

**Measuring Single Nucleotide Polymorphism in the
Vegetable Pathogen *Phytophthora capsici***

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Abstract

Phytophthora capsici is a eukaryotic plant pathogen that causes root, fruit, and foliar disease on a variety of important vegetables including pepper, tomato, eggplant, snap and lima beans, and essentially all cucurbits. At some locations, populations utilize sexual and asexual reproduction, whereas at others, populations appear to be entirely asexual. The differing population structure has important implications for developing control strategies. Our objective was to develop SNP markers to characterize natural populations and laboratory crosses. In chapter two, a novel technique to assay SNP genotypes based on high-resolution DNA melting analysis is presented. In chapter three, the genetic diversity for *P. capsici* isolates collected at diverse locations in Argentina is summarized. Clonal reproduction dominated over a wide geographical area and multiple years. The genetic diversity for *P. capsici* isolates collected on Long Island, New York, USA is summarized in chapter four, suggesting that both sexual and clonal reproduction play important roles in the epidemiology. And lastly, the genetic diversity of *P. capsici* isolates collected from peppers grown in France is discussed in chapter five. The results from France are similar to the USA and it appears that both sexual and asexual reproduction play an important role in the epidemiology.

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Chapter One

Introduction

The genus *Phytophthora* is comprised mainly of plants pathogens (Erwin and Ribeiro 1996; Judelson and Blanco 2005). Many cause significant economic losses (Rizzo et al. 2005; Tyler 2007; Sekimoto et al. 2008; Wolinska et al. 2008). *Phytophthora infestans* is the best known species because of the destruction it caused to the potato crop in Ireland during the mid-19th century. Consecutive years of heavy losses triggered mass emigration and it has since been dubbed the Irish potato famine (Kamoun et al. 2009). Recently, *Phytophthora ramorum* which causes Sudden Oak Death has emerged as a serious threat to forest ecosystems in the United States (Rizzo et al. 2005). Worldwide loss due to *Phytophthora sojae*, which causes seedling ‘damping off’ and root rot of older soybean plants, are about 1-2 billion USD per annum (Tyler 2007). *Phytophthora capsici* is another important soilborne plant pathogen that causes crown, fruit and root rot on diverse vegetables including peppers, pumpkin, squash, and tomato (Hausbeck and Lamour 2004; Gevens et al. 2007; Hurtado-Gonzales et al. 2008; Dunn et al. 2010). More recently, the host range of *P. capsici* has expanded to legume crops such as snap beans and lima beans (Gobena et al. 2011; Lamour et al. 2011).

Phytophthora belongs to the phylum Oomycota and the kingdom Stramenopila (Forster et al. 1990; Sogin and Silberman 1998; Petersen and Rosendahl 2000). Evolutionarily it is more closely related to algae and plants than to fungi (Figure 1.1) (Baldauf and Palmer 1993; Tyler et al. 2006).

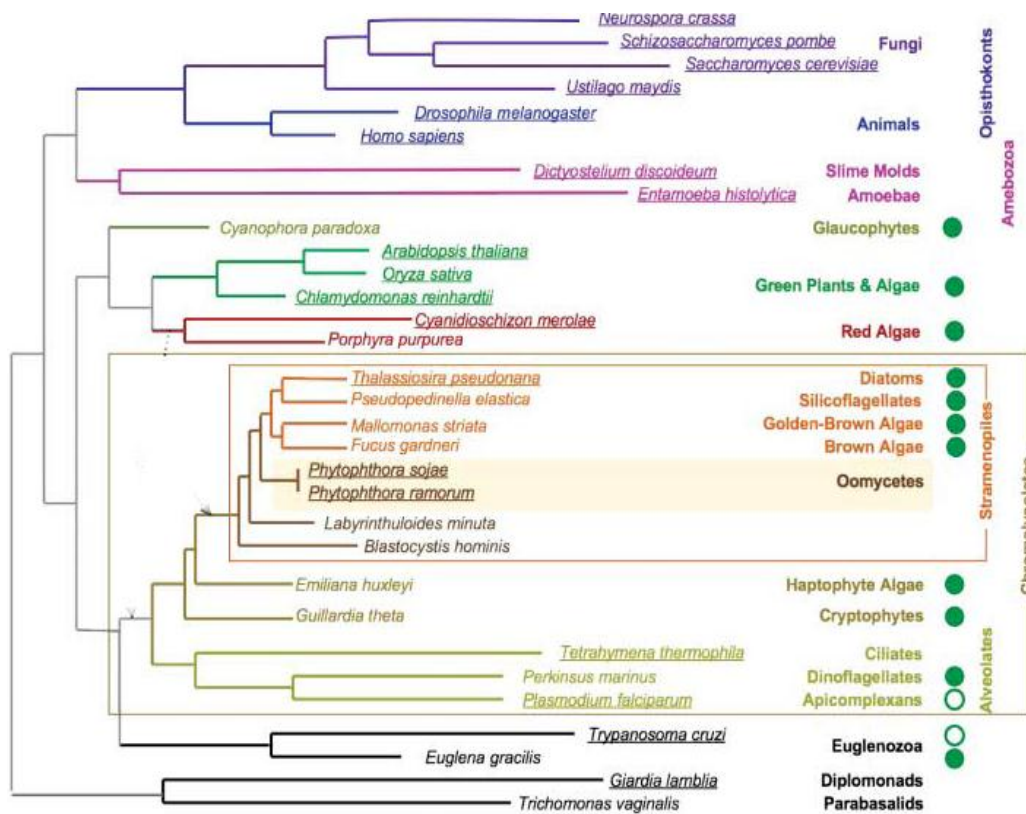


Figure 1.1 Phylogenetic tree based on six highly conserved proteins. Filled green circles indicate photosynthetic species while open green circles indicate species with vestigial photosynthetic origin (Found in Tyler et al. 2006).

Unlike filamentous fungi, which are dominated by a haploid life cycle, the majority of the *Phytophthora* life cycle is diploid. In addition, *Phytophthora* hyphae contain relatively few septa while fungal hyphae are regularly septate. The cell walls are primarily composed of 1,3- β - and 1,6- β - glucans, and contain little chitin, an important component of fungal cell walls (Adams 2004). Oomycetes use α - ϵ diaminopimelic acid pathway to synthesize lysine whereas fungi use the α -amino adipic acid pathway (Latijnhouwers et al. 2003). Finally, most chemicals useful to control fungi are not effective against oomycete pathogens.

Despite the above differences, *Phytophthora* cause infection similar to fungi. Infection starts when spores or hyphae come in contact with susceptible host tissue as outlined in Figure 1.2. Spores that can germinate and cause infection include the thick-walled sexual oospore. The asexual deciduous sporangia can release 20-40 swimming zoospores when there is free water. Once a swimming zoospore comes into contact with a host plant they lose their flagella and form walled cysts (Hardham and Hyde 1997). *Phytophthora* cysts germinate immediately without the requirement of any particular stimuli (Judelson and Blanco 2005). The germ tube emerges and often develops small appressoria that are not melanized or pigmented like other true fungi (Enkerli et al. 1997; Kramer et al. 1997; O'Connell et al. 2001). The combination of cell wall degrading enzymes and mechanical pressure allow the pathogen to breach the epidermal cell wall (Bircher and Hohl 1997). In addition, infection may occur through natural openings and wounds.

At the growing edge of a lesion, infection by *Phytophthora* is often biotrophic and the host tissue is not killed (Figure 1.2). During biotrophy *Phytophthora* obtains nutrients from the infected host through a specialized feeding structure known as a haustoria (Hohl and Suter 1976). However, as the infection progresses, the older part of the lesion switches to a necrotrophic growth phase and the host tissue is killed (Latijnhouwers et al. 2003). This is known as a hemibiotrophic life style and is common for oomycetes in the genera *Phytophthora* and *Pythium* and other fungal genera including *Colletotrichum* and *Venturia* (Deising et al. 2008; Templeton et al. 2008).

In recent years draft genome sequences have been developed for multiple *Phytophthora* species, including *P. capsici*. These resources have been crucial to our understanding of the evolutionary

history of *Phytophthora* and have also prompted the discovery of gene families important to pathogenesis and virulence. One of the most interesting gene families revealed thus far is a large group of secreted proteins known as effectors (Tyler et al. 2006; Kamoun 2007; Lamour and Kamoun 2009). Many effectors share a conserved motif RxLR-dEER at the N-terminal end and variable C-terminal end (Morgan and Kamoun 2007; Birch et al. 2008). The recognition of the RxLR-dEER motif as a signature has led to the rapid identification of candidate avirulence proteins (proteins that helps the infection process) from many sequenced genomes. The RxLR-dEER motif is thought to be involved in effector protein entry to the host cells (Birch et al. 2006). The mechanism is similar to the malaria parasite, *Plasmodium* which uses a similar targeting sequence RxLRE/Q to traverse membranes including the parasite membrane, parasitophorous vacuole membrane, and erythrocyte membrane (Hiller et al. 2004; Marti et al. 2004).

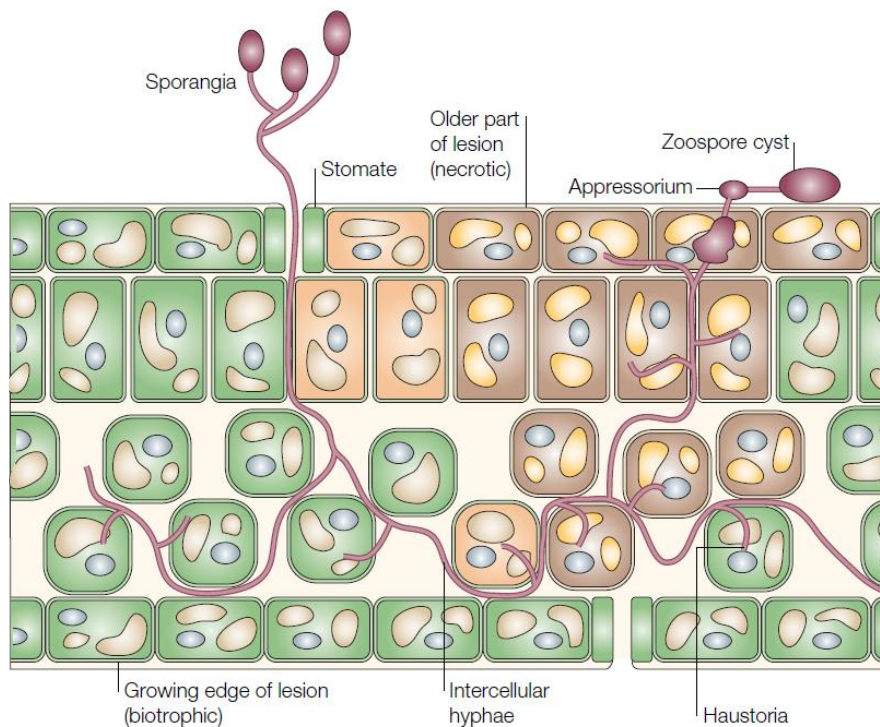


Figure 1.2 Typical course of infection as shown by *Phytophthora infestans* on potato (Found in Judelson and Blanco 2005).

Both homothallic and heterothallic sexual reproduction systems exist within the genus *Phytophthora* and other oomycetes. Heterothallic species like *Phytophthora capsici* require two compatibility types (also known as mating types) designated as A1 and A2 to complete the sexual life cycle. However, homothallic species such as *Phytophthora sojae* produce sexual oospores from a single isolate. Meiosis takes place in the differentiated male organ known as the antheridium and the differentiated female organ known as the oogonium. Mating type hormones trigger the production of the male and female organelles. The gametangia are fused and the haploid nuclei migrate from the antheridia to the oogonia to produce thick-walled sexual oospores. Oospores can survive outside the host for extended periods and for some species are important for over-wintering (Hausbeck and Lamour 2004). Both asexual and sexual reproduction plays a significant role in the disease epidemiology of many *Phytophthora* species.

The work presented here is focused on the vegetable pathogen *P. capsici*. Populations of *P. capsici* are dynamic and characterized by rapid adaptation (Leonian 1922; Erwin and Ribeiro 1996; Lamour and Hausbeck 2001; Hausbeck and Lamour 2004; Gevens et al. 2008; Hurtado-Gonzales et al. 2008; Lamour and Kamoun 2009; Meitz et al. 2010; Gobena et al. 2011; Lamour et al. 2011). Both the A1 and A2 mating type have been recovered from field populations worldwide and the pathogen often features annual meiosis between the two mating types, which produce thick-walled oospores that remain dormant for years (Erwin and Ribeiro 1996; Gobena et al. 2011; Gobena et al. 2012). In the USA, this results in highly dynamic field populations and disease management is challenging (Lamour and Hausbeck 2003). Although populations contain significant variation, the diversity observed at the beginning of the growing season may result in only a few clonal lineages at the end of the growing season (Lamour and Hausbeck 2001).

Phytophthora capsici populations outside the USA, however, may be quite different. As outlined previously and in this document, populations in Argentina and Peru are dominated by single clonal lineages that are widely dispersed and persist over many years (Hurtado-Gonzales et al. 2008; Gobena et al. 2012).

Understanding the epidemiology of *P. capsici* is important to develop appropriate management strategies. An example is plant breeding. There are many current efforts to develop pepper varieties resistant to *P. capsici* and knowing the composition of populations is crucial for adequate testing of potential resistant lines. Since there are few phenotypic characters, molecular markers such as amplified fragment length polymorphism (AFLPs) have been used previously to assess genetic variation (Lamour and Hausbeck 2001; Lamour and Kamoun 2009). Although powerful, AFLPs are a dominant marker system in which heterozygous and homozygous genotypes cannot be directly determined. The recent development of a reference genome for *P. capsici* opened the door to develop co-dominant single nucleotide polymorphism (SNP) markers. SNPs are attractive for a number of reasons. They are simple to analyze and allow accurate determination of genotypes and allele frequencies, which is vital for investigating field populations. SNPs are abundant in many genomes including *P. capsici*.

Analyses based on re-sequencing single copy genes indicate that *P. capsici* may be one of the most SNP-rich eukaryotic organisms reported to date. This high level of polymorphism makes the process of identifying informative markers challenging and ultimately, quite expensive. The second chapter of this dissertation presents a strategy for SNP genotyping in *Phytophthora* using high-resolution DNA melting analysis (HR-DMA). HR-DMA, as outlined here, provides a

robust and relatively low cost way to assess SNP genotypes and this technique is utilized in chapters 3-5 allow the assessment of genetic diversity for populations of *P. capsici* in Argentina, US, and France. The current situation, implications based on our findings, and future directions are addressed in each chapter.

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Chapter Two

A strategy for SNP genotyping in *Phytophthora* using high-resolution DNA melting analysis.

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Will be submitted to BioTechniques soon

My primary contributions to it were all of the writing, the DNA sequencing, data analysis, much of the DNA melting analysis and experimental design.

Abstract

Single nucleotide polymorphism (SNP) markers are abundant in many *Phytophthora* genomes.

Although valuable for genetic analysis, SNPs present challenges for routine analysis. Here we present a technique that utilizes high-resolution DNA melting analysis (HR-DMA) to assess SNP genotypes. The approach includes standard PCR in the presence of an intercalating fluorescent dye followed by heteroduplex formation and a melting analysis to determine hetero-homozygous fragments. The homozygous fragments are further analyzed by mixing with a known homozygous amplicon and re-melting to determine the correct homozygous genotype. Data is presented for *P. capsici* field isolates and sexual progeny from the vegetable pathogen *P. capsici*. Protocols and the advantages and limitations of this approach are discussed.

Introduction

Single Nucleotide Polymorphisms (SNPs) are the most common type of genetic variation in eukaryotic organisms and are increasingly used to investigate biological questions; from population genetic and evolutionary studies, to molecular assisted breeding and association mapping (Berger et al. 2001; Wicks et al. 2001; Morlais et al. 2004). *Phytophthora* is an important eukaryotic fungal-like plant pathogen that is diploid throughout its life cycle. Species are either self-fertile (homothallic) or require pairing of the A1 and A2 mating types (heterothallic) to produce thick-walled sexual oospores. Oospores play an important role in survival outside of host tissue and in generating novel genotypic variation. Not surprisingly, the inbreeding of homothallism and the outbreeding of heterothallism profoundly impact genetic variation within and between individual isolates.

Over the last decade we have analyzed more than 10,000 *Phytophthora* isolates (crosses and field populations) using anonymous Amplified Fragment Length Polymorphism (AFLP) markers. Although these markers are powerful, they have significant shortcomings (Liu and Cordes 2004). Unambiguous co-dominant markers, such as single nucleotide polymorphisms (SNPs), are highly desirable. As genome resources are increasingly available for *Phytophthora*, our research has focused on SNPs for measuring genetic diversity in natural populations and for analyzing sexual progeny in laboratory crosses.

Unfortunately, many SNP genotyping platforms require substantial investment and are not always easily adapted to smaller projects or non-model organisms (Lai et al. 2007; Van Orsouw

et al. 2007; Van Tassell et al. 2008). High-resolution DNA melting analysis (HR-DMA) is one of various options that can be used to detect PCR product sequence variation and has been applied for mutation scanning and genotyping (Dobrowolski et al. 2009; Nguyen-Dumont et al. 2009). It relies on the differing thermostability of homologous PCR amplicons containing single (or multiple) base substitutions. For HR-DMA, discrimination is highest with small amplicons, becoming less sensitive as the length of the amplicon increases. Here we overview a technique for assaying bi-allelic SNP genotypes in *Phytophthora* using HR-DMA and a LightScanner device. We present data for SNPs in an in vitro cross as well as isolates recovered across a wide area of Mexico. The major limitations and advantages are discussed.

Materials and Methods

Gene selection strategy

The SNPs analyzed here are from full (or nearly full) length complementary DNA (cDNA) sequences generated as part of the recent genome sequencing project for *P. capsici*. This project is ongoing. For the genome project, more than 55,000 Expressed Sequence Tags (EST's) were generated from a mixed sample of mRNA derived from key life stages including spores (sporangia and zoospores), growth in rich media (V8 broth), and starvation conditions (water). In addition to the regular single pass EST sequences, 1100 large insert cDNA sequences were selected for high resolution sequencing to produce full length or near full length DNA's. These large insert cDNAs were positioned on a preliminary *P. capsici* assembly using GMAP alignments and the gene sequences are publically available in GenBank (Wu and Watanabe 2005).

Isolates and DNA extraction

Four field isolates were selected for re-sequencing. Isolate LT51 (CBS 121656) was recovered from an infected cucumber fruit in northeast Michigan in 1997, LT263 (CBS 121657) was recovered from a pumpkin plant in east Tennessee in 2004, LT41 was recovered from a yellow squash fruit in central Pennsylvania in 2000, and LT2135 was recovered from a bell pepper plant in the state of Libertad in Peru in 2005 and is representative of the main clonal lineage dominating the population structure of *P. capsici* in Peru (Hurtado-Gonzales et al. 2008). A collection of *P. capsici* isolates from diverse locations in Mexico, New Mexico, USA and progeny from a cross between LT263 and LT51 parent's genotype data is presented. Oospore progenies were generated by transferring mycelial mats between the parents and blended in 20 ml MQ water for about two minutes. The blended suspension was filtered through sterilized Kimwipes. Lysing enzyme was added to the filtered solution and incubated overnight on a shaker. Germinating oospores were manually selected with a pipettor under a microscope and transferred to water agar or dilute V8 agar. Hyphal tips from the growing edge of the plate were used for sub-culturing to generate oospore progeny. Isolates were grown in PARP-V8 broth and genomic DNA was extracted as previously described (Lamour and Finley 2006). Any type of DNA extraction method can be used for HR-DMA although it should be noted that the type of buffer used for extraction may impact the DNA melting temperature (Montgomery et al. 2007).

Sequencing, SNP identification, and primer design

Twenty eight putative single copy genes were amplified with PCR from the panel of four field isolates and the electropherograms aligned and manually inspected for polymorphic sites using the CodonCode Aligner program (CodonCode). Polymorphic sites that contain at least 25 bp on

either side free from additional nucleotide variants' are suitable for marker development as outlined in Figure 2.1. HR-DMA primers were designed to produce a 45-55 bp amplicon that spanned a single polymorphic site using the LightScanner primer design software 1.0 at default settings (Idaho Technology, Salt Lake City, UT).

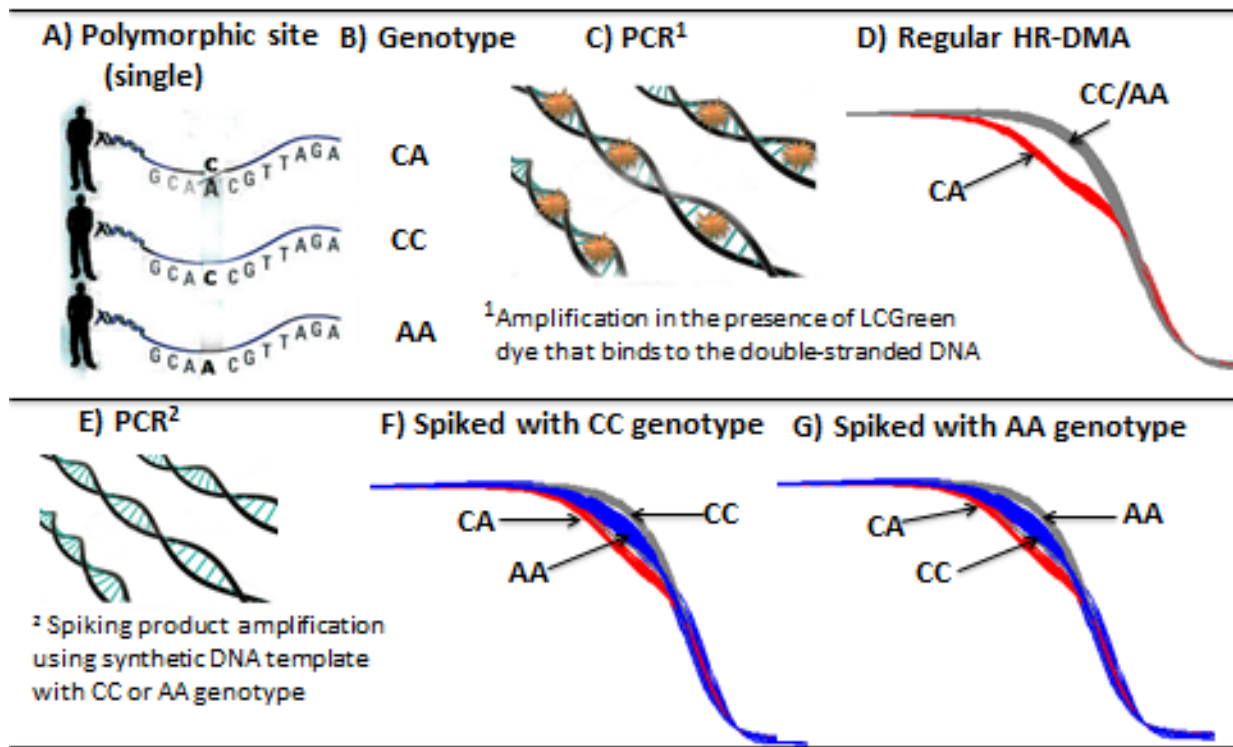


Figure 2.1 Overview of bi-allelic SNP genotyping in *Phytophthora* using HR-DMA.

A, Sequence alignment and identification of appropriate polymorphic sites. B, Genotypes of the targeted SNP locus. C, PCR amplification in the presence of the intercalating DNA binding dye LCGreen Plus. D, an initial melting to resolve heterozygous (CA) and homozygous (CC & AA) melt curves. E, PCR amplification of a known homozygote to add to each of the previously melted reactions. F, HR-DMA after spiking with CC genotype pushing the AA genotype towards the CA genotype. G, HR-DMA after spiking with AA genotype pushing the CC genotype towards the CA genotype.

PCR amplification and HR-DMA

PCR was accomplished in a 384-well plate in 5 µl reaction volumes with a 15 µl mineral oil overlay. Individual PCR reactions contained 3 µl of DNA template (10-20 ng), 0.5 µl 10x LCGreen plus dye (Idaho Technology), 0.5 µl 10x buffer, 0.2 µl 5mM dNTPs, 0.05 µl 50mM MgCl₂, 0.1 µl Taq Polymerase, 0.025 µl 100 µM forward and reverse primers and 0.6 µl water to make 5 µl final volume. The LCGreen Plus fluorescent dye binds double stranded DNA. The PCR conditions were as follows: 2 minutes at 95 °C, 30 seconds at 94 °C, and 35 cycles of primer annealing and extension at 64 °C for 30 seconds. The samples were then heated to 94 °C for 30 seconds and cooled to 25 °C to form hetero- and homo- duplexes. PCR reaction for each isolate was done in two replicates.

High-Resolution DNA melting analysis (HR-DMA) was accomplished using a 384-well LightScanner device (Idaho Technology, Salt Lake City, UT) according to the manufacturer's instructions. This device measures the loss of fluorescence signal as the double-stranded PCR products are heated from 62-98 °C. In general, the sequence length and GC content determine the melting temperature for each amplicon (Lipsky et al. 2001). The loss of fluorescence is visualized as a melt curve and the melt curves are analyzed using the LightScanner 2.0 software. Often it is quite simple to discriminate amplicons containing homozygous vs. heterozygous genotypes, but, it can be difficult to clearly differentiate wild-type vs. the alternate allele homozygous genotypes.

Addition of a known homozygous fragment to differentiate unknown homozygotes

To distinguish homozygous wild-type and the homozygous alternate allele genotypes, a known homozygous amplicon is mixed into each of the reactions and the samples are re-melted. The PCR product used for mixing is amplified separately without LCGreen plus dye. The template for this reaction can be either genomic DNA from an isolate with a known homozygous genotype, or a synthetic amplicon. The PCR conditions to produce the amplicon for mixing are the same as the HR-DMA amplification except the primer annealing is changed to 60 °C. The two amplicons were mixed in a one-to-one ratio and heated at 95 °C for 30 seconds and cooled to 50 °C for 30 seconds for three cycles and analyzed using the LightScanner. The melt curves will shift if the known homozygous amplicon has a different genotype from the amplicons being analyzed.

Results

SNP genotyping and segregation pattern analysis

Genotype data is presented from a progeny set developed by crossing two parents, LT263 and LT51. SNP markers for 28 loci were developed and applied and both progeny set and parental isolates were genotyped with HR-DMA. The segregation pattern was determined for each loci included in our survey (Table 2.1). All of the genes and genomic regions were inherited in our crosses as expected for simple Mendelian characters with a Chi-square p-value of 0.05 – suggesting these are single copy genes or unique loci in the genome (Table 2.1).

Field isolates SNP genotyping

Sequence data from 33 isolates recovered from pepper, *Capsicum* sp., *Cestrum* sp., *Myrthus* sp. and tomato host in Mexico and two isolates from New Mexico, USA has been generated to

explore the polymorphic sites of the target locus in the natural populations (Table 2.2). The locus was found on the second scaffold from position 854251 to 85455 on the *P. capsici* genome assembly. A total of 29 SNPs were detected from a 300-bp PCR amplicon that was re-sequenced. The position is defined as polymorphic if there is a nucleotide difference in a single isolate or between two or more isolates. A single polymorphic locus that was spanned by 25-bp homozygous stretches was selected for assaying using HR-DMA. The targeted locus is found on the second scaffold at position 854455 of the *P. capsici* genome assembly. From 35 isolates that have been genotyped; 18 were TC genotype, 11 were CC genotype and 6 were TT genotype, which complements the data from direct sequencing of the fragment.

Discussion

Development of a rapid and cost effective SNP assay for understanding the population structure of *P. capsici* has paramount importance to our laboratory. Our goals for *P. capsici* SNP markers are to utilize them for investigating populations' genetic diversity and also tracking them in successful crosses for linkage analysis. As we have demonstrated, HR-DMA can be utilized to identify segregation pattern and genetic diversity analysis of natural population of *Phytophthora* species. HR-DMA has been chosen for SNP assays due to its attractive features like, simplicity, low cost and ease of use in genotyping (Vossen et al. 2009). HR-DMA is a closed-tube assay with simple PCR performed under slightly modified conditions in the presence of a DNA binding dye. SNP genotyping using HR-DMA has been applied as a reliable and effective method for analyzing large populations.

In HR-DMA, melting curves are generated during melting of the PCR sample using a LightScanner instrument. As temperatures increase, the fluorescence signal decreases generating a characteristics melting profile for each sample included in the analysis. Sensitivity of the detection is affected by PCR product size and other intrinsic factors. The sensitivity of detection is better for shorter fragments, and sequence variant G to A is predicted to have the largest melt shift (Reed and Wittwer 2004). PCR product size determination primarily depends on the possible number of polymorphic sites in the target sequence. For assaying SNPs in *P. capsici* field populations, 45-55 bp PCR fragments have been targeted to minimize the chance of having extra polymorphic sites that makes normalization and data acquisition challenging. Normalization and data analysis is still possible with targets with multiple polymorphic sites, however, it considerably increases the number of samples flagged for sequencing.

The polymorphic nature of the target amplicon is not the only factor affecting quality data generation. Addition of 1 µl high salt buffer [1.0M KCL, 0.5M Tris-HCl (pH=8.0)] improved the resolution of non-uniform DNA concentration (Vossen et al. 2009). High quality DNA template is also required as efficient PCR amplification is very important to obtain melt curves with clear resolution.

Like many other techniques, HR-DMA has its limitations. Any base substitution, irrespective of its position on the amplicon, could produce duplexes that generate classical heterozygous and homozygous melt curves. Therefore, there is a possibility that melt curves could be generated from a non-target polymorphic locus, but it could be assumed that they are from the targeted

locus. Sequencing selected individual samples from the melt group would provide further confirmation that the genotype is indeed due to the locus under investigation.

Table 2.1 Segregation analysis for single nucleotide polymorphism markers within *Phytophthora capsici* genes.

Gene Model	SNP ID ¹	² Ob 1	Ob 2	Ob 3	³ Exp 1	Exp 2	Exp 3	df	χ^2 value
Scaffold_29:196912-198459	Sc29_197362	15	19		17	17		1	0.47
Scaffold_158:41466-42254	Sc158_41670	23	22		22.5	22.5		1	0.02
Scaffold_27:545590-547311	Sc27_546679	21	13		17	17		1	1.88
Scaffold_19:880709-882275	Sc19_881188	23	22		22.5	22.5		1	0.02
Scaffold_19:634212-634424	Sc19_634212	16	18		17	17		1	0.12
Scaffold_15:160557-162666	Sc15_161928	9	22	12	10.75	21.5	10.75	2	0.44
Scaffold_22:653551-657040	Sc22_655685	7	18	8	8.75	17.5	8.75	2	0.43
Scaffold_7:1254360-1255783	Sc7_1255709	27	18		22.5	22.5		1	1.80
Scaffold_39:342193-344049	Sc39_343284	13	22		17.5	17.5		1	2.31
Scaffold_2:1048829-1053328	Sc2_1051268	19	14		16.5	16.5		1	0.76
Scaffold_43:318720-320777	Sc43_319143	18	26		22	22		1	1.45
Scaffold_7:1000216-1002404	Sc7_1000456	18	29		23.5	23.5		1	2.57
Scaffold_71:156154-158367	Sc71_157951	21	25		23	23		1	0.35
Scaffold_82:33595-36807	Sc82_34429	13	22		17.5	17.5		1	2.31
Scaffold_112:62346-65858	Sc112_62856	23	22		22.5	22.5		1	0.02
Scaffold_23:62378-63583	Sc23_62968	21	27		24	24		1	0.75
Scaffold_17:428762-430721	Sc17_429757	24	21		22.5	22.5		1	0.20
Scaffold_72:70985-74119	Sc72_73177	26	19		22.5	22.5		1	1.09
Scaffold_63:256692-259740	Sc63_257740	24	21		22.5	22.5		1	0.20
Scaffold_17:22114-25606	Sc17_25167	26	19		22.5	22.5		1	1.09
NA	Sc28_418045	29	16		22.5	22.5		1	3.76
NA	Sc5_85922	24	21		22.5	22.5		1	0.20
Scaffold_7:1018621-1020309	Sc7_1019920	12	13	8	8.5	17	8.5	2	2.41
Scaffold_30:142830-144963	Sc30_144325	21	13		17	17		1	1.88
Scaffold_25:678612-681126	Sc25_680731	4	21	8	8.25	16.5	8.25	2	3.42
Scaffold_9:148852-150633	Sc9_149212	18	17		17.5	17.5		1	0.03

Gene Model	SNP ID ¹	² Ob 1	Ob 2	Ob 3	³ Exp 1	Exp 2	Exp 3	df	χ^2 value
Scaffold_121:54032-56025	Sc121_54954	21	11		16	16		1	3.13
Scaffold_4:415240-417604	Sc4_416123	24	21		22.5	22.5		1	0.2

*The critical value for 0.05 Chi-square P value is 3.84 at 1 df and 5.99 at 2 df. ²=Observed genotype, ³=Expected genotype

Table 2.2 *Phytophthora capsici* isolates recovered from various hosts in Mexico and New Mexico,USA.

Year	Specific location	Host	Total
2002, 2009	Michoacán, Mexico	Chile pepper, Tomato, <i>Capsicum</i> sp., <i>Cestrum</i> sp., <i>Myrthus</i> sp.	12
2002, 2006	Guanajuato, Mexico	Chile pepper	6
2005	Edo. México, Mexico	Tomato	1
2002, 2009	Chihuahua, Mexico	Chile pepper	3
1998, 2003, 2005	Aguascalientes, Mexico	Chile pepper	11
NA	New Mexico, USA	Chile pepper	2

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Chapter Three

Genetic diversity of *Phytophthora capsici* isolates from pepper and pumpkin in Argentina.

This chapter is published in Mycologia

Gobena, D, Roig, J, Galmarini, C, Hulvey, J and Lamour, K (2012). Genetic diversity of *Phytophthora capsici* isolates from pepper and pumpkin in Argentina. Mycologia 104(1): 102-107.

My primary contributions to it were all of the writing, DNA melting analysis, data analysis, most of the DNA sequencing, mating type analysis and mefenoxam sensitivity screening.

Abstract

Phytophthora capsici is a soilborne oomycete plant pathogen that limits pepper production worldwide. The population structure varies significantly depending on the location (e.g. Peru vs. USA) and very little is known about the diversity of *P. capsici* in Argentina. Our objective was to assess the diversity of *P. capsici* in Argentina at key pepper production areas. From 2006 to 2009, 40 isolates were recovered from pepper and one isolate from pumpkin at eleven locations. Isolates were assessed for mating type, mefenoxam sensitivity, and multi-locus single nucleotide polymorphism (SNP) genotype profiles. Ten isolates with identical SNP profiles were also genotyped using amplified fragment length polymorphism (AFLP) markers. All 41 isolates had the A1 mating type and were sensitive to mefenoxam. Genotypic analysis using eight polymorphic SNP markers indicated 87% of the isolates had the same multi-locus genotype which is fixed for heterozygosity at seven of the eight SNP sites. AFLP analyses confirmed these findings, and overall it appears that clonal reproduction drives the population structure of *P.*

capsici in Argentina. The implications for breeding resistant peppers and overall disease management are discussed.

Key words: clonal reproduction, genotyping, population genetics, SNP

Introduction

Phytophthora capsici limits pepper and cucurbit production in many areas of the world (Hausbeck and Lamour 2004; Tian and Babadoost 2004; Hurtado-Gonzales et al. 2008).

Phytophthora capsici was first described as the causal agent of chili pepper (*Capsicum annuum* L.) blight in New Mexico in 1922 (Leonian 1922). Since the species description, *P. capsici* has been reported on a wide range of cucurbit and solanaceous crops as well as snap and lima beans (Hausbeck and Lamour 2004). All parts of the plant can be infected by *P. capsici* and the spores are spread via water (Gevens et al. 2007). Unlike many other *Phytophthora* species, which thrive under cool wet conditions (e.g. *P. infestans*, *P. ramorum*, and *P. sojae*), *P. capsici* thrives under warm (25-28°C) and wet conditions.

Phytophthora capsici is heterothallic requiring A1 and A2 mating types to produce thick-walled sexual oospores and in many populations it appears that dormant oospores can provide a source of inoculum for an extended period of time (Erwin and Ribeiro 1996; French-Monar et al. 2007). *Phytophthora capsici* produces deciduous sporangia on infected plants, both above and below ground, and in the presence of free water the sporangia release biflagellate motile zoospores or germinate directly to cause infections. Epidemics can develop rapidly (within days) under suitable environmental conditions (Hausbeck and Lamour 2004).

In the United States, the A1 and A2 mating types are often recovered from within fields, and typically field populations are comprised of many isolates with unique genotypes. In addition to a high level of genotypic diversity there are often clonal lineages that are spread within single fields or contiguous cropping areas during a single year (Hausbeck and Lamour 2004; Gevens et al. 2007; Dunn et al. 2010). The overall population structure is consistent with a strong selection for thick-walled sexual oospores that serve as dormant inoculum (Lamour and Kamoun 2009). The population structure in Peru is significantly different and a single clonal lineage has been dispersed across a wide geographical area and is able to survive multiple years (Hurtado-Gonzales et al. 2008). The apparent genetic homogeneity in Peru may be useful for developing resistant vegetables because breeders can test candidate pepper varieties using a limited number of isolates and thus focus their efforts on varieties that are resistant to this clonal population.

In Argentina, *P. capsici* was reported in 1940 as the causal agent of red or sweet pepper (*Capsicum annuum* L.) seedling damping-off (Godoy 1940). In 1950, *P. capsici* was reported on the pods and stems of lima bean (*Phaseolus lunatus* L.) (Frezzi 1950). Root rot of pepper caused by *P. capsici* is one of the most serious factors limiting pepper production in Argentina, especially in irrigated zones. In some years nearly 40% of the production has been reduced due to this disease (Galmarini 1997). At La Consulta Experiment Station of the National Institute of Agricultural Technology (INTA), a breeding program has been conducted since 1966 to introduce resistance against local strains of *P. capsici* in both bell and heart-shape-type peppers. As a result of the program, the resistant bell-type cultivars Fyuco INTA (Galmarini and Senetiner 1986) and bell-type cultivar Lungo INTA (Galmarini et al. 1995) as well as resistant heart-type cultivars for the cannery industry, Calafyuco INTA and Don Humberto INTA, have

been released (Galmarini et al. 1991, Galmarini et al. 1996). In all cases, an accession from Dr. P. Smith of the University of California, Davis was used as the initial source of resistance. The evolutionary potential of a pathogen population can affect the durability of disease resistance (McDonald and Linde 2002). For *P. capsici*, the evolutionary potential is highest in populations that are outcrossing and maintaining a reservoir of genotypic and genic diversity (e.g. at many locations in the USA). Sexual recombination will rearrange existing and new variation into novel genotypes that may be able to overcome the host resistance. Very little is known about the population structure of *P. capsici* in Argentina and our main objective was to examine the phenotypic and genotypic diversity of *P. capsici* at key locations in major areas of production. This knowledge will be used to further refine our breeding strategies against this pathogen.

Materials and Methods

Isolates and mating type analysis

From 2006 through 2009, *P. capsici* isolates were recovered from pepper and paprika hosts at 11 locations (Figure. 1). For pathogen isolation, host plants showing typical symptoms of *Phytophthora* root rot were thoroughly washed with tap water. Small roots and crown pieces were excised from the edge of expanding lesions, transferred to V8 agar plates amended with PARP (100 ml V8 juice, 2.5 g CaCO₃, 18 g agar per liter amended with 100 ppm of pimaricin, 250 ppm of ampicillin, 100 ppm of rifampicin, and 100 ppm of pentachloronitrobenzene) and incubated for 3 days at 25°C. Hyphal tips were then sub-cultured from actively expanding mycelium and transferred to V8 agar plates without additions. For long-term storage, agar plugs of mycelium were stored in 2 ml screw cap tubes with 1 ml of sterile distilled water.

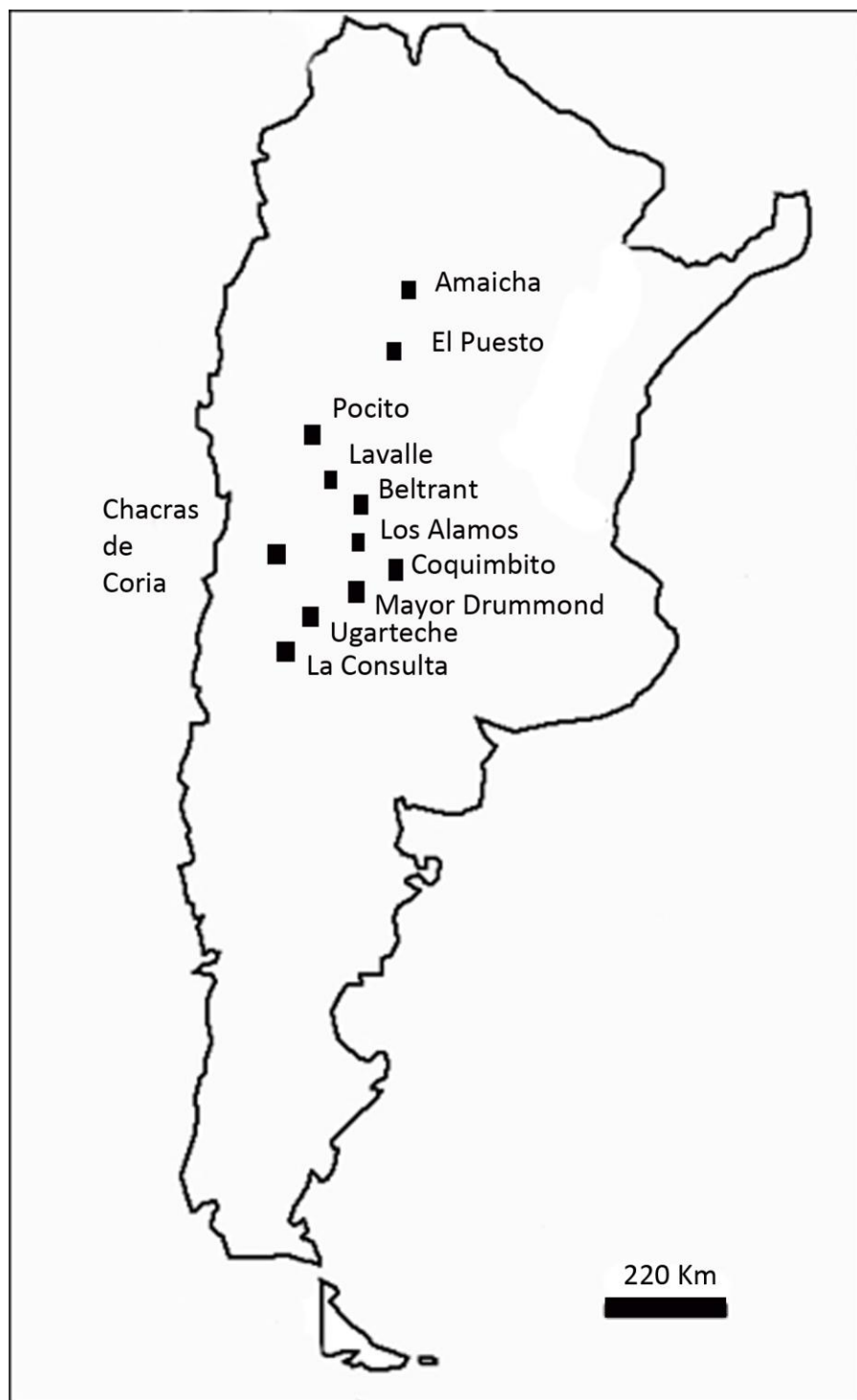


Figure 3.1 Locations where *Phytophthora capsici* was collected in Argentina.

The locations span a broad geographic range with more than 1100 km between the two most distant locations (Roig et al. 2009). Mating type was determined using *P. capsici* tester isolates A1 (CBS 121656) and A2 (CBS 121657). The query and tester isolate were grown together on small agar squares prepared from dilute V8-PARP (40 ml V8 juice, 3 g CaCO₃, 15 g Bacto agar per liter amended with 25 ppm pimaricin, 100 ppm ampicillin, 25 ppm rifampicin, and 25 ppm pentachloronitrobenzene). Four to seven days post inoculation, the colony intersections were observed for oospore production by examination under a light microscope at 400X magnification.

Genomic DNA extraction and AFLP analysis

Isolates were grown for seven days in PARP amended V8 juice broth, the mycelium was harvested and freeze-dried, and high molecular weight DNA was extracted using the protocol of Lamour and Finley (Lamour and Finley 2006). For the AFLP reactions, the protocol of Vos et al. (1995) was followed with minor modifications. *EcoRI* and *MseI* restriction enzymes and adapters were used for digestion and ligation reactions respectively. The modified DNA fragments were subjected to pre-selective PCR reactions using *EcoRI* + 0 and *MseI* + 0 primers. The pre-selective product was diluted and used for selective PCR amplifications with Eco-CG and Mse-CG primer pairs. The selective amplification products were diluted and labeled in a separate PCR reaction using a labeling PCR reaction as described previously (Habera et al. 2004). The resultant fluorescently labeled amplified fragments were visualized and sized using a Beckman-Coulter CEQ8000 capillary instrument and accompanying software. Peaks between 100 and 600 bp were scored for absence or presence.

SNP identification and genotyping

Polymorphic SNP sites were identified by resequencing 20 putative single copy genes in a panel of five *P. capsici* isolates from Argentina, Michigan, Tennessee, Pennsylvania and Peru (data not shown). The SNPs selected for further analysis are heterozygous in the Argentina isolate and, when considering the sequences from the other isolates, had flanking sequences of at least 40 bp on either side of the SNP site free of additional polymorphisms (Table 3.1).

Table 3.1 Single Nucleotide Polymorphism (SNP) markers used to characterize *P. capsici* from Argentina.

SNP ID ^a	Gene model	Scaff size	Protein ID	Predicted Change
Sc29_197561(2)(6)(9)	PHYCAscaffold_29:196912-198459	707326	507640	C>T Thr297Thr A>C Gly298Gly C>T Pro299Pro C>T Leu300Leu C>T Ser313Ser
Sc27_546541	PHYCAscaffold_27:545590-547311	705730	507461	C>T Ser313Ser
Sc19_634212	PHYCAscaffold_19:634212-634424	944885	110976	C>T Asp71Asp
Sc22_655493	PHYCAscaffold_22:653551-657040	843021	534326	C>T Arg363Cys
Sc39_343450	PHYCAscaffold_39:342193-344049	591971	508875	C>T Gly200Gly
Sc7_1019850	PHYCAscaffold_7:1018621-1020309	1368812	102611	T>A Ser154Thr
Sc25_680731	PHYCAscaffold_25:678612-681126	841216	507189	C>T Gly685Gly
Sc121_54954	PHYCAscaffold_121:54032-56025	70414	512063	A>G Asn358Ser

^a Nucleotide location in the *P. capsici* reference genome. Sc = scaffold followed by the SNP location.

High resolution DNA melting analysis (HR-DMA) was used to assay the SNP genotypes. The HR-DMA assay is accomplished with PCR amplification of a small amplicon (45-60 bp total length) that contains the polymorphic site (or sites) in the presence of the fluorescent dye LCGreen Plus. LCGreen Plus fluoresces brightly when bound to double-stranded DNA. Following amplification, the amplicons are heated to 99°C and cooled to form heteroduplex DNA and then analyzed using a Lightscanner machine (Idaho Technologies, Salt Lake City, UT). Heteroduplex DNAs are formed when the amplicon has a heterozygous SNP. The homo-heteroduplex DNAs have differential stabilities due to the sequence mismatches in the heteroduplexed DNA, and the Lightscanner instrument produces distinctive melting profiles (curves) for each (Montgomery et al. 2007).

Our assays were designed to differentiate homozygous and heterozygous alleles for markers spanning either a single polymorphic site, or, for one marker, multiple closely linked polymorphic sites. All the assays were replicated at least twice. Data analysis and normality parameters were performed using LightScanner 2.0 software (Idaho Technologies, Salt Lake City, UT). Despite the high efficiency in discriminating between homozygous and heterozygous alleles, both the wild-type and mutant homozygous genotypes can be difficult to resolve with regular HR-DMA. In order to properly assign genotypes to the two homozygous genotypes a known homozygous amplicon is added to the reactions in a 1:1 ratio and the mixed amplicons are reanalyzed. If the amplicons are identical (e.g. AA and AA at the SNP site) then the melt curve remains the same. If they are different (e.g. AA and TT), then the curves for those isolates are destabilized by heteroduplex formation and a distinctive curve is produced. DNA sequencing was performed on isolates having a unique melting profile, and this was typically found to be the

result of additional polymorphic loci not identified in the panel of isolates. For the marker covering four heterozygous loci, the genotypes for different melting profiles were confirmed by sequencing a representative isolate from the group.

The LightScanner primer design software 1.0 (Idaho Technologies, Salt Lake City, UT) was used to design HR-DMA primers. The PCR amplification was accomplished in 384 well hard-shell PCR plates (Bio-Rad, Hercules, CA). The reaction consisted of the following: 10-20 ng genomic DNA, 0.5 µl 10x buffer, 0.2 µl 5mM dNTPs, 0.05 µl 50mM MgCl₂, 0.025 µl 100 µM forward and reverse primers, 0.1 Units Taq Polymerase, 0.5 µl 10x LCGreen plus dye (Idaho Technologies, Salt Lake City, UT) with 5 µl final PCR reaction volume. The PCR amplification protocol was as follows: initial denaturation at 95° C for 2 min., then 35 cycles of 95° C for 30 s and 64° C for 30 s, and then a final step for homo-hetero-duplex formation at 95° C for 30 s followed by 25 °C for 30 s. HR-DMA was carried out according to manufacturer's instructions on a LightScanner instrument (Idaho Technologies, Salt Lake City, UT).

Mefenoxam sensitivity screening

Plugs (7 mm diameter) from the edge of fresh cultures were placed in the center of 5 cm diameter V8 agar plates amended with either 100 parts per million (PPM) mefenoxam (Ridomil Gold EC, 48% a. i.) or V8 plates without mefenoxam. The plates were incubated at room temperature for three days. Colony diameter was measured and an isolate was considered resistant (insensitive), intermediately sensitive and sensitive when growth on 100-PPM plate was more than 90%, 30-90% and less than 30% of the control, respectively (Lamour and Hausbeck 2000).

Results

A total of 40 isolates of *P. capsici* were recovered from pepper and one isolate from pumpkin (Table 3.2). Of the 41 isolates, 23 were collected in 2009, 13 in 2008, 1 in 2007 and 4 in 2006. All of the isolates were the A1 mating type. The isolates were assayed for seven markers that target a single SNP site and one marker which contained four heterozygous loci concentrated in a 10-bp base pair region (Table 3.3). All of these sites were heterozygous in the representative Argentina isolate that was used for our initial sequencing. Of the 41 isolates, 36 have an identical multi-locus SNP genotype designated as ArPcc-1. The ArPcc-1 genotype is fixed for heterozygosity at seven of the eight markers loci (Table 3.4). The remaining five isolates had a total of three genotypes which are designated as ArPcc-2, ArPcc-3, and ArPcc-4. Two isolates, designated ArPcc-2, were similar to the ArPcc-1 genotype except for homozygous loci at the Sc27_546541 and Sc29_197561 markers. Two isolates, designated ArPcc-3 were homozygous at the Sc19_634212, Sc39_343450, Sc7_1019850 and Sc121_54954 markers. And finally, a single isolate, designated ArPcc-4, was homozygous at the Sc25_680731 and Sc29_197561 markers. Ten isolates of the ArPcc-1 clonal lineage were analyzed by AFLP using the Mse+CG/Eco+CG primer pair. This revealed identical fragment profiles containing 40 markers (data not shown). The ArPcc-1 genotype was recovered from all eleven locations as well as during the 2006, 2008 and 2009 growing seasons (Table 3.2 and Figure 3.1). All of the isolates were sensitive to mefenoxam.

Table 3.2 Summary information for *P. capsici* isolates collected in Argentina.

Year	Regions	Approximate distance from La Consulta (Km)	No of isolates	Mating type	Host	Genotypes
2009	Amaicha	1100	6	A1	paprika	ArPcc-1, ArPcc-2
	El Puesto	1100	5	A1	paprika	ArPcc-1
	Lavalle	140	4	A1	pepper	ArPcc-1
	Beltrán	110	2	A1	pepper	ArPcc-1
	Los Álamos	110	6	A1	pepper	ArPcc-1
2008	Mayor Drummond	80	4	A1	paprika	ArPcc-1, ArPcc-4
	Chacras de Coria	80	1	A1	paprika	ArPcc-1
	Ugarteche	60	2	A1	pepper	ArPcc-1
	Pocito	230	6	A1	pepper	ArPcc-1
2007	Chacras de Coria	80	1	A1	paprika	ArPcc-2
2006	Chacras de Coria	80	1	A1	paprika	ArPcc-1
	La Consulta		3	A1	pumpkin (1), paprika (2)	ArPcc-1, ArPcc-3

Table 3.3 High resolution DNA melting analysis (HR-DMA) primers used to genotype isolates of *Phytophthora capsici* from Argentina.

SNP ID	SNP	Forward Primer	Reverse Primer
Sc29_197561(2)(6)(9)	C/T, C/A, C/T/, C/T	CGCCTTCAAGAACACGG	CTTGATGCCATCGTGCG
Sc27_546541	C/T	GTACTGGACGCGTGCAA	CGCATGATCACGAGGTAG
Sc19_634212	C/T	CTGATCCGGATAAACGTAAAG	CTCCAAACATACGAGCGTC
Sc22_655493	C/T	ATTGACACAGCGCTATTC	CAGCTCCTTGTACCGCTTA
Sc39_343450	C/T	TCGACCGGACGGAGATGA	GTGGGAACACTGTACGTTG
Sc7_1019850	A/T	GGTTTTGTTAGTGGTGAT	GCGCGTCTTCCTGCTGCAG
Sc25_680731	C/T	CAGTGCGCAGTGTGGGA	ATCGTCCATCTCCTTCACT
Sc121_54954	G/A	TCCAAATCGAGCGGCTACA	TTCGAGGACTCACCAAGAAT

Table 3.4 Summary of SNP genotypes for *Phytophthora capsici* recovered from Argentina.

Genotype	Sc29_197561(2)(6)(9)					Sc27_5 46541	Sc19_63 4212	Sc22_6554 93	Sc39_3434 50	Sc7_10198 50	Sc25_6807 31	Sc121_549 54	No of isolates
ArPcc-1	C/C	C/A	C/T	C/T	C/T	C/T	C/T	C/T	C/T	A/T	C/T	G/A	36
ArPcc-2	C/T	C/A	C/T	C/T	C/C	C/T	C/T	C/T	C/T	A/T	C/T	G/A	2
ArPcc-3	C/T	C/A	C/T	C/T	C/T	C/C	C/T	C/T	C/C	T/T	C/T	G/G	2
ArPcc-4	C/C	C/C	T/T	T/T	C/T	C/T	C/T	C/T	C/T	A/T	C/C	G/A	1

Discussion

Phytophthora capsici is the most important pathogen limiting pepper production in Argentina. Until now, very little was known about the diversity of the pathogen. This study was initiated to assess variation in isolates from widely separated locations and to gain a baseline understanding of the population structure. Surprisingly, all 41 isolates recovered from eleven locations during 2006-2009 are the A1 mating type and fully sensitive to the fungicide mefenoxam (Table 3.2). If populations were outcrossing, this finding is possible simply due to small sample sizes, but usually at least a few A2 mating type isolates should be found. Further analysis using eight SNP loci indicated that genotypic diversity is limited and that a single clonal lineage, ArPcc-1, is responsible for much of the disease in Argentina. The SNPs utilized in this study are all from different, relatively large, scaffolds of the *P. capsici* genome assembly and likely provide a reasonable assessment of genotypic diversity. A follow-up analysis of 10 randomly chosen ArPcc-1 isolates produced 10 identical AFLP profiles, confirming our findings based on the SNP genotypes. The ArPcc-2 and ArPcc-4 genotypes may be closely related, or even derived from the ArPcc-1 clonal lineage (Table 3.4).

In total, the SNP analyses, AFLP profiles, mefenoxam sensitivity and mating type data all support the hypothesis that clonal reproduction dominates in Argentina and that sexual recombination plays a limited (or absent) role in driving the population structure. Most likely, mutation and mitotic recombination explain the limited genetic variation arising within this large clonal lineage (Goodwin et al. 1995; Dobrowolski et al. 2003). During reverse genetic screening of *P. capsici* after N-ethyl-nitrosourea (ENU) mutagenesis, loss of heterozygosity was observed with varying frequencies at different loci (Hulvey et al. 2010).

It is interesting to note that the ArPcc-1 clonal lineage is distributed widely in Argentina and is able to survive fallow periods between pepper plantings. In a study of Peruvian *P. capsici* populations, a single A2 mating type clonal lineage (PcPE-1) dominated over a wide area and over multiple years, and this was thought to result from continuous cropping of susceptible host material (Hurtado-Gonzales et al. 2008). Unlike Peru and similar to many areas of the USA, severe frost incidences occur in Argentina that limit continuous cropping. At this point, it is difficult to speculate on how the ArPcc-1 lineage has become so widely dispersed or how it is able to survive freezing and/or non-host periods. Further studies comparing isolates from Argentina to those recovered from outcrossing populations in the US and the clonal population in Peru may reveal key differences. We have attempted crosses between isolates of the ArPcc-1 lineage and the PcPE-1 lineage from Peru. Thus far, none have produced viable oospores.

In the United States, cultural practices that limit water are often the most effective for controlling *P. capsici* and the use of disease resistant varieties may be more difficult because populations maintain a high level of diversity (Dunn et al. 2010, Lamour and Hausbeck 2002, 2003). Furthermore, *Phytophthora* root rot and *Phytophthora* blight of pepper seems to be controlled by at least two different resistance genes (Oelke et al. 2003, Walker and Bosland 1999).

Our findings may shed light on the fact that resistant pepper varieties bred using local Argentinian isolates have been useful for limiting infection by *P. capsici*. The resistant cultivars released by INTA La Consulta, such as Fyuco INTA and Calafyuco INTA are still widely used by pepper growers and the limited genetic variation in the pathogen population may be a factor that allows disease resistance to remain viable for extended periods of time. The data presented

here provides a useful baseline of knowledge that will be useful in the future; particularly if there are instances where previously resistant varieties begin to sustain elevated levels of disease.

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Chapter Four

Survival and spread of *Phytophthora capsici* on Long Island, New York.

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My primary contributions to it were all of the writing, DNA melting analysis, data analysis, pathogen isolation, most of the DNA sequencing and mating type analysis

Abstract

Phytophthora capsici is an oomycete soilborne plant pathogen that causes root, fruit and foliar disease on a variety of vegetables. The epidemiology and population structure varies depending on the region surveyed. Our objective was to investigate survival and spread on farms on Long Island, New York using single nucleotide polymorphism (SNP) markers. A total of 373 *P. capsici* isolates were collected from pumpkin, pepper, watermelon and snap bean on 15 farms. Both mating types were recovered from most locations. Genotypic analysis was conducted using 14 SNP loci located primarily within genes. A total of 128 unique multi-locus genotypes were identified. Of these, 54 were clonal lineages ranging in size from 2 to 26 members. Most clonal

lineages were recovered during the same year. Our results indicate that both sexual and clonal reproduction play important roles in the epidemiology of *P. capsici* on Long Island, NY, USA. The implications for managing the disease are discussed.

Introduction

Phytophthora capsici is one of the most important vegetable pathogens worldwide (Erwin and Ribeiro 1996; Hausbeck and Lamour 2004). Unlike other *Phytophthora* species, which thrive under cool conditions (e.g. *P. infestans* on potato), *P. capsici* grows optimally during warm (25-28°C) conditions and is often severe when crops are near harvest (Lamour and Kamoun 2009). At locations where rainfall is limited (e.g. Southwestern USA), infections are often on the roots and crown leading to wilting and plant death. Where rainfall is common (e.g. Eastern, Midwestern, and Southeastern US), *P. capsici* often produces massive quantities of deciduous asexual sporangia on the fruit. Sporangia can germinate directly to cause infection or, if there is free water, release swimming zoospores. The asexual phase of the life cycle can progress rapidly, with just a few days from infection to sporangia production and subsequent spread. *Phytophthora capsici* is heterothallic, requiring the presence of both mating types (A1 and A2) to produce the thick-walled sexual oospores, and both mating types have been found at locations in the United States. In Michigan, it appears that epidemics often progress from initial infection by diverse oospore-derived isolates to the spread of clonal lineages as the season progresses (Lamour and Hausbeck 2001b). Spread of clonal lineages can be substantial at a given location but it does not appear that long distance movement is common (e.g. outside of contiguous or nearby fields) and generally clonal lineages do not appear to survive fallow or winter periods (Lamour and Hausbeck 2001a). Populations of *P. capsici* in Peru are quite different and a single clonal lineage

is widely distributed throughout the country and has persisted for multiple years (Hurtado-Gonzales et al. 2008). This is likely due in part to the presence of susceptible crops all year and high fitness of the dominant genotype.

Vegetable production on Long Island, New York, USA while very diverse, is dominated by crops susceptible to *P. capsici* including significant acreage planted to pumpkins, gourds, and squashes aimed at the fall tourist market (for decoration and not consumption). Other important crops are squashes, melons, peppers, and tomatoes grown organically and conventionally for fresh market sale at farm stands. Within the past two decades *P. capsici* has become the most serious factor limiting vegetable production on Long Island and many producers, particularly of pumpkin, have had years where they have lost 100% of their crops due to infection.

Phytophthora capsici isolates were collected in 2007 and 2008 from vegetables grown on 15 farms on Long Island, NY, USA primarily from pumpkin, but with isolates from tomato, pepper and snap beans as well. Our goals were to determine the contribution of sexual and asexual reproduction in this growing region, to investigate the movement of isolates among farms, and to assess the diversity of isolates recovered from snap beans.

Materials and Methods

Isolate collection and mating type analysis

Infected plant samples were collected from 15 farms found in the eastern end of Long Island, NY, USA. The two most distant locations were about 25 km apart; farms that are adjacent to one another were included also. No specific sampling strategy was followed for sample collection. Disease samples were collected on a regular basis from different grower fields and some of the

farms were sampled more than one time during the study period. The samples were randomly picked from every corner of the field when the diseased hosts are available. Isolates were obtained in the laboratory by plating small sections of infected tissue onto dilute V8-PARP agar medium (40 ml V8 juice, 3 g CaCO₃, 15 g Bacto agar and 960 ml water amended with 25 ppm pimaricin, 100 ppm ampicillin, 25 ppm rifampicin, and 25 ppm pentachloronitrobenzene) and incubated at room temperature (about 25 °C). After 3 to 7 days, single hyphal tips were sub-cultured onto full strength V8-PARP agar (V8-PARP agar medium with 162 ml V8 juice instead of 40 ml). A single isolate was recovered from each sample and used for the subsequent analysis.

Mating type was determined by co-culturing the isolates with A1 (CBS 121656) and A2 (CBS 121657) *P. capsici* tester isolates on agar blocks (approximately 4 cm²) cut from dilute V8-PARP agar. Mycelium from the tester isolates and the sample isolates was placed on opposing corners of the agar squares and incubated for four to seven days. The presence or absence of oospores was observed with a light microscope at 400X magnification. The χ^2 -test was used to test if there was a 1:1 distribution of mating types for each farm with more than 10 unique genotypes. It was also used to test the combined genotypically unique samples from all farms.

Genomic DNA extraction and SNP markers

Mycelium for genomic DNA isolation was prepared by growing isolates in 24-well plates containing 1 ml PARP amended V8 broth for 7 days. The mycelium was harvested, lyophilized, powdered, and high molecular weight DNA extracted as previously described (Lamour and Finley 2006). The SNP loci were selected from single copy genes that are spaced across different

scaffolds in the publically available *P. capsici* genome sequence (<http://genome.jgi-psf.org/Phyca11/Phyca11.home.html>). The SNP loci were tested for Mendelian inheritance in laboratory crosses (data not shown).

High resolution DNA melting analysis (HR-DMA)

HR-DMA assays were used to assess the SNP genotypes. The 14 SNP loci used for this study are from twelve putative single copy genes and one non-coding region found on different scaffolds of the draft *P. capsici* genome sequence (Table 4.1).

Table 4.1 Summary data for single nucleotide polymorphism (SNP) markers.

SNP ID ¹	Gene Model	Scaffold size	Protein ID	Predicted Change
Sc29_197362	Scaffold_29:196912-198459	707326	507640	G>C Pro366Pro
Sc29_197194	Scaffold_29:196912-198459	707326	507640	G>C Ala422Ala
Sc158_41613	Scaffold_158:41466-42254	42459	577597	T>C Asn26Asn
Sc19_881188	Scaffold_19:880709-882275	944885	506449	C>T Leu157Leu
Sc15_161928	Scaffold_15:160557-162666	1032281	564492	T>C Ile215Ile
Sc71_157951	Scaffold_71:156154-158367	252329	510944	A>G Gly577Gly
Sc82_34480	Scaffold_82:33595-36807	207552	556035	C>T Arg516Arg
Sc72_72952	Scaffold_72:70985-74119	230452	510981	5'UTR
Sc63_257680	Scaffold_63:256692-259740	355987	510602	T>C Pro329Pro
Sc30_144322	Scaffold_30:142830-144963	669438	507774	C>T Phe181Phe
Sc30_470182	Scaffold_30:467725-473115	669438	116106	C>T Leu825Leu
Sc33_253377	NA	640966	NA	NA
Sc1_401723	Scaffold_1:401450-402353	2170955	502611	T>C Asp102Asp
Sc16_108605	Scaffold_16:108041-109063	1020712	505847	C>T Asn151Asn

¹ Nucleotide location in the *P. capsici* reference genome. Sc = scaffold followed by the SNP location.

The loci were selected after sequencing these regions in two isolates recovered from Long Island (an A1 and an A2 mating type) and an additional four *P. capsici* isolates recovered from Michigan, Tennessee, Pennsylvania and Peru. The SNP assays were made by designing primers that span a single polymorphic SNP site producing an amplicon between 45 and 65 bp (Table

4.2). Primers were designed using the LightScanner primer design software 1.0 (Idaho Technologies, Salt Lake City, UT). Amplification was accomplished by Peltier thermal cycler (MJ Research) in 384-well hard-shell PCR plates (Bio-Rad, Hercules, CA) with 5 µl final PCR reaction volumes. The PCR reaction consisted of 10-20 ng genomic DNA, 0.5 µl 10x buffer, 0.2 µl 5mM dNTPs, 0.05 µl 50mM MgCl₂, 0.025 µl 100 µM forward and reverse primers (See Table 4.1 for primers), 0.1 Units Taq Polymerase and 0.5 µl 10x LCGreen plus dye (Idaho Technologies, Salt Lake City, UT). The PCR amplification protocol was as follows: initial denaturation at 95 °C for 2 min, then 35 cycles of 95 °C for 30 s and 64 °C for 30 s, and then a final step for duplex formation at 95 °C for 30 s followed by 25 °C for 30 s.

Table 4.2 High resolution DNA melting analysis (HR-DMA) primers used to analyze isolates of *Phytophthora capsici* recovered from Long Island, NY.

SNP ID	Alleles	Forward Primer	Annealing Temperature (° C)	Reverse Primer	Annealing Temperature (° C)
¹ Sc29_197362	C/G	GTGTACGTCTGGTCGATCTT	54	AGTGTCAACAACGCTGTG	53.2
¹ Sc29_197194	C/G	GGCCACTGCTACTCGGA	57.1	CACCGACTCAATGACAGAC	53
Sc158_41613	C/T	ACTTGGGCCTCCGCCTT	60	ATGCTGAGCTCGTTGTCTCG	57.4
Sc19_881188	C/T	ACTGAATTTGCTCAGTATGACA	52.1	TGATCTCGTTACCGGCG	54.5
Sc15_161928	C/T	GCACCACGAGCTTTGAAATC	54.7	GTTGGACGTGGCATTGAG	54.1
Sc71_157951	A/G	CAGCAGCGATATGATCA	48.9	CATCATGGCGTGAAGCG	54.4
Sc82_34480	C/T	CTCGTACGCTAAGATACC	49	GGCAAGGCTGTGGACTG	56.5
Sc72_72952	A/G	CTGTGCTTCAGCGCATATTA	53	CATCTGCTGTTGAAGACGAC	53.6
Sc63_257680	C/T	TGTCAAGGGCAGGTACAG	54.3	GGACATGGACCCGAACA	54.6
Sc30_144322	C/T	GAACAGGTGCGATCAAGG	53.5	TCGGGTCGAATTGGTCT	53.2
Sc30_470182	C/T	GTGCTGATCTATCGTGAGGT	54	GCACTATTCAGTGTGAGCG	53.6
Sc33_253377	C/T	ATGCAATGGCCTGTTCC	53.2	GAGTGCTCGAACCGTGGA	56.9
Sc1_401723	C/T	GGACTACAACATCCAGCG	53	ACTGCGGGATGTTGTTT	52.7
Sc16_108605	C/T	GTTACCCATGGCTCATATATTG	53.7	ACAGTATTGACGTTCTGCAT	51.8

¹SNP from the same gene

All the primers are from 5' to 3' directions

Following heteroduplex formation, amplicons were melted and the change in fluorescence was measured using a LightScanner Instrument (Idaho Technologies, Salt Lake City, UT) and the resulting melt curves analyzed with the LightScanner 2.0 software package according to the manufacturer's directions. The samples were melted at the default LightScanner melting rate ($0.1^{\circ}\text{C s}^{-1}$). The fluorescence signal decreases as temperature increases and a characteristic melting profile is generated for each genotype. The minimum and maximum melting temperature range is determined for each amplicon and is dictated by sequence length, GC content and other inherent factors (Lipsky et al. 2001).

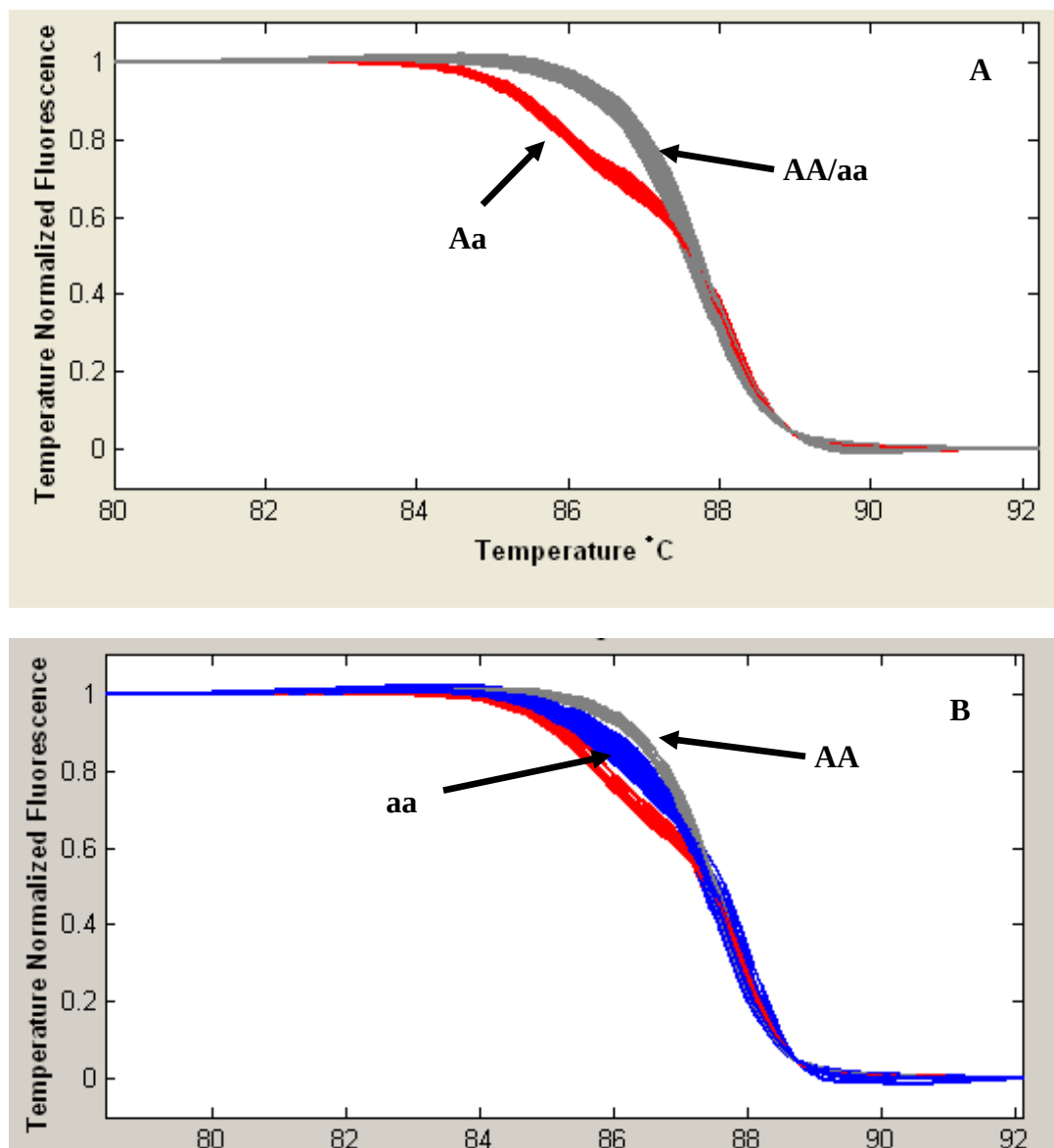


Figure 4.1 Representative high resolution DNA melting analysis (HR-DMA) curves. A, non-spiked normalized curves showing the combined homozygous (AA or aa) and heterozygous (Aa) melt curves. B, normalized curves after spiking in an amplicon with the homozygous alternate allele. Notice the shift for the isolates with the “aa” genotype away from the homozygous “AA” melt curve.

The initial melt curves clearly differentiate homozygous and heterozygous genotypes (Figure 4.1, the upper panel). All of the markers utilized in this study appear to be bi-allelic. Often the two possible homozygous genotypes (e.g. AA and aa) have similar melting curves. In order to determine the correct homozygous genotype, an amplicon which contains a homozygous copy of

the locus under investigation was spiked into the reaction and the curves were re-analyzed (Figure 4.1, lower panel). The homozygous DNA fragment used for spiking was produced by a separate PCR amplification from a synthetic template (45-65 bp in length) purchased from Integrated DNA Technologies, Inc. San Diego, CA. The PCR reaction to produce the spiking amplicon contained 1 μ l 10 μ M template DNA, 3 μ l 10x buffer, 1.2 μ l 5mM dNTPs, 1 μ l 10 μ M forward and reverse primers, 0.3 Units Taq Polymerase and water in a 30 μ l final reaction volume. The PCR amplification protocol was as follows: initial denaturation at 95 °C for 2 min, then 30 cycles of 95 °C for 30 s and 64 °C for 30 s, followed by 4 °C. Two μ l of the amplified fragment for spiking was added to the 5 μ l initial PCR product. The sample was heated to 95 °C for 30 s and cooled to 25 °C for 30 s for three cycles to generate duplex products. Following duplex formation, the sample was re-analyzed to identify melt curves that had changed from the homozygous to the heterozygous melt curve type. Isolates having melt curves that changed are homozygous for the alternate allele.

Departure from Hardy-Weinberg equilibrium (HWE) was analyzed for the 10 SNP markers, which were genotyped for all three possible genotypes using a χ^2 -test (Table 4.4). In addition, allele frequency data was used to estimate the probability of erroneously assigning a unique individual to a clonal lineage. The average heterozygosity for the different populations was calculated. Isolates collected from a single farm during a single year were considered a subpopulation (Table 4.3) and Wright's F_{ST} (Fixation index) was used to quantify the proportion of genetic variation that lies between subpopulations that contain more than 10 unique genotypes.

Results

Isolates and mating type

A total of 373 isolates were recovered from 15 farms: 330 from pumpkin, 17 from snap bean, 11 from pepper, 9 from tomato, and 6 from watermelon (Table 4.3). The farms are all located on the eastern end of Long Island in an area of approximately 150 square kilometers. Some of the farms (e.g. LINY-J, LINY-P, and LINY-Z) have fields that are directly adjacent. Approximately 80% of the isolates were collected in 2008. The remaining samples were collected in 2007 primarily from pumpkin with a few samples from pepper (Table 4.3). Most (approximately 95%) of the samples collected were crossed to A1 and A2 isolates to determine the mating type. The overall distribution of the A1 and the A2 mating types were roughly equal, with 188 A1 mating types and 161 A2 mating types. The total sample set χ^2 -test for mating type based on unique genotype showed that the distribution of the mating type does not deviate significantly from a 1:1 ratio at a P value of 0.05. Both mating types were recovered from all the farms except LINY-ES and LINY-P. However, most of the farms did not have enough unique genotypes to test the distribution using a χ^2 -test. Five farms sampled in 2008 with more than 10 unique genotypes were considered for analysis. Three of them (LINY-H, LINY-M and LINY-S) had a one to one distribution at a P value of 0.05 while two of them (LINY-R with eleven A1 and two A2 and LINY-Z with five A1 and seventeen A2) deviated from the one to one distribution at P=0.05.

Table 4.3 Summary of *P. capsici* isolates recovered from pumpkin, tomato, pepper, watermelon and snap beans on farms in Long Island, NY.

Year	Farms	Clonal lineages	Nº isolates (Nº unique genotypes)	Unique genotypes A1:A2 ratio	Hosts
2007	LINY-H		6 (6)	5: 1	Pumpkin
	LINY-L	CL8-2	4 (3)		Pumpkin
	LINY-MRP	CL21-6	6 (1)		Pumpkin
	LINY-S	CL24-6	6 (1)		Pumpkin
	LINY-W	CL19-2, CL30-11, CL45-2	8 (3)	1:2	Pumpkin
	LINY-P	CL20-2, CL30-11	9 (2)	2:0	Pepper
	LINY-Z	CL3-17, CL4-11, CL16-2, CL29-2	32 (7)	1:6	Pumpkin
2008	LINY-A	CL12-3, CL15-12, CL23-3, CL36-3, CL51-4	30 (10)	2:8	Pumpkin (7), Snap bean (17) and watermelon (6)
	LINY-ES	CL32-5, CL52-2	9 (4)	0:4	Tomato
	LINY-F	CL11-3	3 (1)	0:1	Pumpkin
	LINY-H	CL13-5, CL18-6, CL22-3, CL26-2, CL35-5, CL39-6, CL42-4, CL43-15	43 (19)	11:8	Pumpkin
	LINY-J	CL1-7, CL5-3, CL6-6, CL7-4, CL23-3, CL25-3, CL31-22	15 (7)	2:5	Pumpkin
	LINY-L	CL9-4, CL31-22, CL37-8, CL38-26, CL43-15	59 (8)	6:2	Pumpkin
	LINY-M	CL44-2, CL47-2, CL48-7, CL50-2, CL53-6	33 (19)	8:11	Pumpkin
	LINY-P	CL31-22	18 (2)	0:2	Pumpkin (16), Pepper (2)
	LINY-R	CL28-10, CL46-3, CL49-3, CL54-3	28 (13)	11:2	Pumpkin
	LINY-S	CL27-4, CL33-2, CL34-3, CL40-4, CL41-7	26 (11)	7:3	Pumpkin
	LINY-V		1	1	Pumpkin
	LINY-Z	CL2-2, CL3-17, CL5-3, CL6-6, CL7-4, CL10-5, CL14-5, CL17-2, 25-3	37 (22)	5:17	Pumpkin

Fourteen HR-DMA SNP markers were used to analyze the isolates from Long Island (See Tables

1 and 2 for the position of the SNP loci on the reference genome sequence and the list of primers

used). For ten of the markers (Table 4.4), the three different genotypes expected for a bi-allelic site were determined by HR-DMA analysis as shown in Figure 4.1. The red curves represent all the heterozygous (CG) genotypes at Sc29_197362 marker while the homozygous genotypes (CC and GG) were represented by gray curves as shown in Figure 4.1, upper panel. Re-melting the sample following the addition of homozygous CC genotypes shifts the GG genotypes towards the heterozygous red curves (See figure 4. 1 lower panel). For four markers (Sc29_197194, Sc72_72952, Sc63_257680, and Sc30_144322) only two genotypes (heterozygous or homozygous) were determined with melting analysis.

Table 4.4 Estimated SNP allele frequencies and tests for Hardy-Weinberg equilibrium.

SNP ID	Allele	%	Allele	%	χ^2 - value
Sc29_197362	G	83	C	17	4.00
Sc158_41613	T	79	C	21	2.62
Sc19_881188	C	75	T	25	14.22*
Sc15_161928	T	94	C	6	16.83*
Sc71_157951	G	87	A	13	3.00
Sc82_34480	C	79	T	21	2.05
Sc30_470182	T	77	C	23	0.52
Sc33_253377	C	96	T	4	2.69
Sc1_401723	C	77	T	23	8.35
Sc16_108605	T	72	C	28	1.57

*Significant deviation at 0.05%.

A total of 128 multi-locus genotypes were identified with 54 being present in more than one isolate and 74 present in only a single isolate (Figure 4. 2). Isolates with the same multi-locus genotype are referred to as clonal lineages and are designated as CLX-Y where X is the lineage identifier and Y is the number of isolates. Clonal lineages ranged in size from two to twenty six isolates (Figure 4. 2). Members of a clonal lineage were mostly limited to a single year (either

2007 or 2008) and the same farm (Table 4.3). However, clonal lineage CL3-17 contains isolates recovered from pumpkin in 2007 and 2008 from LINY-Z farm. Clonal lineages CL5-3, CL6-6, CL7-4 and CL25-3 contain isolates collected from farms LINY-J and LINY-Z, which are adjacent to one another. Furthermore, the two adjacent farms LINY-J and LINY-P share one clonal lineage (CL31-22) with LINY-L, which is less than 10 km away. LINY-H and LINY-L, which are approximately 20 km from one another also have members of the same clonal lineage (CL43-15) with 14 isolates from LINY-L and one from LINY-H.

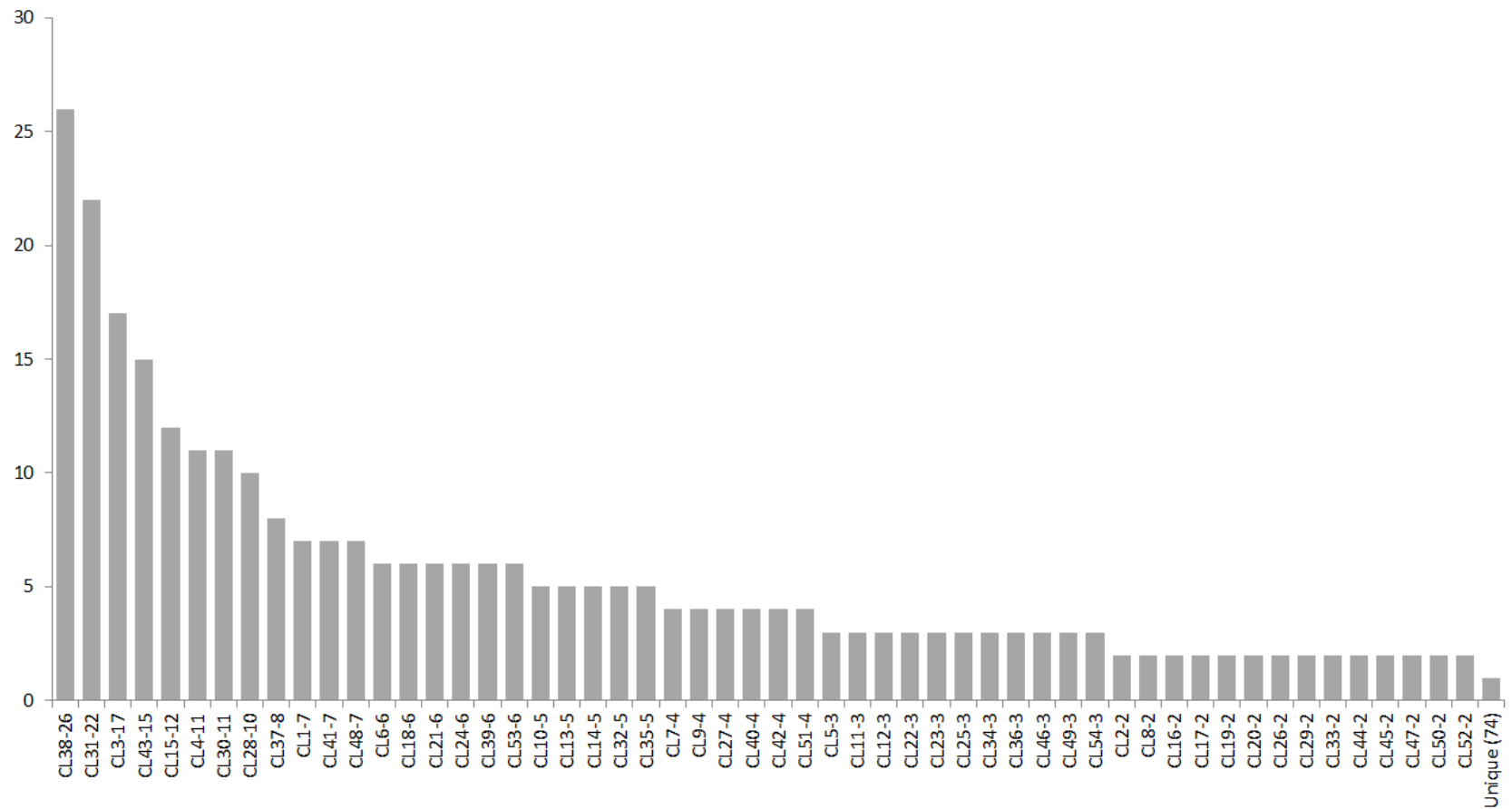


Figure 4.2 Frequency of *P. capsici* clonal lineages obtained in 2007 and 2008 from Long Island, NY. The horizontal axis indicates the clonal lineages with the name and number of members indicated on the bottom of the bar graph.

In 2008, seventeen *P. capsici* isolates were recovered from snap bean pods that developed disease symptoms after harvest at farm LINY-A. Prior to this, *P. capsici* had not been reported on snap bean on Long Island, NY, USA. Two clonal lineages were recovered from the snap bean samples; CL12-3 and CL15-12, having three and twelve members respectively. Two isolates with unique genotypes (but no clonal copies) were also isolated.

Isolates LT6010 and LT6011 were recovered from pepper samples in 2008 from the LINY-P farm where susceptible crops had not been grown for about 15 years. These pepper isolates have the same genotype (CL31-22) as isolates collected from an adjacent planting of pumpkin. Furthermore, another clonal lineage (CL30-11) includes isolates from pumpkin as well as pepper collected from LINY-P and LINY-W in 2007. Two clonal lineages (CL32-5 and CL52-2) and two isolates with unique genotypes were obtained from tomato samples collected in 2008.

Allele frequencies for the SNP markers were calculated from the 128 unique genotypes. The rare allele frequency at each SNP locus ranged from 4-28% and three markers (Sc19_881188, Sc15_161928 and Sc1_401723) deviated significantly from HWE (Table 4.4). The probability of assigning an isolate to a clonal lineage by chance ranged from 1.12% for CL31-22 to 0.0008% for CL8-2. As described above for mating type distribution, only five farms (LINY-H, LINY-M, LINY-S, LINY-R and LINY-Z) have more than ten unique genotypes for average heterozygosity and fixation index calculations. The average heterozygosity of the subpopulations ranged from 0.15 (LINY-H) to 0.2 (LINY-R) with an average value of 0.17. These values fall within the range described for a wide range of obligately outcrossing diploid plant species (Mable and Adam

2007). The overall F_{ST} value was 0.08, which indicates that approximately 8% of the total genetic variation was present among locations.

Discussion

Phytophthora capsici is one of the most important pathogens limiting vegetable production on Long Island, New York. To avoid losses, producers utilize crop rotation when possible, manage soil moisture, and apply fungicides; however, when the weather is conducive (warm and wet), it is very difficult to control the disease. Vegetable producers are concerned about movement of *P. capsici* between fields and farms, as well as survival of *P. capsici* over time. We assessed genotypic and genetic diversity to better understand how *P. capsici* may be surviving and spreading. A novel set of SNP markers was employed that are distributed over 12 large scaffolds of the *P. capsici* genome, predicted to be either silent or non-coding, and have rare allele frequencies ranging from 4-28% (Table 4.4). High-resolution DNA melting analysis provided a relatively inexpensive, medium throughput technique for assaying genotypes and allelic diversity, and we observed a high level of reproducibility between duplicate reactions.

Our results indicate that clonal reproduction contributes significantly (66% of the observed genotypes were clonal in origin) to the population structure, although no single clonal lineage was dominant in the area. Of the 373 isolates analyzed, 34% had a unique multi-locus SNP genotype. Genotypic diversity was high, compared to other outcrossing *Phytophthora* species in the United States (e.g. *P. infestans* and *P. ramorum*), indicating oospore-derived isolates are important and that clonal reproduction most likely stems from infections initiated by overwintering dormant oospores. The detection of both mating types from the same farms

suggests sexual reproduction contributes to multi-locus SNP genotype at Long Island. This pattern has been observed in *P. capsici* populations from other places in the USA as well as Long Island, New York (Dunn et al. 2010; Wang et al. 2009), where both mating types have been detected from the same farm as well as the same diseased plant. A recent population study from South Africa revealed clonal populations as well as high genotypic diversity of isolates recovered from 2000 to 2008 (Meitz et al. 2010).

The high level of genotypic diversity observed in Long Island differs dramatically from that observed in populations of *P. capsici* recovered from pepper and tomato in Peru. In Amazon and coastal areas of Peru, a single clonal genotype dominated over a wide area over multiple years (Hurtado-Gonzales et al. 2008). The lack of a hard winter freeze allows continuous cropping of diverse pepper varieties (e.g. *Capsicum pubescens*, *C. annum*, and *C. baccatum*) in Peru. Lack of pathogen diversity suggested that oospores do not always play a major role in the population biology.

We also analyzed *P. capsici* isolates recovered from snap beans to determine if there was evidence that a unique clonal lineage, possibly adapted to beans, was responsible. Our analysis indicated four genotypes were present on the snap beans. We utilized Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis to generate a graphic representation of the relationships among the isolates, and the bean isolates did not cluster uniquely. It is most likely that the snap bean isolates were derived from the surrounding population of *P. capsici*. It is worth noting that *Phytophthora* blight occurred on pepper the previous year at this same location, which is a low spot with relatively poor drainage. During the two days before the snap

beans were harvested the environmental conditions were highly favorable and included about 76.5 mm/m² of rainfall. *Phytophthora capsici* was reported from lima beans in Delaware, Maryland and New Jersey (Davidson et al. 2002), and snap beans from four commercial farms in Michigan during 2003 to 2005 (Gevens et al. 2008). Snap bean pods have been infected with isolates recovered from pumpkin under laboratory conditions (Tian and Babadoost 2004).

Even though the calculated F_{ST} value of 0.08 indicates moderate genetic differentiation according to the guidelines suggested by Wright (Wright 1978; Hartl and Clark 1997) and a few markers (Sc19_881188, Sc15_161928 and Sc1_401723) deviated significantly from HWE (Table 4.4), it is most likely that the population on Long Island is not structured based on geography or on host. This is supported by the distribution of clonal lineages among sites and among hosts. Though *P. capsici* is considered a soilborne plant pathogen, significant local spread via water has been reported (Ristaino et al. 1993; Gevens et al. 2007) and massive numbers of sporangia can be produced on above-ground plant parts, especially the fruit. Our finding of clonal lineages distributed among farms, even those that are not directly adjacent to each other, suggests that movement of asexual propagules is common and it is likely that this entire area is open to gene flow and that there are no significant barriers to movement.

Thus far, there is no strategy that consistently provides satisfactory control of *P. capsici* on vegetable hosts. Cultural practices such as keeping fields well drained, combined with chemical control methods (fumigation and application of fungicides on a preventive schedule) may be the only viable options to manage *P. capsici* problems on Long Island. Currently there are no resistant varieties for most cucurbits and in Michigan crop rotation has proven to be ineffective

even over extended periods of time (Lamour and Hausbeck 2002, 2003). Different crop rotation practices and fungicide application programs are utilized at locations included in this study. For instance, pumpkins were grown for about 10 years at location LINY-S, while at other locations, (e.g. LINY-Z, LINY-L, LINY-H) susceptible crops are routinely rotated with non-host crops for as many as six years. Despite the differing approaches to disease management, both mating types were recovered from most of the fields (Table 4.3) indicating the sexually produced thick-walled oospores may allow *P. capsici* to overwinter and survive in the soil for extended periods (French-Monar et al. 2007). The development of alternative management strategies to control *P. capsici*, especially on cucurbit crops, is of paramount importance (Lamour and Hausbeck 2001; Parra and Ristaino 2001; Keinath 2007).

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Chapter Five

Characterization of *Phytophthora capsici* recovered from pepper in France.

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Will be submitted to Mycologia.

My primary contributions to it were all of the writing, DNA melting analysis, crossing, mefenoxam sensitivity screening, data analysis, DNA sequencing and mating type analysis

Abstract

Phytophthora capsici is a soilborne oomycete plant pathogen that attacks important vegetable crops. Both sexual and asexual reproduction plays a significant role in *P. capsici* population dynamics and disease epidemiology. The genetic structure varies depending on the region and our objective was to assess genetic diversity at two locations in Southern France. A total of 63 *P. capsici* isolates were recovered from roots or crowns of pepper grown in high tunnels in the Provence-Alpes-Côte d'Azur region. Both mating types were obtained from both locations and none of the isolates showed resistance to mefenoxam. Genotypic analysis using 10 SNP loci revealed a total of 35 multi-locus genotypes, of which ten were clonal lineages and 25 were unique genotypes. Clonal lineages were confined to the same locations and collection period and it appears that sexual and clonal reproduction play an important role in *P. capsici* disease

epidemic development. The implications for pathogen survival and disease management are discussed.

Introduction

Phytophthora capsici is a soilborne oomycete plant pathogen that causes crown, fruit and root rot on various vegetables which includes pepper, pumpkin, tomato and many other cucurbits (Erwin and Ribeiro 1996; Hausbeck and Lamour 2004; Tian and Babadoost 2004). *Phytophthora capsici* was first described as the causal agent of chili pepper (*Capsicum annuum* L.) blight in New Mexico in 1922 (Leonian 1922). Many *Phytophthora* species, like *P. infestans*, *P. ramorum* and *P. sojae* thrive best under wet and cool conditions. *Phytophthora capsici*, however, performs best under warm (25-28 °C) and wet conditions. Large scale disease epidemics can easily be initiated from massive production of asexual deciduous sporangia from limited number of hosts infected by the pathogen (Schlub 1983; Ristaino 1991). Sexual reproduction requires two compatible mating types (A1 and A2), which results in the production of thick-walled sexual oospores.

In the USA, *P. capsici* populations are characterized by high genetic diversity and a highly dynamic population structure where sexual and asexual reproduction play a significant role and oospores are thought to be the main overwintering mechanism (Hausbeck and Lamour 2004). Very high genetic diversity and survival of the oospore inocula outside host tissue make disease management strategies challenging. There is no single control strategy that is effective in controlling *P. capsici*. Currently, disease management relies on a combination of cultural practices, crop rotation and chemical control methods (Ristaino and Johnston 1999). The fungicides metalaxyl and mefenoxam are sometimes used to try to control *P. capsici* and in these

instances resistant isolates and populations are common (Parra and Ristanio 2001; Pennisi et al. 1998; Lamour and Hausbeck 2000; Lamour and Hausbeck 2001).

Population dynamics of *P. capsici* appears to be influenced by the different agroecologies. Our investigation of populations from Argentina and Long Island, New York showed contrasting population structure with very little genetic or genotypic diversity in Argentina and diverse populations in New York (Gobena et al. 2011; Gobena et al. 2012). In this chapter we report preliminary data on the genetic structure and biology of *P. capsici* populations in France. Our main objectives were; a) to examine genotypic diversity and mefenoxam sensitivity of the isolates obtained from infected pepper plants and b) to generate sexual progeny that will be useful in developing genetic and genomic resources for this pathogen.

Materials and Methods

Field isolates recovery.

In 2010, *P. capsici* isolates were recovered from *Capsicum annum* (pepper) grown in plastic covered high-tunnels at two locations in France. Host plants with typical *Phytophthora* root rot symptoms were uprooted and the roots were washed with water. For pathogen isolation, small piece of roots were excised from the edge of expanding lesions and transferred to dilute V8-PARP agar media (40 ml V8 juice, 3 g CaCO₃, 15 g Bacto agar and 960 ml water amended with 25 ppm pimaricin, 100 ppm ampicillin, 25 ppm rifampicin, and 25 ppm pentachloronitrobenzene) and incubated at room temperature. Hyphal tips were transferred to V8-PARP agar medium (same as above except 162 ml V8 juice was added).

Mefenoxam sensitivity screening

Isolates were tested for sensitivity to mefenoxam by placing 7 mm plugs of actively expanding mycelium at the center of a V8-PARP agar plate, amended with either 100 parts per million (PPM) mefenoxam (Ridomil Gold EC, 48% a. i.) or a control plate with no mefenoxam. The plates were incubated at room temperature for three days. Colony growth was measured and an isolate was considered resistant (insensitive), intermediately sensitive and sensitive when growth on the 100-PPM mefenoxam amended plate was more than 90%, 30-90% and less than 30% of the control, respectively (Lamour and Hausbeck 2000).

Crossing, oospore preparation and generating single oospore progeny

Sexual progeny were recovered from a laboratory cross between an A2 mating type (LT263) recovered from pumpkin in Tennessee and an A1 mating type (LT7395) recovered in 2010 from pepper in France. The cross was prepared by placing axenic plugs from each parent on the surface of V8-PARP medium 2 centimeters apart (Figure 5.1). The plates were wrapped with parafilm and incubated at room temperature in a dark cabinet for at least eight weeks.

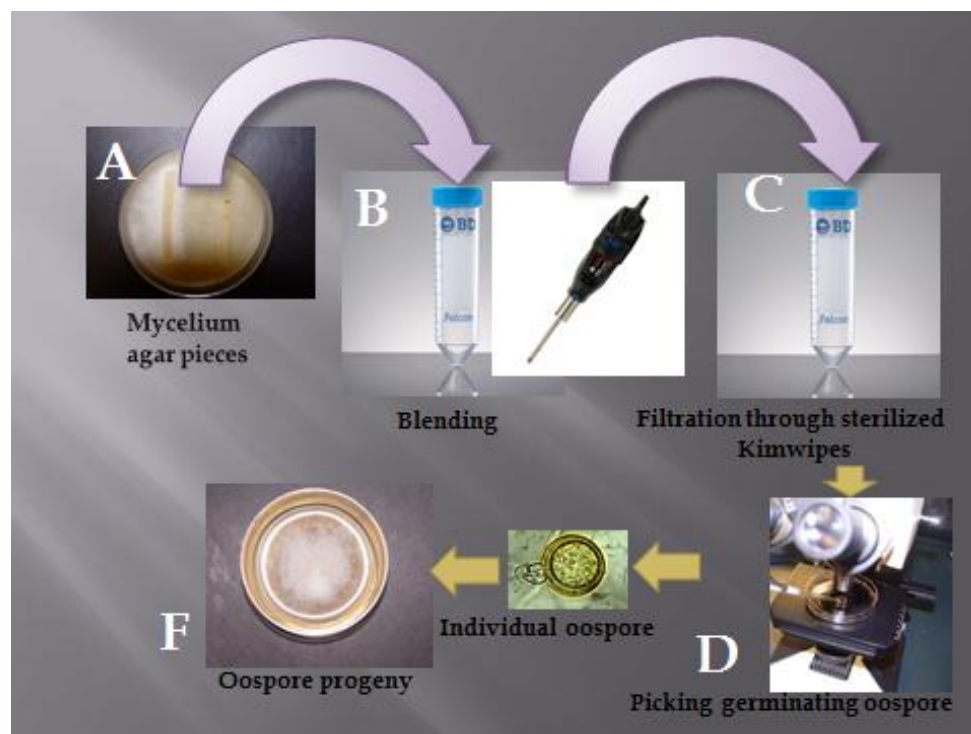


Figure 5.1 Crossing and oospore progeny preparation scheme in *Phytophthora capsici*. A, plates are incubated in the dark at room temperature for at least 8 weeks. B, oospores are sheared from the subtending mycelium using a disruption device. C, the oospore slurry is filtered and treated with enzymes for 16 – 24 hours and single germinated oospores individually transferred (D) to V8-PARP (F).

Oospore preparation

With a sterile scalpel, mycelium mats were scraped from the top layer between the two parents (Figure 5.1 A). The scrapings were put into a 50-ml disposable centrifuge tube containing 20 ml sterile water. A sterile Tissuetearor (Fisher Scientific Inc, Hampton, NH) was used to blend the oospore/mycelium/agar mixture for 1 to 2 minutes until there were no visible agar chunks in the homogenized mixture (Figure 5.1 B). The oospores were filtered through a funnel lined with a single layer of sterile Kimwipes® paper into another 50-ml tube (Figure 5.1 C). The filtered sample was checked for the presence of oospores using a microscope. Crude enzymes of *Trichoderma harzianum* (20 mg/ml) was added to the oospores mixture and incubated overnight at room temperature on a rotator or oscillating tray. Following overnight incubation, the samples

were poured into a sterile Petri dish and checked for germinated oospores (Figure 5.1 D). Germinated oospores were transferred under a stereo-microscope using a pipette to dilute V8-PARP plates or water agar amended with PARP antibiotics. After three to four days incubation at room temperature, a single hyphal tip was excised from the edge of the expanding mycelium.

Mating type and DNA extraction

Mating type was determined using *P. capsici* tester isolates A1 (CBS 121656) and A2 (CBS 121657). The statistical significance of mating type distribution for the total sample set was analyzed with a χ^2 -test. The query and tester isolate were grown together on small agar fragments prepared from dilute V8-PARP plates. Four to seven days post inoculation, the colony intersections were examined for the presence or absence of oospores using a light microscope at 400X magnification. The presence of oospores with an A1 tester parent and the absence with an A2 parent and the vice-versa was used to determine the query isolates mating type. High molecular weight genomic DNA was extracted from mycelium by the method of Lamour and Finley (2006).

SNP markers identification and primer design

Candidate Single Nucleotide Polymorphism (SNP) markers were selected from re-sequencing of single copy genes in a panel of *P. capsici* isolated that includes isolates from Peru, Argentina, France, and the USA. The sequence data was aligned and inspected manually for polymorphic positions. High resolution DNA melting analysis (RH-DMA) primers that span a single polymorphic position were designed using the LightScanner primer designing software 1.0 (Idaho Technology, Salt Lake City, UT). At least 25 bp upstream and downstream of the target

locus for HR-DMA primer should be monomorphic to avoid additional SNP in the PCR amplicon.

PCR amplification and HR-DMA

PCR amplification was accomplished in a 384-well plate (Bio-Rad, Hercules, CA) in 5- μ l reaction volumes in duplicate with a 15- μ l mineral oil overlay. Individual PCR reactions contained 10-20 ng DNA template, 0.5 μ l 10x LCGreen plus dye (Idaho Technology), 0.5 μ l 10x buffer, 0.2 μ l 5mM dNTPs, 0.05 μ l 50 mM MgCl₂, 0.1 μ l Taq Polymerase, 0.025 μ l 100 μ M forward and reverse primers and water to make a 5- μ l final volume. The PCR reactions were performed with the following conditions: the sample was melted for 2 minutes at 95°C followed by 30 second hold at 94 °C, and primer annealing and extension was at 64°C for 30 seconds followed by 35 cycles. At the end of the PCR reactions, the samples were heated to 94°C for 30 seconds and cooled to 25°C to form hetero-homoduplexes. Following duplex formation, the samples are analyzed using a LightScanner instrument (Idaho Technologies, Salt Lake City, UT) according to the manufacturer's instructions. The melting data was normalized and analyzed using LightScanner 2.0 software (Idaho Technologies, Salt Lake City, UT) and the genotypes assigned.

Results

Field isolates mating type and sensitivity to mefenoxam

Phytophthora capsici isolates were recovered from symptomatic pepper in July and November 2010. The peppers were grown in high tunnels in the Provence-Alpes-Côte d'Azur region, southern France. About 68% of the sample set included in the analysis was collected in July, 2010 and 22% was obtained in November, 2010, while the rest (10%) had been used in the

laboratory for many years. There were 34 A1 mating types and 22 A2 mating types obtained during the 2010 sampling survey. The overall χ^2 -test for mating type showed that the distribution of the mating type did not significantly deviate from a one to one ratio at a P value of 0.05. There were 18 A1 mating types and 15 A2 mating types that were unique, based on 10 multi-loci SNP markers. Furthermore, both mating types were sampled from three tunnels with more than five isolates recovered from the sampling survey. All of the isolates were tested for mefenoxam sensitivity and no resistant isolates were obtained from the screening.

Genotypic analysis with SNP markers

The genetic diversity of field isolates was analyzed with 10 SNP markers found on the different scaffolds of the *P. capsici* genome assembly (See Tables 5.1 and 5.2 for the position of the SNP loci on the reference genome sequence and the list of primers used). Based on analysis with 10 SNP markers, 35 multi-locus genotypes were identified from a total of 63 isolates. There were 25 unique genotypes that are represented by only one genotype and 10 clonal lineages with a minimum of two isolates and a maximum of 15 isolates (Figure 5.2). Two isolates with identical multi-locus SNP genotypes are considered clonal lineage and designated as CLX-Y where X is the lineage identifier and Y is the actual number of isolates. The clonal lineages obtained appear to be limited to the same tunnel, location and collection period. However, CL10-4 contains isolates from two different tunnels at the same location (Table 5.3).

F1 progeny obtained from France and Tennessee parent.

Of the 176 F1 progeny recovered and analyzed, there were 64 with the A1 mating type, 96 with the A2 mating type, and 16 progeny, which appeared to produce oospore with A1 and A2 tester

isolates. From multi-loci SNP genotyping using 9 markers, 37 unique progenies and 27 lineages with same multi-locus genotype were revealed.

Discussion

Although *P. capsici* is one of the most important diseases of pepper in France, very little is known about the epidemiology. This study was conducted to obtain a baseline understanding regarding the genetic diversity and survival mechanisms for peppers grown commercially in plastic covered high tunnels. Both A1 and A2 mating types were recovered from most tunnels. In typical field situations (e.g. Long Island, NY) this is the hallmark of outcrossing populations (Dunn et al. 2010; Gobena et al. 2011). Although our sample sets from individual tunnels are small, the overall mating type distribution was not significantly different from a 1:1 ratio. Crossing an isolate from this area of France with a known fecund isolate from Tennessee resulted in copious oospore production, many of which germinated. The relatively few SNP markers applied to this cross clearly showed that many of the progeny are products of meiosis and sexual recombination. The progenies are recombinant and will be valuable resource for dense linkage map construction and differential reaction to host infection that could lead to identification of important genes for virulence mechanisms.

Further analyses of the isolates recovered from the high tunnels using 10 SNP markers revealed that 35 of the 63 isolates had unique multi-locus genotypes (Table 5.1). This suggests that sexual recombination and oospore production are an important part of the epidemiology in this area. In addition, it is clear that clonal reproduction also plays an important role. Clonal spread in this scenario is particularly interesting because the importance of environmental factors, particularly

free water, is well understood (Geuens et al. 2007; Granke and Hausbeck 2010). Compared to a field situation where rainfall is impossible to control, the high tunnel should allow a more precise monitoring and distribution of water. Tighter control of water in the high tunnels may allow dispersal to be limited.

Table 5.1 Single Nucleotide Polymorphism (SNP) markers used to characterize *P. capsici* isolates. The top four markers were used for both field isolates and laboratory progeny sets while the middle six rows and the bottom five rows were used for the field isolates and the progeny sets respectively. The F1 progenies are from a cross between LT7395 (A1, France) and LT236 (A2, Tennessee).

SNP ID ¹	Gene model	Scaff size	Protein ID	Predicted Change
*Sc22_655493	Scaffold_22:653551-657040	843021	534326	C>T Arg363Cys
*Sc71_157951	Scaffold_71:156154-158367	252329	510944	A>G Gly577Gly
*Sc72_72952	Scaffold_72:70985-74119	230452	510981	5'UTR
*Sc25_680731	Scaffold_25:678612-681126	841216	507189	C>T Gly685Gly
**Sc121_54954	Scaffold_121:54032-56025	70414	512063	A>G Asn358Ser
**Sc1_85501	Scaffold_1:84814-86097	2170955	531455	G>A Arg248Arg
**Sc1_118179	Scaffold_1:117955-119032	2170955	537964	G>A Ser75Ser
**Sc3_1585784	Scaffold_3:1584751-1586162	1611504	503450	T>C Arg346Arg
**Sc17_25167	Scaffold_17:22114-25606	985188	505928	G>T Ser997Ile
**Sc28_334146	Scaffold_28:332888-334752	757753	507525	G>T Pro402Pro
***Sc19_634212	Scaffold_19:634212-634424	944885	110976	C>T Asp71Asp
***Sc39_343615	Scaffold_39:342193-344049	591971	508875	C>T Ser145Ser
***Sc82_344529	Scaffold_82:33595-36807	207552	556035	T>C Pro500Pro
***Sc7_1020180	Scaffold_7:1018621-1020309	1368812	102611	T>C Val177Ala
***Sc6_1069343	Scaffold_6:1067057-1069710	1423605	526040	A>G Arg114Arg

¹ Nucleotide location in the *P. capsici* reference genome. Sc = scaffold followed by the SNP location.

*The Markers used for analyzing the field isolates and the F1 progenies

**Markers used only field isolates

***Markers used only for F1 progenies

Table 5.2 High resolution DNA melting analysis (HR-DMA) primers used to genotype isolates of *Phytophthora capsici* from France.

SNP ID	Forward primer	Reverse primer	Annealing Temp in °C Forward/Reverse
Sc22_655493	ATTGACACAGCGCTATTC	CAGCTCCTTGTACCGCTTA	50.1/54.4
Sc71_157951	CAGCAGCGATATGATCA	CATCATGGCGTGAAGCG	48.9/54.4
Sc72_72952	CTGTGCTTCAGCGCATATTA	CATCTGCTGTTGAAGACGAC	53.0/53.6
Sc25_680731	CAGTGCGCAGTGTGGGA	ATCGTCCATCTCCTTCACT	58.5/52.7
Sc121_54954	TCCAAATCGAGCGGCTACA	TTCGAGGACTCACCAAGAAT	56.5/53.3
Sc1_85501	GAAGAGGCCTCAGGGAT	CAAACCTCATAAAGGACTTGCCA	53.5/53.3
Sc1_118179	CTTTGCCGGTGTTTTCAAT	GTATCCCTTGGGCAGCTC	51.7/55.5
Sc3_1585784	CGAAACTCACGGACTTTGG	TCATGGTCGTGGTGTCCGAG	53.7/54.1
Sc17_25167	CGAACGACTTAACGGGT	CTTCATCTTCCACGTCCT	51.9/51.8
Sc28_334146	ACCCGTCAGCAACACAT	TCGAACGGTACAGCTTA	54.4/50.0
Sc19_634212	CTGATCCGGATAAACGTAAA	CTCCAAACATACGAGGGTC	50.8/53.3
Sc39_343615	GAAGCTCAGTTCTCGTCATTT	GTCTACGTGCATGGACATC	52.8/53.1
Sc82_344529	TGGGCACATGCGAATAC	GCCGTCCTTCATAATCAGT	52.8/52.0
Sc7_1020180	GTGGCTTTATGGCAGGC	GCCTTTACCATCACAAGTACC	54.2/53.4
Sc6_1069343	GACAAGAAGACGAAGGAGG	CATGACGTAAATCTGATTACATTG	54.1/52.6

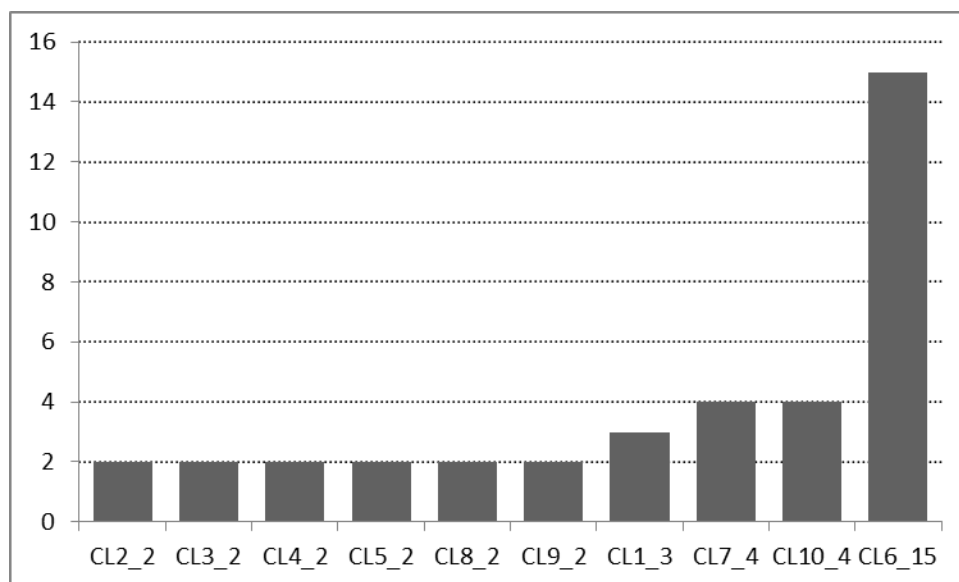


Figure 5.2 Clonal lineages of *Phytophthora capsici* isolates recovered from pepper in France. Clonal lineages are designated as CLX-Y where X is the lineage identifier and Y is the actual number of isolates.

Table 5.3 Summary of *P. capsici* isolates recovered from pepper in France.

Location	Specific Location	Clonal lineages	Nº isolates (Nº unique genotypes)	Unique genotypes A1:A2 ratio
Eygalieres	DRT1	CL9-2 & CL10-4	3(2)	0:1
Eygalieres	DRT3	CL2-2 & CL10-4	14(11)	2:9
Eygalieres	DNT9	CL6-15, CL7-4 & CL8-2	25(7)	4:4
Eygalieres	PC	CL1-3	3(2)	2:0
Sénas	HCT1	Unique	1(1)	1:0
Sénas	HCT3	CL1-3	7 (7)	6:1
Sénas	HCT4	CL5-2	3(2)	2:0
Laboratory	NA	CL3-2, CL4-2	7(5)	5:0

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Chapter 6

Conclusions

The heterothallic oomycetes pathogen *Phytophthora capsici* causes significant economic losses on many vegetable crops. Once introduced to a new location, it is very difficult to control and essentially impossible to eradicate. In the US, the persistence is thought to be due to production of thick-walled sexual oospores. This has been shown primarily using molecular data, although oospores have been found in the tissue of infected fruit. A first detailed analysis of populations in Southern France suggests the situation there may be similar to that in the US and oospores may play an important role in survival. This is not the case everywhere and despite similarly invasive and persistent life history, the molecular data suggests the oospore is not always necessary to allow long term survival. This is illustrated by previous studies in Peru and, outlined here, Argentina. Further work is needed to determine how clonal lineages are able to be spread on such large scales and are able to persist for multiple years. Even without knowing how clonal lineages are surviving and spreading in South America this data may prove useful to vegetable breeders who are working to develop novel varieties useful in these areas. Screening will focus the extant lineages.

One of the objectives of this dissertation was to develop an efficient and effective way to assay single nucleotide polymorphisms (SNPs) markers for population studies and linkage mapping. Even though SNPs are highly abundant in the *P. capsici* genome, identifying useful marker for populations' genetic analysis is challenging. The biggest challenge is presented by their abundance. All SNP typing approaches require assessment of a discrete polymorphic site – something not always easy to find in the *P. capsici* genome. Although this needs to be tested

further by assessing the SNP sites in additional populations, it appears that *P. capsici* is one of the most SNP-rich eukaryotic organisms reported to date. We developed a strategy that utilizes high-resolution DNA melting analysis (HR-DMA) to assess genotypes. HR-DMA is cost effective and fast, it is nonetheless challenging due to the fact that not all polymorphic sites are known and analysis of new populations often requires the development of new markers. For sexual progeny this approach is very useful as the markers can be selected without the problem of unknown SNPs in a field population. It is likely that the cost of DNA sequencing may become so cost-effective that any type of SNP genotyping approach will be unnecessary and the whole genome can be assessed for all members of a cross or population.

Vita

Daniel Jara Gobena was born and raised in Najo in western Oromia regional state, Ethiopia. He completed his elementary and high school education in his native country. He also received a BSc. degree in Plant Sciences in 2000 from Haramaya University (used to be Alemaya University), Ethiopia. In March 2001, he joined The Ethiopian Institute of Agricultural Research (EIAR) and worked on Tef improvement research at Debre Zeit center for four years. In August 2004, he joined Wageningen University, The Netherlands and graduated in MSc in Plant biotechnology in 2006. He worked again for a year at EIAR. He began his Ph.D. in the Genome Science and Technology graduate program at the University of Tennessee in August 2007. He completed his studies in May 2012.