linkage group 3 of CBS121657 with seven AFLP markers covering 15.6 cM. Overall, the genetic linkage map for CBS121657 had a better coverage and expanded 268 cM whereas the framework map for CBS121656 spanned 141 cM. This is the first genetic linkage analysis for the diploid fungus-like *P. capsici*.

**INTRODUCTION**

*Phytophthora capsici* (L.) is a diploid fungus-like plant pathogen with a broad and expanding host range that includes crops such as tomato, peppers, pumpkin, cucumber, watermelon, squash, and snap beans (3, 4, 6). *Phytophthora capsici* causes root, crown, and fruit rot, causing economic losses of millions of dollars annually for the vegetable

industry (6, 16). Due to the unique biology of oomycetes, most fungicides do not effectively control outbreaks of *P. capsici* (3).

In recent years, a better understanding of the epidemiology of *P. capsici* has provided useful information about fungicide resistance, dispersal of the pathogen, and the importance of sexual reproduction for the long term survival of *P. capsici* (10-14). However, little is known about the genetics of this devastating pathogen. The identification of races or pathotypes within *P. capsici* has been elusive, but laboratory crosses could be used for this purpose, as they have generated a range of pathogenic and non-pathogenic isolates on pepper, cucumber, tomato, and watermelon plants (19, 21). More recently, pepper breeders have started to focus their efforts on creating a set of differential hosts similar to those resources existing in other pathosystems (*P. infestans* and potato or *P. sojae* and soybean) in order to elucidate the genetic bases of pathogenicity (2, 18). Oelke et al. identified 13 physiological races of Phytophthora root rot and foliar blight using a limited collection of isolates from New Mexico (18).

Progress in identifying the molecular basis of pathogenicity and other traits such as sporulation, organogenesis, heterothallism, etc., can be accomplished via functional analyses of candidate genes genetically associated with the phenotype under study. There are several examples in which map-based cloning strategies have led to the identification of the gene of interest. Genetic analyses in *P. sojae* have shown that single dominant avirulence genes corresponding to the resistant *Rsp* gene in soybean were organized in clusters and that the *Avr1b* locus contained a secreted effector protein (17, 22, 34). Bulked segregant studies using AFLP markers in *P. infestans* identified markers closely linked to five *Avr* genes (30). The *Avr3a* avirulence gene from *P. infestans* was identified

using a combination of linkage disequilibrium and candidate gene approach of ESTs (expressed sequence tags) encoding secreted proteins (1). A mapping interval study using AFLP, combined with a physical spanning of a BAC library from a $F_2$ cross of *Hyaloperonospora parasitica,* was the starting point for cloning the *ATR1Nd* avirulence gene (20). DNA markers have also been used to study the mating type locus in *P. infestans* (8, 9).

The abundance of molecular markers for the construction of saturated genetic linkage maps enables these strategies to effectively identify markers linked to the trait of interest. Despite the utility of the strategies mentioned above, very few genetic linkage maps have been developed in oomycetes. This is due in part to the challenges in generating laboratory crosses, low oospore germination, and the biotrophic dependence of a group of oomycetes (downy mildew). In oomycetes such as the homothallic *P. sojae*, the number of DNA markers is also limited. Out of 400 decanucleotide primers used for generating RAPD (random amplified polymorphic DNAs) markers in $F_1$ populations of *P. sojae*, 224 did not generate polymorphic fragments between the parental isolates (33). Inspection of the whole-genome sequence revealed ~500 SNPs for *P. sojae* compared to ~13000 SNPs in the outcrossing *P. ramorum* (28). In addition, in the heterothallic *P. infestans*, trisomy in oospore-derived progenies can hamper genetic studies (31).

*Phytophthora capsici* is an outcrossing species, requiring both mating types (A1 and A2) to complete the sexual cycle (11, 12). In the U.S., both mating types are found at most locations and oospores are thought to play an important role in the epidemiology (6). Consequently, U.S. populations of *P. capsici* carry a large amount of genetic variation which can be exploited for the generation of saturated genetic linkage maps.

The generation of a genetic linkage map is also an important component of the *P. capsici* genome project. The linkage maps provide independent information on the genomic organization, playing a crucial role especially in areas of the genome with repetitive sequences where a definitive assembly is hard to predict. Our goal was to generate the first genetic linkage map for *P. capsici* using AFLP and SNP markers and to provide preliminary information about the genomic organization of *P. capsici.*

## MATERIALS AND METHODS

### *Phytophthora capsici* mapping population

The mapping population was generated from a cross of *P. capsici* between two field isolates: CBS121656 (mating type A1) isolated from a cucumber fruit in Michigan in 1997 and CBS121657 (mating type A2), isolated from a pumpkin fruit in Tennessee during 2004. To generate oospores, the parent isolates were plated approximately 2 cm apart on UCV8 juice agar amended with PARP (100 ppm of pimaricin, 100 ppm of ampicillin, 30 ppm of rifampicin, and 100 ppm of pentachloronitrobezene). Plates were wrapped with Parafilm®, kept in the dark at room temperature and incubated for at least 2 months before the oospores were separated from the parental material and stimulated to germinate. Oospore germination was performed as previously described (10). Germinated oospores ("octopus" shape) were retrieved from the solution using a constructed device using a glass Pasteur pipette. Individual germinated oospores were transferred to water agar plates and after 1-2 days, single hyphal tips were transferred to V8 amended agar. A total of 225 isolates were recovered using this method. To determine mating type of *P. capsici* isolates, plugs of actively expanding mycelium were placed at the center of V8

juice agar plates approximately 2 cm away from "tester" isolate CBS121656 (mating type A1) or CBS121657 (mating type A2). Plates were wrapped with Parafilm and incubated in the dark at room temperature for at least one week after which observations were made for the production of oospores at the interface using a light microscope. Isolates able to produce oospores when crossed against CBS121656 were determined as A2 mating type. Conversely, isolates that were able to form oospores when crossed against CBS121657 were determined as A1 mating type.

**DNA isolation and AFLP fingerprinting**

AFLP profiles were generated for all 41 oospore-derived isolates. Isolates were grown and treated as previously described for DNA isolation (15). To assess the quantity and quality of the DNA, 3 µl of DNA per isolate were resolved on a 1% agarose gel alongside a dilution series of lambda DNA (10 and 5 ηg) (Takara Bio Inc, Shiga, Japan).

AFLP profiling was done using *Eco*RI and *Mse*I restriction endonucleases, adapters, and primers as described by Vos et al. (32). Pre-selective amplification was performed using the primer combinations Eco +0/MseI +0, Eco+A/Mse+A, and Eco+A/Mse+C. Selective amplification was conducted with 16 selective primer combinations (Table 5.1). Selective amplifications were diluted and labeled in a separate reaction according to Habera et al. (5). AFLP fragments were resolved and analyzed on a CEQ$^{TM}$ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) following the manufacturer's protocols. A 600 bp DNA size standard was used to resolve AFLP fragments (Beckman Coulter, Fullerton, CA). AFLP marker sizes ranged from 70 to 500 bp.

**SNP genotyping with Hi-resolution DNA melting analysis**

SNP genotyping was performed using the hi-resolution DNA melting analysis of small amplicons (7, 35). Full-length complementary DNA sequences (FL-cDNA) were generated as part of the genome project for *P. capsici* (unpublished) and used as template for designing primers to amplify 700 to 1100 bp in both parental isolates (unpublished data). Subsequently this sequence data was used to design primers to amplify small genomic regions (65-150 bp) flanking one to three SNPs. Primers were designed using the LightScanner primer design software (Idaho Technologies, Salt Lake City, UT) (Table 5.2). Small amplicons were generated using PCR parameters: 95°C for 5 min, 45 cycles of denaturing at 95°C for 30 s, annealing at 74°C for 30 s, synthesis at 72°C for 30 s with a final extension at 72°C for 5 min. After PCR amplification and heteroduplex formation, PCR products were subjected to melting analysis using a light scanner instrument (Idaho Technologies, Salt Lake City, UT). Parameters were adjusted accordingly using the LightScanner 2.0 software and melting analyses were performed twice using DNA from two biological replicates.

**Marker name and scoring**

Each AFLP marker was named according to the primer combination and its position given according to the molecular ladder (Beckman Coulter, Fullerton, CA). The presence of a marker was scored as 1 and absence as 0. A binary matrix was constructed using only clearly resolved markers. Markers were confirmed visually by one person throughout the whole experiment. The format for naming the markers consisted of three

elements: Eco primer followed by the number of the selective nucleotide combination (e.g.: E22, means adapter sequence plus two selective nucleotides, [C=2]), Mse primer (e.g: M21) and the corresponding size marker (e.g: 350 bp). The final name for the marker in this example would be E22M21.350.

Markers generated using the hi-solution DNA melting analysis (DMA) were named according to their primer's name. For haplotype combinations P1=H1H1 x P2=H1H2, and P1=H1H1 x P2=H2H3, parents with the coupled haplotype (H1H1) were scored as 0 and the uncoupled haplotypes (H1H2 and H2H3) were scored as 1 for mapping purposes. Haplotype identification relied on sequence information previously generated from both parents (unpublished). Haplotypes H1H2 and H2H3 were scored "1" (presence of the "band") only in sequence trace data that contained the heterozygous site (SNP) in both strands of the parental isolate.

**Markers segregation**

A binary matrix with polymorphic markers for each parent was generated and all markers were analyzed for their goodness of fit to the appropriate segregation ratio. Observed segregation ratio (presence vs absence) in the progeny was analyzed for the Mendelian segregation 1:1 with a chi-square ($P$=0.01) and one degree of freedom.

**Linkage analysis and map construction**

Data files with segregating markers were used as input files in the software JoinMap 3.0 (24) and individually analyzed for each parental isolate. The similarity of loci was calculated using JoinMap and loci with similarity values larger than 0.98 were

excluded from the analysis. JoinMap calculates linkage between two markers based on the Logarithm of Odd (LOD) and estimated LOD values are compared to specific threshold value (stepwise approach). After the grouping of markers, the order of the markers was calculated. Mapmaker/EXP 3.0 was used to confirm the order of markers using commands 'order', 'compare' and 'map' with the Kosambi mapping function. One map was generated for each parental isolate. Linkage groups were exported as text files and a compiled file containing all linkage groups was generated for use in MapChart 2.1 (Plant Research International, Wageningen, The Netherlands).

## RESULTS

### Marker segregation

AFLP profiling using 16 primer combinations yielded 189 reliable markers. From this data set, 87 markers were polymorphic for CBS121656, 102 markers were polymorphic for CBS121657, and 57 markers were present in both parents but segregated in the progeny. Chi-tests on all AFLP markers revealed that 95% (83 markers) and 94% (96 markers) segregated as 1:1 for CBS121656 and CBS121657, respectively ($P$=0.01). Similarly, 85% of AFLP markers (49 markers) in both parents segregated as 3:1 ratio ($P$=0.01). Hi resolution DMA (DNA melting analysis) generated six markers for CBS121656 and 12 markers for CBS121657. All but one marker (FL35), fit a 1:1 ratio at $P$=0.01.

**Linkage analysis**

JoinMap revealed two loci with identities equal to 1.0 for parent CBS121657 and therefore one of these loci was excluded from further analysis. No loci with identity larger than 0.98 were detected in isolate CBS121656. Five $F_1$ isolates (LT976, LT1001, LT1052, and LT1060) were excluded from the genetic analysis because they contained more than 50% loci with missing scores and all genetic analyses were performed with only 41 isolates. Linkage analysis was performed at LOD threshold values 4, 5, 6 and 8.

Linkage groups were generated between LOD 4 and LOD 6. At these conditions, 15 linkage groups for CBS121656 parent were generated and 32 AFLP markers did not link to any linkage group (Fig. 5.1). Similarly, 15 linkage groups were generated for isolate CBS121657 and 39 markers did not link to any linkage group (Fig. 5.2). Further analysis of unlinked loci showed that at least 20% were missing between 20 to 30% of their scores. The remaining loci (51 AFLP markers and 6 FL markers) did not have a significant distortion in their segregation 1:1 ($P$=0.01), and after a second round of linked analysis, they could be linked to other linkage groups under LOD threshold value of 2 (data not shown).

The largest linkage group (LG) for CBS121656 was LG8. LG8 was comprised of 6 markers (4 AFLPs and 2 FLs) and spanned ~20 cM, whereas the smaller LG was LG7 with two markers and spanned 2.6 cM (Fig. 5.1). The largest LG in CBS121657 was LG7, comprised of 10 AFLP markers and two FLs markers, spanning 44.7 cM. The smallest LG in CBS121657 was LG12 with two AFLP markers (Fig. 5.2).

## DISCUSSION

This is the first genetic linkage analysis of the oomycete *Phytophthora capsici*. We used a combination of two types of markers (AFLP and SNPs) and obtained loci data for 41 $F_1$ oospore-derived isolates. Initially, we generated AFLP profiles on 93 isolates derived from the cross under study.

We decided to investigate how the SNP data linked into the context of a mapping population because the SNP data could provide relevant information regarding the genome assembly. The mapping population size was thus reduced to 46 $F_1$ isolates to fit the SNP data. We mapped a total of 10 FL markers (four FLs in CBS121656 and six FLs in CBS121657). A comparison between the genetic linkage data and the assembly information for SNP markers FL5 and FL6 was possible due to their location in the same scaffold of assembly FORGE7. FL5 and FL6 were also mapped in the same LG (LG9) of CBS121657. Mapping data indicated that both markers were separated by 7.7 cM at LOD 5. The assembly FORGE7 indicated that SNPs FL5 and FL6 were located in scaffold 1 in the relative positions of 86 and 95% in the scaffold, respectively (~2.6 Mb and ~2.9 Mb) (Fig. 5.2). Calculations demonstrated that 1cM is equivalent to 38 Kb, and because the genetic linkage framework of CBS121657 resulted in 268 cM, the genome of isolate CBS121657 should be ~10Mb. The size of the *P. capsici* genome has been previously calculated at 65Mb (27); however, this discrepancy could be due to the lack of more markers. We concluded that there is 1 cM per 38 kb. This value is comparable to that of *P. sojae* (38/56 kb/cM) (17) but considerably smaller than that of *P. infestans* (~200 kb/cM) (29). The remaining FLs markers mapped within one LG (pairs FL18-FL24 for

LG7 and FL22-FL25 for LG8) and they were located at different scaffolds in the assembly, therefore it was not possible to corroborate our previous calculations.

The number of linkage groups per parent isolate was considerably higher than the estimated basic number of chromosomes. Observation of chromosome number under a light microscope is difficult due to their small size in oomycetes. For *P. capsici*, the number of chromosomes has been suggested as n=6 (25, 26). Therefore in a framework map built only with dominant markers, the number of linkage groups generated will be the double of the basic chromosome number (mirror map).

We generated 15 groups per parental isolates but there were at least three groups per parental isolate composed by two or three markers covering a distance larger than 8 cM (Figs. 5.1 and 5.2). There was a significant number of AFLP markers unlinked at LOD 4. Additional markers are needed to merge these markers into other linkage groups and probably the number of linkage groups would be reduced. The use of co-dominant markers will enable us to integrate both linkage framework maps and reduce the number of LGs. In our case, all SNPs generated in this study behaved as dominant markers with a Mendelian segregation of 1:1. The generation of SNP markers based on coding region (our case) will also be useful for the identification of gene distribution in the genome and for future syntheny studies.

In general, AFLP profiling of parental isolate CBS121657 yielded more heterozygous markers that enabled a better coverage of its linkage groups. DNA markers were not evenly distributed evenly in and between linkage groups per parental isolate. There were several distinct clusters of markers observed in parental CBS121657 that

could be due to reduced recombination or unequal detection of markers (23). Moreover, the map saturation was not sufficient for an indicative location of the centromeric region.

We attempted to describe the first linkage analysis in *P. capsici* using dominant markers. The generation of additional markers (SNPs) with power to bridge both parental maps is underway. Functional maps based on information generated from ESTs or high-throughout technologies at each life stage will increase our understanding about which areas of the genome are active during specific developmental processes.

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## APPENDIX 5

### Tables

Table 5.1. Summary of primer combinations used for generating amplified fragment length polymorphic (AFLP) markers. Cero is equivalent to the absence of selective nucleotide.

| Pre-selective PCR | Selective PCR | No markers |
|---|---|---|
| Eco+0/Mse+0 | EcoRI+12/MseI+212 | 10 |
| | EcoRI+21/MseI+212 | 7 |
| | EcoRI+12/MseI+211 | 17 |
| | EcoRI+21/MseI+213 | 12 |
| | EcoRI+12/MseI+213 | 11 |
| | EcoRI+21/MseI+222 | 8 |
| | EcoRI+22/MseI+21 | 13 |
| | EcoRI+12/MseI+211 | 9 |
| | EcoRI+21/MseI+21 | 19 |
| | EcoRI+22/MseI+23 | 19 |
| | EcoRI+23/MseI+23 | 20 |
| Eco+1/Mse+1 | EcoRI+14/MseI+13 | 26 |
| | EcoRI+11/MseI+14 | 22 |
| Eco+1/Mse+2 | EcoRI+11/MseI+24 | 19 |
| | EcoRI+12/MseI+24 | 19 |
| | EcoRI+13/MseI+21 | 15 |

A=1, C=2, G=3, and T=4

Table 5.2. Primer list designed for generating SNP marker in F1 population of *Phytophthora capsici*

| Locus ID | Forward Primer | Reverse Primer | Amplicon Size | $T_m$ °C | # of sites |
|---|---|---|---|---|---|
| FL-3 | GGGGCGTTCTCAACAGTATGC | CAGCGTCGGGTGCGTC | 227 | 64 | 2 |
| FL-6 | CCTCATTTCTCAATTTACCACG | GTGGAATTGTGCACCATTT | 67 | 59 | 3 |
| FL-5 | GTGATGATCTGCTGACCAC | TCACGGTCGATGAATACCC | 67 | 64 | 2 |
| FL-9 | TGCAGAAGAAGCCGGAG | TCACATCTGCAGCGCTGA | 54 | 64 | 2 |
| FL-13 | CTCTGCACGTTGTGCTC | TGACGACAATGGATGTACG | 90 | 64 | 1 |
| FL-14 | AGAAGGCTCTCGTTACG | CACAAACACAGCAGCAA | 83 | 64 | 1 |
| FL-18 | CCTGCTAATGCTGAAGTCAA | GTTTCCTTAGTCTCTGTCTCAC | 56 | 64 | 2 |
| FL-20 | TCATTCCGCTTATGACAGAAC | ATACTGCTTACTCAGATTGGG | 56 | 64 | 1 |
| FL-22 | CCTGCAGCGACATCAAC | TAGCCGTCCATCACATCT | 53 | 64 | 2 |
| FL-23 | ATACCCTCCGACATATCTTCA | CAGCATCGAATCGTTACTG | 69 | 64 | 1 |
| FL-24 | CCTGCCCTTGACAAGGA | CCCACCTTGACCATCTG | 95 | 64 | 3 |
| FL-25 | CGAACGACTTAACGGGT | CTTCATCTTCCACGTCACT | 53 | 64 | 1 |
| FL-26 | GGAAATGGCAGAAGAAATTGG | CGCAGCACCTTCAAACTA | 51 | 64 | 2 |
| FL-27 | CTGGGTCAACTTGGTCTTT | GCAGTACATTACTCTCTACCTCA | 70 | 64 | 2 |
| FL-32 | TCGTGTGTTTTCTGCTGT | GTAAAAATGAGAGTAATCTCACCGA | 52 | 64 | 2 |
| FL-34 | CGAACAGCTTCATACGGA | GATGGCGTGTTCAAGGT | 55 | 64 | 2 |
| FL-35 | CATCTTCGGCAGTGACG | GTAACAATAGCAACACACAGAAC | 110 | 64 | 1 |
| FL-37 | AAACCCCAATAGCTGAGAAA | TCAACGTCTCCTAAAGGTGT | 55 | 64 | 1 |

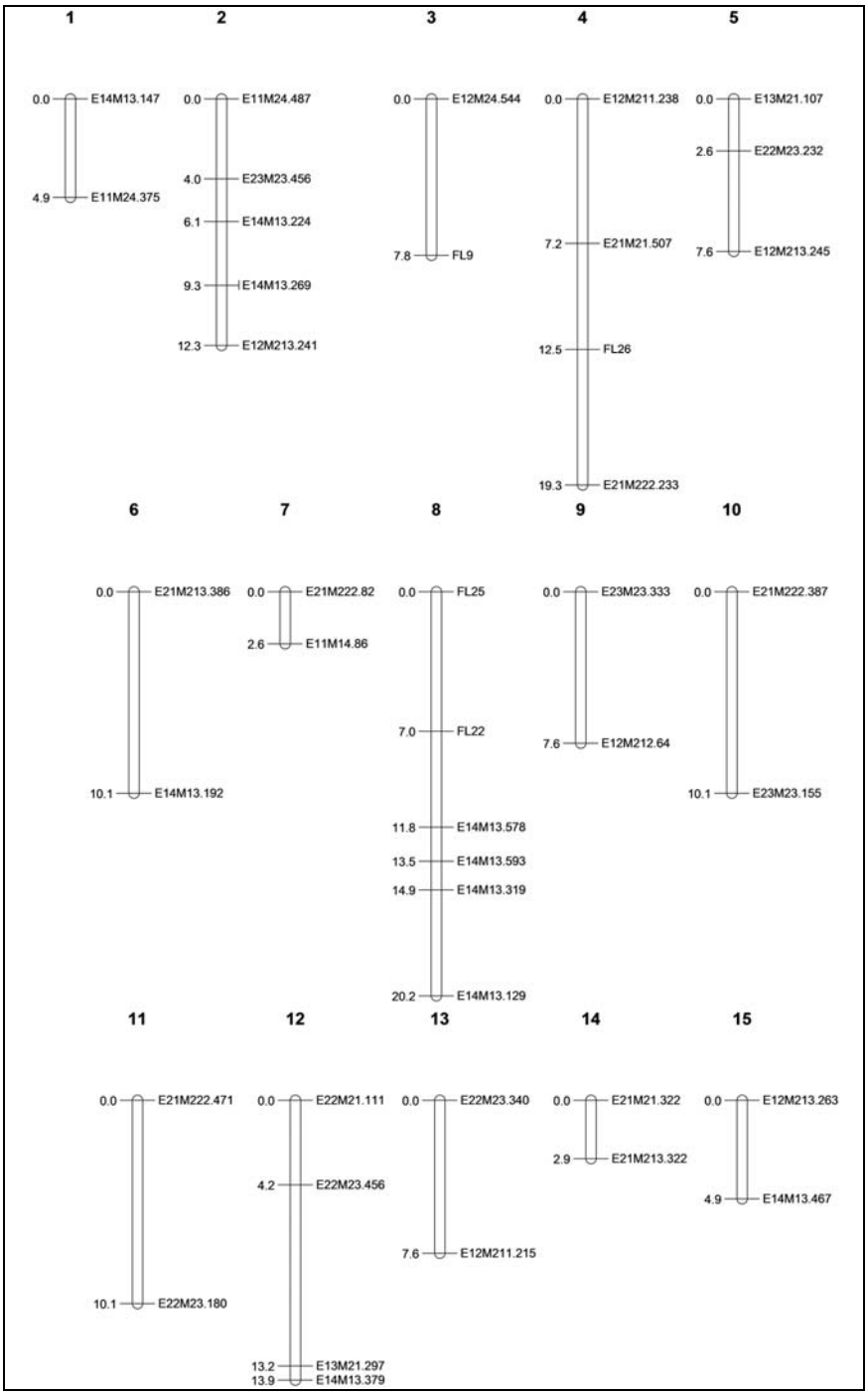


Figure 5.1. Genetic linkage map for CBS121656. Markers are indicated on the right according to marker code. Genetic distance is indicated at the left side (cM).

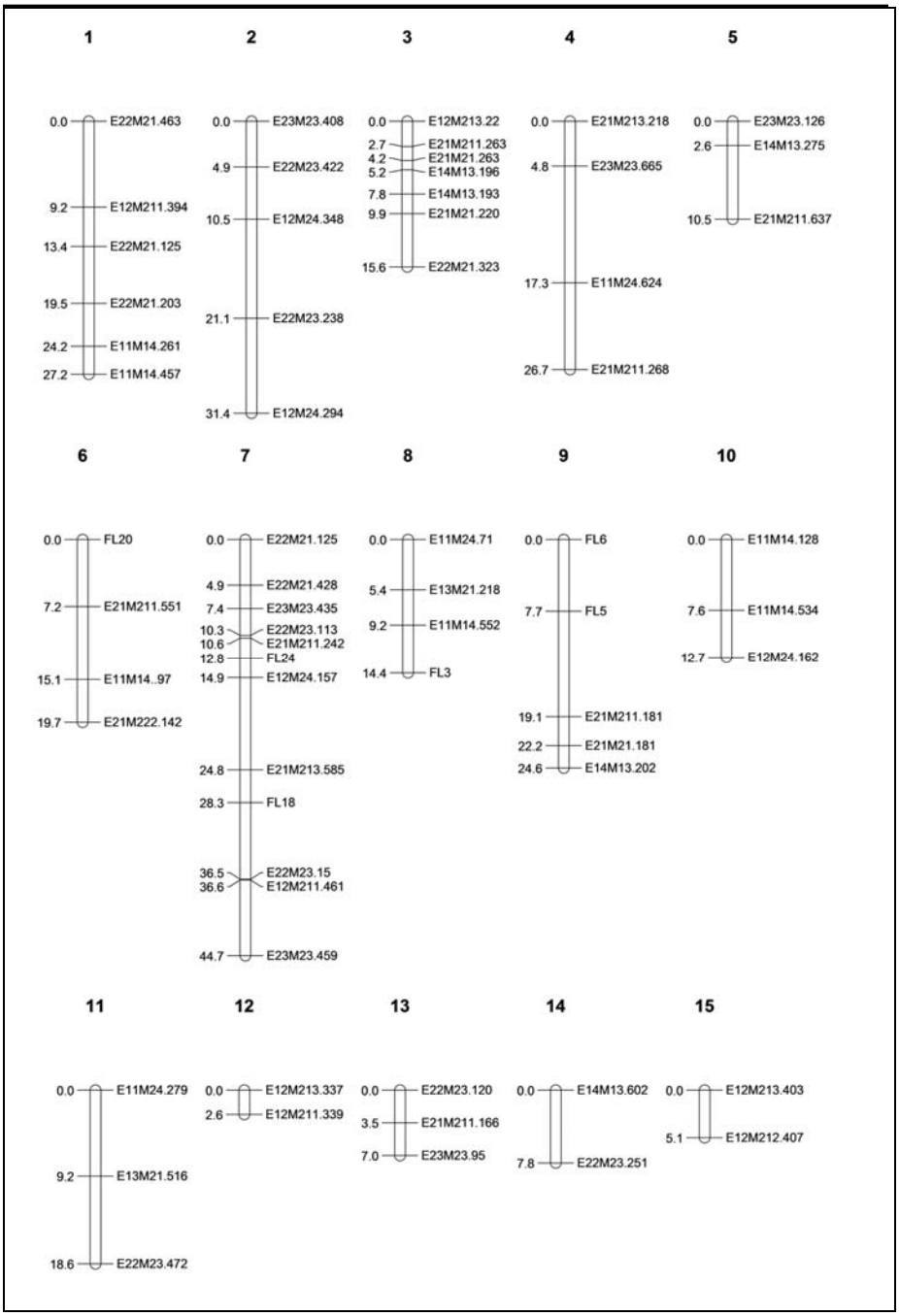


Figure 5.2. Genetic linkage map for CBS121657. Markers are indicated on the right according to marker code. Genetic distance is indicated at the left side (cM).

# Chapter Six Polyethylene glycol-mediated transformation in *Phytophthora capsici*

**ABSTRACT**

Transformation for drug resistance (G418) in conjunction with constructs for green fluorescent protein production was tested using protoplasts and polyethylene glycol (PEG) for *Phytophthora capsici*. The protocol for PEG-mediated transformation was adapted from a modified version of a protocol described for *P. infestans* and *P. sojae*. Protoplasts were generated (3 x $10^7$ protoplast/ml) using a cocktail of enzymes (crude lyzing enzyme from *Trichoderma harzianum* and cellulase), and isolates resistant to G418 (50 µg/ml) were subcultured at least five times in selective media for three months. Approximately 65% of the transformed isolates were not able to recover in selective medium (G418 50 µg/ml) after subculturing on UCV8 medium. Our observations agree with previous reports of PEG-mediated transformation for other *Phytophthora* spp. in which a combination of stable and nonstable transformants were recovered.

**INTRODUCTION**

The genus *Phytophthora* is comprised of more than 90 described species (5, 13, 17). *Phytophthora* species infect many economically important crops such as potato, soybean, and tomato (5, 8). The genomics era has placed oomycete researchers in a privileged position: two draft genome sequences are available (*P. sojae* and *P. ramorum*) (24) and the genome sequences for *P. infestans*, *P. capsici* and *Hyaloperonospora parasitica* will be available soon. Thus, devising approaches for studying gene function are in demand.

A wide array of transformation methods have been developed, such as the PEG-mediated transformation of protoplasts (9), electroporation of zoospores (16),

*Agrobacterium tumefaciens* mediated transformation (26), and microprojectile bombardment (3). In addition, triggering of gene silencing has been demonstrated via incubation with double stranded RNA (29). Each method has its drawbacks; some are not efficient for obtaining homokaryotic stable transformants and others do not reach a completed silencing of the gene under study. Currently, the PEG-mediated protocol remains the favorite transformation method although promising new techniques are emerging, such as the generation of gene knockouts based on chemical mutagenesis (15).

The overall objectives of this study were to adapt a *P. sojae* PEG-mediated transformation protocol for *P. capsici,* and to study the stability of transformants during continuous subculturing on selective and non-selective medium (G418 50 µg/ml) .

## MATERIALS AND METHODS

### *Phytophthora* growth conditions

Isolate CBS121657 was used for the transformation experiments. Four plugs of expanding mycelium growing in V8 juice agar plates amended with PARP (100 ppm of pimaricin, 100 ppm of ampicillin, 30 ppm of rifampicin, and 100 ppm of pentachloronitrobezene) were transferred to 100-mm Petri plates containing nutrient pea agar medium (described in Appendix 6). Plates were incubated in the dark for four days at room temperature. Four plugs of expanding mycelium were then transferred to 250-ml Erlenmeyer flasks containing 50 ml of sterile nutrient pea broth (described in Appendix 6). Flasks were incubated at room temperature in darkness for three days.

**Isolation of protoplasts**

The protocol described below was kindly provided by Dr. B. Tyler at Virginia Bioinformatic Institute (VBI) and is currently used for transformation in *P. sojae*. Mycelium mats from three Erlenmeyer flasks were harvested and rinsed with 50 ml of mannitol solution (0.8 M) followed by a 10 min wash in 0.8 M mannitol on shaker (50 RPM) at 28ºC. After mannitol incubation, mycelium was transferred to a sterile 100 mm sterile Petri dish and incubated with 60 ml of a cocktail enzyme [0.4 M mannitol, 20 mM KCl, 20 mM 2-morpholinoethanesulfonic acid (MES) pH 5.7, 10 mM $CaCl_2$, 0.25% (w/v) lyzing enzyme *Trichoderma harzianum*, 0.5% (w/v) cellulase] at 28ºC on shaker (50 RPM) for 40 minutes. Observations for protoplast release were done at least twice and protoplast concentration was determined using a hemocytometer. Protoplast solution was filtered through two layers of sterile 50-µm nylon mesh (BioDesign, Carmel, NY) into 50-ml Falcon tubes to remove any mycelial fragments. Protoplasts were centrifuged for three minutes at 1500 RPM and supernatant was decanted. The protoplast pellet was gently washed with 35 ml of W5 buffer (5 mM KCl, 125 mM $CaCl_2$, 154 mM NaCl, 177 mM glucose) during one minute and immediately centrifuged for 4 minutes at 1500 RPM. The pellet was resuspended in 10 ml of W5 buffer and the concentration was adjusted to 2 x $10^6$ protoplasts/ml. Protoplast solution was incubated on ice for 30 min, centrifuged for 4 minutes at 1500 RPM and the supernatant was discarded. Protoplast pellet was resuspended with 10 ml of MMg solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES pH 5.7) and incubated for 10 min at room temperature before PEG (polyethylene glycol)-mediated transformation.

**PEG-mediated transformation of protoplast and regeneration**

A total of 30 µg of plasmid DNA from construct pNC-GFP (described below) or a mix of plasmid pHA and pUBlN (described below) were used in a 3:1 proportion for PEG-mediated transformation.

Plasmid DNA was placed in 50-ml Falcon tube and mixed gently with ~ 1 ml of protoplast solution and incubated in ice for 10 min. Three aliquots of 580 µl of fresh filter-sterilized PEG solution [40% (w/v) PEG 4000, 0.3 M mannitol, 0.15 M $CaCl_2$] were added to the protoplast/DNA mix and incubated on ice for 20 min. Two milliliters of pea/mannitol solution (described in Appendix 6) were added to the protoplast solution, the tube was gently inverted once, and incubated in ice for 2 min. Eight milliliters of pea/mannitol solution were added and the tube was incubated for 2 min. Protoplast solution was poured in 100-mm Petri dishes containing 10 ml of pea/mannitol solution amended with ampicillin (100 µg/ml) and incubated at 18ºC for 15 h in order to allow protoplast regeneration.

**Selection of stable transformants**

The solution of regenerated protoplasts was spun for 5 min at 2000 RPM and then decanted carefully without disturbing the pellet. The pellet was gently dissolved by flicking the tube on the sides and kept on ice. Fifteen milliliters of warm (40ºC) amended (30 µg/ml geneticin) pea/mannitol agar solution (described in Apendix 6) were added to regenerated cells and mixed gently. The solution was immediately poured into 100 mm Petri dishes and incubated at 25ºC for one day or until mycelial growth appeared on the agar surface. An overlayer with 5 ml of amended (50 µg/ml geneticin) pea/mannitol

solution was poured after 24 to 48 h. Plates were kept at 25ºC until mycelial colonies appeared on the agar surface. Hypahal tips were taken from colonies with fast growth and transferred into 60-mm Petri dishes with amended (50 µg/ml geneticin) pea agar medium, then incubated at 25ºC. Colonies that survived 7 days in amended pea agar medium were considered to be primary transformants. After 7 days, colonies were transferred into amended (50 µg/ml geneticin) V8 juice agar plates, incubated at room temperature and subcultured to fresh amended geneticin (50 µg/ml)-PARP V8 juice agar plates every 3 weeks. Colonies were also transferred into V8 juice agar plates without the selective antibiotic. For long term storage, three agar plugs of expanding mycelium of each primary transformant were placed into 96-well plates containing three hemp seeds and 1 ml of sterile distilled water. Plates were sealed with a sterile cap mat and stored at room temperature.

**DNA isolation and PCR confirmation of stable transformants**

Isolates were grown and treated as previously described for DNA isolation (14). Isolates transformed with plasmid pNC-GFP were tested with *GFP* primers, Forward: 5'-ctacggaaagctcaccctga-3' and Reverse: 5'-catgtggtccctcttctcgt-3' (expected PCR product size= 541 bp). An additional pair of primers was tested to amplify a region between the promoter and the terminator region of the GFP gene. The sequences of the primers were Forward: 5'-aagcctcgcccgactcgcccacg-3' and Reverse: 5'-aaatctgcaacttcgcactca-3' with a 948 bp PCR product size. Isolates transformed with pHA/pUBIN vectors were tested with primers flanking the region between the *Ham34* promoter and *Avr1b* gene (pHA construct) or with primers amplifying the antibiotic resistance gene (pUBIN construct).

Primer sequences for the *Ham34-Avr1b* region were Forward: 5'-aaagctgtcactgcgcttgttcag -3' and Reverse: 5'-acgcaactgagtactccgacgaaa-3' with an expected PCR product size equal to 622 bp. PCR parameters were: 95°C for 5 min, 34 cycles of denaturing at 95°C for 30 s, annealing at 56°C for 30 s, synthesis at 72°C for 30 s with a final extension at 72°C for 5 min. Positive (plasmid DNA) and negative controls (wild type DNA and non-template DNA) were included in each PCR amplification. Amplification was repeated twice using DNA from two technical replicates.

**Plasmid source and isolation**

Plasmids pHA and pUBIN were kindly provided by Dr. Brett Tyler (VBI) and construct pNC-GFP was provided by Nicolas Champouret (The Netherlands, NCBI accession EU257522). Construct pHA was designed to express the *Avr1b* gene in a antisense orientation (23) driven by the transcriptional regulatory promoter sequence *Ham34* from *Bremia lactucae* (*ham34::Avr1b::ham34*) (9). Construct pUBIN contains the gene for resistance to geneticin (G418). Construct pNC-GFP harbors the green fluorescent protein flanked at the 3' region by the *Ham34* promoter and at the 5' region the terminator *Ham34* (*han34::GFP::ham34*). pNC-GFP also contains the gene for conferring resistance to geneticin. Large quantities of each plasmid DNA (0.5 mg) were obtained with the Qiagen plasmid Maxi kit (Qiagen, CA) following the manufacturer's instructions and quantified with a spectrophotometer.

**RESULTS**

Isolate CBS121657 grew well in nutrient pea agar medium and mats of mycelium were readily generated by day three. Protoplast release was achieved as soon as 30 min after incubation with the enzyme cocktail. The protoplast concentration was 1.4 x $10^7$ and adjusted according to the protocol from the Virginia Bioinformatic Institute for *P. sojae*. Four 50-ml Falcon tubes containing enough protoplast solution (a total of 1 x $10^8$ protoplasts per 250- ml flask) were further treated. Each falcon tube represented one single 100-mm Petri dish and up to 20 *Phytophthora* colonies were observed in each Petri dish before the first overlay of selective medium. After the first overlay, only 40% of the colonies were able to grow on the surface and survive for more than three days. Once transferred into amended pea agar medium, 90% of the remaining colonies survived for more than one week. Only colonies that survived through this step were subjected to DNA isolation and PCR confirmation.

PCR using genomic DNA of transformants showed that all transformants generated with constructs pNC-GFP (13 isolates) and/or pHA/pUBIN (16 isolates) were positive for the antibiotic resistant gene (Fig. 6.2). However, only 14 isolates of the latter group (pHA/UBIN) were confidently identified as PCR positive with primers amplifying the *Ham34* to *Avr1b* region (Fig. 6.3). Three isolates resulted in a smear PCR product with a size similar to the expected size for primer *Ham34-Avr1b* region (Fig. 6.3). All isolates transformed with pNC-GFP were also PCR positive for the GFP flanking primers (data not shown).

Isolates were subcultured five times (3 months) on amended (50 µg/ml G418) V8 juice agar plates. Isolates were also growing in V8 agar plates without the selective agent

and kept for one month, then transferred into selective medium (50 µg/ml G418). Approximately 53% of the isolates continued growing in the selective medium.

## DISCUSSION

Genetic transformation in oomycetes was first described for *Phytophthora infestans* in 1991, representing a significant breakthrough in oomycete research (9, 10). Several other species, such as *P. sojae*, *P. palmivora*, *P. parasitica*, *Pythium aphanidermatum*, and *Saprolegnia monoica*, have been transformed using the PEG-mediated protocol (2, 6, 10, 11, 20, 28). However the PEG method has sometimes resulted in protoplasts with a low regeneration rate (7-10%) and requires the preparation of a large amount (30-40 150 mm Petri dishes for sporangia production) of starting material. In our experience, only a small amount of starting material (one mat of mycelium) was required for producing enough protoplasts ($1.4 \times 10^7$ protoplast/ml).

The coenocytic mycelium of oomycetes harbors multiple nuclei, and regenerated transformants may contain transformed and untransformed nuclei (heterokaryotic transformants). This could explain why gene silencing triggered by overexpression of the transgene is sometimes not fully accomplished in the organism (25). Our results are consistent with previous observations from other transgenic *Phytophthora* species. In *P. parasitica*, repeated subculture under nonselective medium resulted in the loss of transformed nuclei, stopping their growth in selective medium (7). Moreover, intensive manipulation of *Phytophthora* during protoplast generation is thought to provoke changes in the normal morphology and function of the isolate. Previous investigations have described the inability of some transgenic oomycete isolates to generate sporangia (28).

In our experience, 30% of the recovered antibiotic resistant isolates had a slightly different growth rate and morphology compared to the wild type (data not shown).

The initial PEG protocols for oomycete transformation needed modifications because the enzyme used to generate protoplasts was no longer commercially available. The current protocol used reagents that are commercially available (lyzing enzyme from *Trichoderma harzianum* and cellulase) (18). The lyzing enzyme from *T. harzianum* is described as a combination of β-glucanase, cellulase, and protease enzymes (22). Other laboratories have described the use of lipofectin instead of PEG (29) as it has proven to increase transformation efficiency by 35-fold in *Schizosaccharomyces pombe* (1). More recently, McLeod et al. adapted a protoplast protocol from *Arabidopsis* to generate stable transformants in four oomycetes (18). The protocol includes an enzyme cocktail of β-D-glucanase and cellulase (aided with 10 mM $CaCl_2$) and the species with the highest rate of regeneration was *Pythium aphanidermatum* (18).

The development of new transformation vectors will contribute to functional studies in oomycetes because traditional vectors used in other fungi have not produced satisfactory results in the past (9, 10, 12). McLeod tested 17 novel vectors for expression using the PEG-mediated protocol and identified pDBHAMT35G as the only promising vector with GUS expression in *P. infestans* and *P. citricola* (18).

The search for novel gene disruption procedures is critical for oomycete research. Two recombination pathways have been identified in eukaryotes: homologous recombination (HR) that requires interaction between homologous sequences; the second pathway, nonhomologous end-joining (NHEJ) that involves direct ligation of the strand ends independent of DNA homology (19). In *Neurospora crassa*, disruption of genes

homologous to *Ku70* and *Ku80* from humans, heterodimer key proteins involved during NHEJ of double-stranded DNA breaks, has demonstrated a high rate of homologous integration of exogenous DNA (21). More recently this strategy to enhance gene disruption has been successfully applied to other organisms such as *Magnaphorthe grisea* and *Aspergillus fumigatus* (4, 27) and it could probably be adapted for *Phytophthora*.

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**APPENDIX 6**

**Solutions**

**Pea/0.5M mannitol (Broth or agar for overlays)**

Pea Broth (see pea media protocol)
91.1 g Mannitol
1 g $CaCl_2$
2 g $CaCO_3$
Make up to 1 L and autoclave in 250-ml bottles
For overlays add 10 g agar

**Pea Agar Medium**

Autoclave 120 g frozen peas in 1 L of distilled water. Filter through four layers of cheesecloth. Squeeze the cheesecloth gently to remove residual broth.
Bring volume of broth up to 1 L add 2 g $CaCO_3$, 15 g agar and autoclave.

**Nutrient Pea Broth and Agar Medium**

| **Chemical** | **Amount** |
|---|---|
| $K_2P04$ | 1.0 g |
| $KH_2P04$ | 1.0 g |
| $KN0_3$ | 3.0 g |
| $MgS0_4$ | 0.5 g |
| $CaCl_2$ | 0.1 g |
| $CaCO_3$ | 2.0 g |
| D-sorbitol | 5.0 g |
| D-mannitol | 5.0 g |
| Glucose | 5.0 g |
| vitamin stock | 2.0 ml |
| trace elements | 2.0 ml |
| yeast extract | 2.0 g |

| **Vitamin stock** | | **Trace elements** | |
|---|---|---|---|
| Biotin | 0.0002 g | $FeC_6H_5O_73H_2O$ | 0.215 g |
| Folic acid | 0.0002 g | $ZnSO_4$ $7H_2O$ | 0.150 g |
| l-inositol | 0.0120 g | $CuSO_4$ $5H_2O$ | 0.030 g |
| Nicotinic acid | 0.0600 g | $MnSO_4$ $H_20$ | 0.015 g |
| Pyridoxine-HCl | 0.1800 g | $H_3BO_3$ | 0.010 g |
| Riboflavin | 0.0150 g | $MoO_3$ | 0.007 g |
| Thiamine-HCl | 0.3800 g | | |
| Coconut milk | 50 ml | | |
| H20 to make | 300 ml | $H_20$ to make 400 ml | |

Autoclave 120 g frozen peas in 1 L distilled water. Filter through four layers of cheesecloth. Squeeze the cheesecloth gently to remove residual broth.
Amend with the chemicals above and bring the volume up to 1 L with distilled water.
For agar medium, add 15 g/L Difco Bacto Agar.

**Figures**

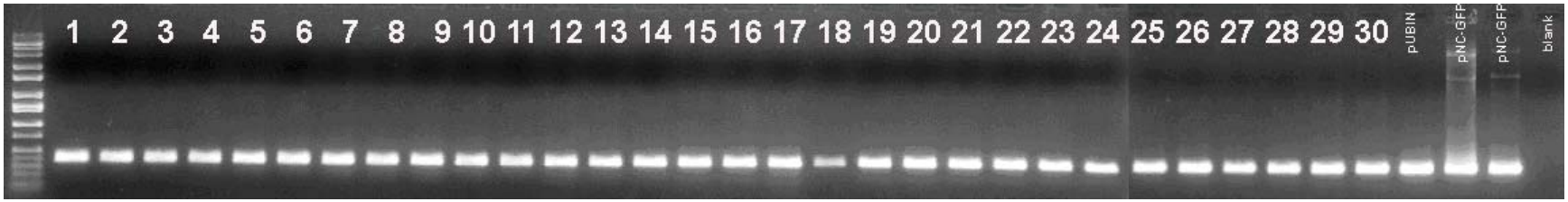


Figure 6.1. PCR screening of CBS121657 isolates (1 through 30) resistant to G418 (50 µg/ml) using primers flanking the antibiotic resistant gene (PCR size~500 bp). Positive controls were included in the PCR reaction (plasmid dilution for pNC-GFP and pUBIN).

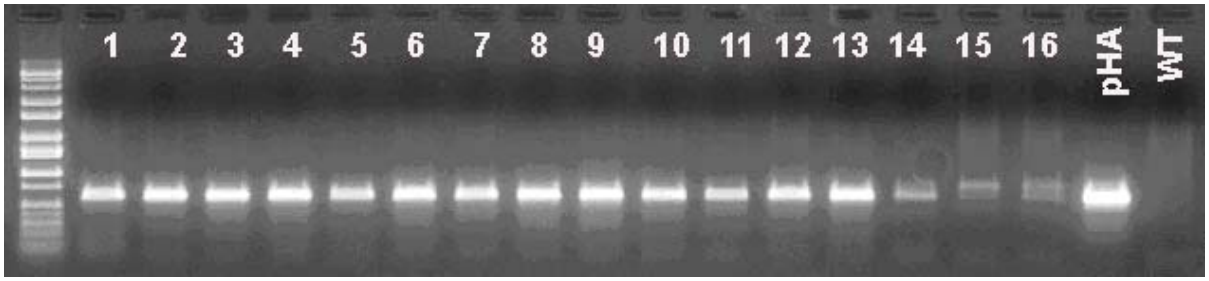


Figure 6.2. PCR screening of regenerated isolates growing in G418 (50 µg/ml). Isolates were transformed with a mix of plasmid pHA and pUBIN. PCR correspond to primers flanking the Ham34-Avr1b region (PCR size ~622 bp).

## Vita

Mr. Oscar Pietro Hurtado-Gonzales was born in Lima, Peru in 1975. He received a B.Sc. in Biology from the Universidad Nacional Agraria La Molina in 1998. He was awarded a fellowship at the International Potato Center (CIP) to conduct his undergraduate thesis research for a professional title. He earned his professional title in biology in 2000, and he continued working in the Molecular Biology laboratory of the CIP as a research assistant. In May 2002, he was awarded a graduate assistantship to pursue a M.Sc. degree at Virginia Polytechnic Institute and State University in the Plant Pathology, Physiology and Weed Science Department. Mr. Hurtado completed his M.Sc. in August 2004. In September 2004, he started his Ph.D. studies at The University of Tennessee in the Entomology and Plant Pathology Department. He finished his studies in May 2008.