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To the Graduate Council:

I am submitting herewith a dissertation written by Jonathan Patrick Hulvey entitled "Investigating Genetic Diversity of Phytophthora spp. and Related Oomycetes." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Kurt Lamour, Major Professor

We have read this dissertation and recommend its acceptance:

Albrecht VonArnim, Alison Buchan, Todd Reynolds

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

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

To the Graduate Council:

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We have read this dissertation
and recommend its acceptance:

Alison Buchan

Albrecht Vonarnim

Todd Reynolds

Accepted for the Council:

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Investigating Genetic Diversity of *Phytophthora spp.* and Related Oomycetes

A Dissertation Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Jonathan P. Hulvey

August 2010

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Abstract

Oomycetes, like fungi, are filamentous heterotrophs, but unlike true fungi are diploid and share a photosynthetic ancestor. Many of these organisms are plant and animal pathogens, and members of the genus *Phytophthora* cause devastating disease on a diverse array of agricultural plant hosts. Several diverse topics in oomycete biology are investigated in this dissertation. Chapter 2 is a report on loss of heterozygosity in *Phytophthora capsici* in response to chemical mutagenesis.The research presented in Chapters 3 and 4 are centered on documenting biodiversity and genetic diversity of populations of *Phytophthora* species obtained from infected plant hosts. The final chapter (Chapter 5) involves determining genetic diversity, ecology, and enzymatic activities of Pythiaceous oomycetes from marsh wetlands of the southeastern US.

Table of Contents

Chapter 1- Introduction…..……………………………………………………………………1

Chapter 2- Loss of heterozygosity in *Phytophthora capsici* following N-ethyl-nitrosourea Mutagenesis…………………………………………………………………………………...16

Chapter 3- Co-occurrence and genotypic distribution of *Phytophthora* species recovered from watersheds and plant nurseries of East Tennessee ……………..……………………………….35

Chapter 4- A clonal genotype of the pepper pathogen *Phytophthora capsici* dominates pepper pathosystems in Peru across the Andes Mountains (In progress)………………………………..60

Chapter 5- Pythiaceae of southeastern estuarine marsh grasses: Occurrence, genetic characterization, and degradative capabilities…………….....………………………………….78

Vita…………………………………………………………………………………………..100

List of Tables

Table 2-1. Summary of LOH events for three loci screened in LOH mutants…………………..23

Table 2-2. Oligonucleotide primers used in this study……………..…………………………25

Table 3-1. Summary results for stream baiting and nursery survey……………….…………47

Table 3-2. Isolates from *Phytophthora* species exhibiting overlap among watershed and nursery sites……………………………………………………………...………………………49-50

Table 4-1. Summary information for high resolution DNA melting analysis (HR-DMA) markers used to genotype isolates of *Phytophthora capsici*…………………………………………....66

Table 4-2. Isolate summary information………………………………………………………...68

Table 4-3. Summary of the distribution of *Phytophthora capsici* genotypes……………………69

Table 4-4. Summary of SNP genotypes for nine clonal lineages of *Phytophthora capsici* recovered from Peru……………………………………………………………………………...71

Table 5-1. Summary of collection locations for isolates characterized in this study……………83

Table 5-2. Summary of taxa isolated in this study………………………………………………87

List of Figures

Figure 1-1. Stepwise (1 through 7) evolutionary pathway demonstrating the chromalveolate hypothesis……………………………………………………………………………………3

Figure 1-2. Life cycle of *Phytophthora infestans*...5

Figure 2-1. PAGE TILLING gel image of the locus 3 amplicons of ENU treated *P. capsici* mutants……………………………………………………………………………………...21

Figure 2-2. Electropherograms that display bidirectional switching to homozygosity at locus 3 in *P. capsici* ENU mutants…………………………………………………………………27

Figure 2-3. DNA melt curves of locus 3 PCR amplicons from 192 *P. capsici* ENU mutants…..28

Figure 3-1**.** Tennessee state map with watershed sample sites TN1-TN8 indicated by stars……41

Figure 3-2. DNA melting analysis curves of ITS1 amplicons from three frequently encountered oomycetes in this surveys……………………………………………………………………..45

Figure 4-1. Map of collection sites surrounding Oxapampa…………………………………….64

Figure 4-2. (A) Healthy rocoto fruit and (B) fruit infected with *Phytophthora capsici*................73

Figure 5-1. Map of collection sites one through seven, from Darien, GA and three barrier islands of coastal Georgia……………………………………………………………………………..84

Figure 5-2. Neighbor joining phylogenetic tree of *Pythium* and *Halophytophthora* taxa and top BLAST hits……………………………………………………………………………………...88

Figure 5-3. Agar plate assay plates after staining………………………………………………..89

Figure 5-4. Sporangium of *Halophytophthora sp*.1 isolate……………………………………...…92

Chapter One

An Introduction to the Oomycetes and *Phytophthora*

In 1865 using crude microscopy techniques, Anton DeBary discovered that the etiology of late blight of potato involved a fungal-like microbe, and aptly named it *Phytophthora*, greek for “plant destroyer”. This was the first of the oomycetes to be formally described, and since then nearly 100 species of these plant pathogens have been added to the genus *Phytophthora* (Erwin and Ribeiro 1996, Blair et al. 2008). Over 800 species total have been added to the oomycetes, some of which are also economically important pathogens of agricultural plants, and also algae, fish, crustaceans, mollusks, amphibians, and insect larvae (Marino et al. 2009, Sekimoto et al. 2008, Woliska et al. 2008, Wicker et al. 2001, Martin 1981, Muraosa et al. 2009, Vennerstrom et al. 1998). Most oomycetes exhibit filamentous growth, and thus were considered true fungi from the time of their discovery up until advancements in microscopy, DNA sequencing technologies and molecular phylogenetics allowed their evolutionary affinity with algae to be determined. More recently, the kingdom Stramenopila was erected to accommodate these organisms in a kingdom separate from fungi, and united with the algae (Dick 2001). Subsequent revision resulted in a new Superkingdom, the Chromalveolates, which included the oomycetes along with the chlorophyll a and c containing algae and the apicomplexans (Tyler et al. 2006). An abundance of evidence in the form of genome sequence data also exists to support a shared ancestory between the oomycetes and algae (Fig. 1-1). Tyler et al. 2006 summarize the presence and sequence similarity of hundreds of genes involved in photosynthetic pathways contained in the genomes of *Phytophthora* species. These gene sequences serve as the evolutionary footprints of an algal progenitor, and are thought to have originated from a red algal symbiont, the genome of which gave rise to the nucleomorph which many chromalveoaltes still possess (Fig. 1-1). The analysis of Tyler et al. 2006 supports an evolutionary hypothesis whereby photosynthetic capabilities of an oomycete progenitor were lost in favor of an exclusively heterotrophic lifestyle

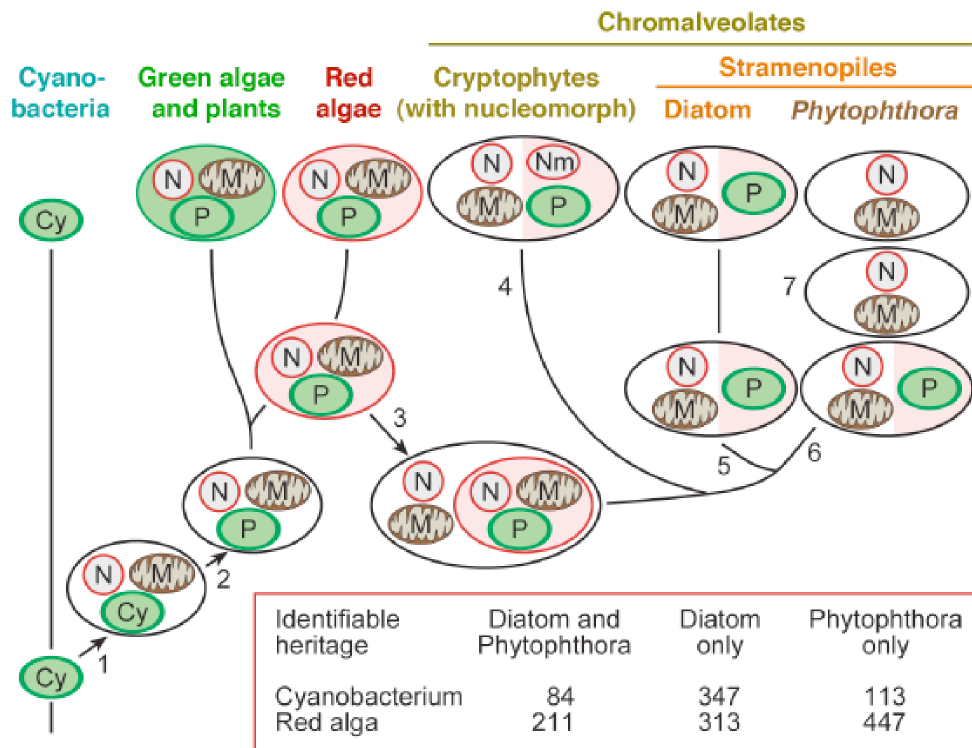

Figure 1-1. (from Tyler et al. 2006 supplementary material). Stepwise (1 through 7) evolutionary pathway demonstrating the chromalveolate hypothesis. Genome sequence data indicate that the ancient ancestor of *Phytophthora* once harbored a red algal photosynthetic symbiont (step 5), confirming the chromavelate hypothesis for the oomycetes. Evidence of gene transfer from an algal symbiont to *Phytophthora* is indicated by the number of genes shared by major lineages of chromalveolates with cyanobacterium and red algae (indicated in the box outlined in red). Subcellular structures are indicated with abbreviations: M for mitochondria, P for plastid, N for nucleus, and Nm for nucleomorph.

(Fig. 1-1).

The asexual life cycle of *Phytophthora* and many algae involves the production of a biflagellate zoospore, with a forward flagellum covered in mastigonemes (tubular hairs), and a posterior whiplash flagellum that beats in a sinusoidal wave. Given the proper environmental cues sporangia and zoospores can be produced in great abundance, with one estimate indicating three million asexual spores could be produced on a single squash infected by *P. capsici* (Lamour 2009). The presence of this stage in the life cycle stresses the importance of water in the dispersal of these pathogens. *Phytophthora* zoospores have been demonstrated to be chemotactic and electrotactic, allowing them to detect molecular components of plant host tissues along concentration gradients in the surrounding environment (Tyler 2002, VanWest 2002). Upon zoospore release, time is limited as zoospores have limited food reserves, do not possess cell walls, and are thus not impervious to dessication. Once an appropriate food source such as host tissues, are located, the spores encyst, and germinate. Germination involves the production of a germ tube hypha, which is capable of penetrating and further ramifying through plant host tissues with which it comes in contact (Fig. 1-2). Turgor pressure generated by hyphal growth contributes from 1 to 50% of the force of invasive growth into host tissues by oomycete hyphae, and thus, digestive enzymes are also required to weaken and overcome host cellular barriers (Money et al. 2004). Oomycetes like *Phytophthora* do share with fungi a similar mode of heterotrophy, known as absorptive nutrition. This means that plant pathogenic oomycetes, like plant pathogenic fungi, not only exhibit filamentous growth, allowing them to invade and grow within host tissues, but also excrete digestive enzymes, such as proteases, cellulases, pectinases (and a multitude of other degradative enzymes) into the surrounding host tissues (Boudjeko et al. 2006). The degradation products that these enzymes aid in forming from host tissues are next

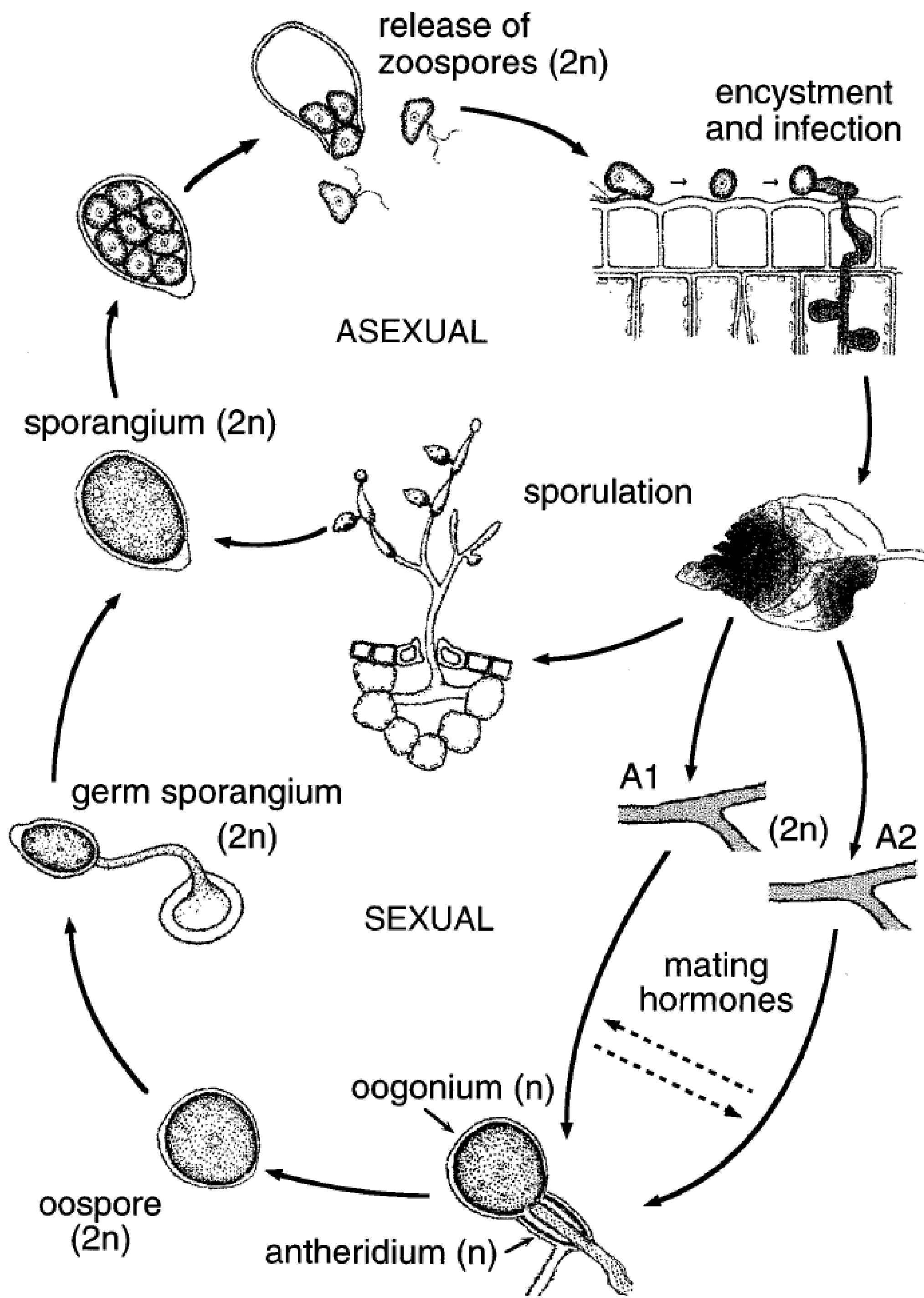

Figure 1-2. Life cycle of *Phytophthora infestans* (from Judelson et al. 1996).

(and a multitude of other degradative enzymes) into the surrounding host tissues (Boudjeko et al. 2006). The degradation products that these enzymes aid in forming from host tissues are next absorbed into the hyphae via transport proteins and passive diffusion. A number of these secreted enzymes have shown significant sequence similarity to those of true fungi and actinobacteria, leading to speculation that these genes originated from lateral gene transfer events between the ancestors of oomycetes and fungi and bacteria (Andersson et al. 2006, Belbahri et al. 2008).

Early investigations of *Phytophthora* and oomycete sexual morphologies also indicated an evolutionary affinity with the alga, and like some algae, the sexual portion of the Oomycete life cycle involves the production of male and female gametangia, the antheridium and oogonium, respectively. The production of these organs is stimulated by the presence of a recently characterized mating hormone and metabolite of the isoprenoid pathway (Qi et al. 2005). Meiotic divisions occur in the antheridia and oogonia (Fig. 1-2), to give rise to haploid nuclei. During plasmogamy of self-infertile species, the antheridium and the oogonium of compatible mating types A1 and A2, are united, allowing karyogamy to occur between the haploid nuclei of the oogonia and antheridia (Fig. 1-2). The result is a recombinant progeny cell in the form of the diploid oospore, which may persist in the environment through the winter season, and withstand dessication. These features are not shared by the asexual zoospore stage and offer oomycetes such as *Phytophthora* the ability to persist in the environment over winter and also the ability to generate novel genotypic diversity. In turn, recombinant genotypes may display superior fitness with regards to enhanced fecundity, infection capabilities, and pesticide resistance (Lamour 2009). There are a number of oomycete species that are not known to complete the sexual portion of the life cycle, and appear to rely exclusively on zoospore, mycelium, and/or resistant asexual spores for reproduction. These include more recently diverged species such as

Phytophthora citrophthora, as well as all members of the basal clades of oomycetes (Edwin and Ribeiro 1996, Beakes and Sekimoto 2009).

Documenting the genetic diversity of oomycete pathogens is currently an expanding realm of plant pathology research. Understanding trends in genetic diversity of oomycetes is important for hypothesizing mechanisms of evolution and speciation and can also be informative for hypothesizing on anthropomorphic and environmental vectors of dispersal of these destructive pathogens. Genotyping techniques have allowed investigators to track the dissemination of clonal lineages from source points, such as for *P. ramorum* in the nursery trade in the U.S. (Ivors et al. 2006). Nationwide efforts are underway to document the spread of *P. ramorum* in the US via nursery plants, since its discovered arrival from infected plant material from Europe (Stokstad 2004). *Phytophthora ramorum* has caused oak forest die back in California, and also is known to infect a very broad host range of woody shrubs and trees (Yakabe 2009). Efforts to track this pathogen have also resulted in survey data of *Phytophthora* species and genotypes that are distributed in the nursery trade throughout the US (Donahoo et al.2008, Yakabe et al. 2009, Chapter 3). These surveys have indicated several species to be common on woody ornamental plants, and genotyping has indicated that there are mefenoxam resistant putative clonal genotypes circulating through the nursery trade and within native ecosystems of East Tennessee (Hulvey et al. 2010B, Chapter 3).

The generation of novel genotypes via sexual selection gives oomycete pathogens the ability to overcome selective factors in the environment which serve to limit their establishment in the field. These factors include plant host resistance and pesticide (typically mefenoxam) application (Lamour 2009). Oomycetes such as *Phytophthora capsici* have been shown to undergo high levels of sexual recombination in North American field settings (Lamour and

Hausbeck 2001A, Lamour and Hausbeck 2001B, Ristaino 1990). It is therefore curious to find that a clonal genotype of *P. capsici* is distributed all along the coast of Peru over the course of at least three years (Hurtado-Gonzales et al. 2008). This is the only report of a clonal lineage of *Phytophthora capsici* exhibiting widespread occurrence, and raises questions pertaining to how this pathogen has been dispersed. Detecting clonality in geographically disparate populations of *Phytophthora* species has been an area of interest for some time, though the molecular markers that have been utilized differ widely. Previous studies have incorporated microsatellite markers, amplified fragment length polymorphism (AFLP), as well as randomly amplified polymorphic DNA (RAPD) markers in tracking clonal lineages of *P. ramorum* (Ristaino 1990, Ivors et al. 2006, Lamour and Hausbeck 2001A). More recently, a genotyping tool of several population studies of *Phytophthora* species, including *P. capsici*, has been done using SNPs (single nucleotide polymorphisms) (Hurtado-Gonzales and Lamour 2008, Niepold 2005, Chapter 3). Population studies have found SNP genotyping of six markers to concord with genotype designations made with over 40 AFLP markers (Hurtado-Gonzales et al. 2008, Chapter 4). Though clonal lineages exist, and appear to be relying on the asexual reproduction exclusively, asexual clonal lineages may also give rise to novel genetic variation. This has been shown for *P. sojae,* which undergoes mitotic gene conversion following strain specific crossing (Chamnanpunt et al. 2001). Additionally, loss of microsatellite alleles has been reported for *P. cinnamomi* following repeated subculturing (Dobrowski et al. 2002). Mutagenizing agents present in the environment may also result in novel genotypes, as it has been shown that chemical mutagenesis of *P. capsici* results in loss of heterozygosity (Hulvey et al. 2010A).

Incorporating a large number of SNP markers into a *Phytophthora* species population study is only feasible in the post genome era in which we find ourselves. Genome sequences

have been published for four diverse *Phytophthora* species, *P. ramorum*, *P. sojae*, *P. infestans*, and *P. capsici* (Lamour et al. 2007). These genome sequences have allowed for not only enhanced marker development capabilities, but also rendered this organism amenable to global protein analysis, termed proteomics. Proteins have been identified that are expressed during specific life stages and during the infection process, and conserved and divergent proteins identified among the species for which genome sequence is available (Savidor et al. 2008). With continued successes in post genomic era investigations of *Phytophthora*, it is hoped that this group of plant pathogens will reach the status and priority afforded to of other model organisms, such as *Drosophila* and *Arabidopsis*.

The following studies aim to further current understanding in several areas of *Phytophthora* and related oomycete research. These areas include response to mutagenesis (Chapter 2), diversity of Pythiaceae from marsh grasses (Chapter 5), and genotypic diversity within natural populations of *Phytophthora* plant pathogens (Chapters 3, 4).

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Chapter Two

Loss of heterozygosity in *Phytophthora capsici* following N-ethyl-nitrosourea mutagenesis

This chapter is modified from a paper published in Mycologia:

Hulvey, J., Young, J., Finley, L., K. Lamour. 2009. Loss of heterozygosity in *Phytophthora capsici* following N-ethyl-nitrosourea mutagenesis. Mycologia, 102: 27-32.

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

Abstract

Loss of heterozygosity (LOH) occurs in a variety of diploid organisms following chemical mutagenesis and was observed in the vegetable pathogen *Phytophthora capsici* following N-ethyl-nitrosourea (ENU) mutagenesis at three loci during reverse genetic screening. Our objectives were to determine (i) the frequency of LOH among mutants, (ii) the directionality of the LOH events, and (iii) the length of the genomic tracts exhibiting LOH. Of the 1152 ENU mutants screened, LOH was most frequent at the locus 3 (99 ENU mutants), with locus 1 (10 ENU mutants) and locus 2 (9 ENU mutants) undergoing LOH at similar frequencies. The LOH was bi-directional for all three loci, with locus 3 mutants biased towards one haplotype. Analysis of upstream and downstream heterozygosity indicates that the LOH events spanned up to at least 4.6 kb. The implications of mitotic recombination and LOH for reverse genetics and natural variation in *Phytophthora* are discussed.

Introduction

Loss of heterozygosity (LOH) is reported to occur in the genomes of a variety of diploid organisms in response to chemical mutagenesis (Tsang et al. 1999, Helleday 2003). Mitotic recombination following exposure to genotoxic agents can result in LOH by mitotic crossing

over or mitotic gene conversion, which is the non-reciprocal transfer of DNA sequence between homologous DNA duplexes during mitotic recombination (Petes *et al*. 1991). In *Saccharomyces cerevisiae* and the pathogenic yeast *Candida glabrata*, mitotic gene conversion results in mating type switching (Wu *et al*. 2005) and also occurs as a result of exposure to mutagenizing agents (Freeman *et al*. 2007). Mitotic recombination has been routinely induced via N-ethyl-nitrosourea (ENU) mutagenesis in the genomes of fruit fly, zebra fish, and mouse (Ayaki et al. 1990, Moore et al. 2006, Chen et al. 2002).

Mitotic recombination following chemical mutagenesis has not been reported for *Phytophthora spp*., but LOH has been documented and is thought to be responsible for some of the genetic diversity found in *Phytophthora spp.* and *Pythium ultimum* (Carter et al. 1999, Francis et al. 1994, Dobrowlski 2002). Most reports of LOH in *Phytophthora* and *Pythium* involve aberrent segregation of alleles following crosses in *Pythium ultimum, Phytophthora parasitica, P. infestans, and P. cinnamomi* (Carter et al. 1999, Francis et al. 1994, Dobrowlski 2002). There are fewer published reports of LOH occurring in *Phytophthora* following mitosis. Loss of microsatellite alleles during the course of successive subculturing is reported for *P. cinnamomi* (Dolbrowski et al. 2001). Mitotic instability is suggested to account for the LOH, and a colony sectoring phenotype was linked to loss of alleles. A similar phenomenon was reported for *P. sojae*, following strain specific crossing and the generation of recombinant progeny. Sectoring was observed in single spore derived progeny and it was shown that markers surrounding the avirulence locus, *Avr1b* had switched to homozygosity (Chamnonpunt et al. 2001).

There are no published reports on the genomic consequences of chemical mutagenesis in *Phytophthora*. Ultraviolet (UV) irradiation has been utilized to mutagenize *P. dreschleri* and *P.*

capsici, giving rise to auxotrophic mutants, though the genetic basis for the auxotrophic phenotypes remains unknown (Castro 1971). Mutagenesis by ENU has recently been applied to *Phytophthora spp*. to introduce point mutations for reverse genetics (Lamour et al. 2006). ENU is a genotoxic DNA alkylating agent frequently used for reverse genetics, as it is known to induce point mutations with high frequency. As with other DNA alkylating mutagens, ENU also induces mitotic recombination and LOH as a consequence of DNA damage and repair mechanisms (Ayaki et al. 1990, Moore et al. 2006, Chen et al. 2002). The reverse genetics technique, TILLING (Targeting Induced Local Lesions In Nuclear Genomes), utilizes a single strand specific endonuclease (CELI) to cleave heteroduplexed PCR amplicons at the sites of heterozygosity, and this technique is performed as a high throughput strategy for detecting ENU mutagenesis-induced single nucleotide point mutations in targeted genes of *Phytophthora spp*. (Lamour and Finley 2006). Thus far only three loci have been screened from *P. capsici* using TILLING and LOH was observed for each locus: locus 1, a proposed Phospholipase D homolog (*PLD*), locus 2 encoding a gene exhibiting up-regulated expression during the germinating cyst life stage, and locus 3, encoding a gene with homology to the *Dicer* gene involved in the eukaryotic RNAi pathway of mRNA degradation.

Our objective was to characterize the rate and breadth of ENU-induced LOH events in *P. capsici*. We report the rates of LOH in ENU mutagenized and non-mutagenized *P. capsici* isolates at three loci, the length of genomic tracts that convert to homozygosity following ENU mutagenesis, and finally, the directionality of conversion to homozygosity.

Materials and Methods

Mutagenesis, TILLING, and Detection of LOH

Two *Phytophthora capsici* isolates were used in this study: CBS121656, and LT 1534. The isolate CBS121656 was recovered from a cucumber fruit in Michigan in 1997, and is an A1 mating type. Isolate LT 1534, an A2 mating type, was obtained through crosses and backcrosses involving CBS121656 and CBS121657 (Hurtado and Lamour 2009). Zoospores of *Phytophthora capsici* CBS121656 and LT 1534 were treated with ENU and libraries of mutants and mirror libraries of genomic DNA were produced as described previously (Lamour and Finley 2006). Primers were designed to amplify approximately 700 to 900 base pair targets from the three loci presented here. These genes were selected by members of the Oomycete molecular genetic research community. The genomic DNA library was screened as described previously (Table 1-1) (Lamour and Finley 2006). An assembly of the *P. capsici* genome is available from the Joint Genome Institute (v1.0), although due to the high level of heterozygosity a finalized assembly and detailed annotation is still in progress. On the version 1.0 assembly locus 1 is found on scaffold 5 (Gene ID: CBOT4083, base pair coordinates 571655 to 572458) locus 2 is found on scaffold 23 (Gene ID: CBOU4545) base pair coordinates 126094-126815), and locus 3 is found on scaffold 5 (No gene ID, base pair coordinates 1509630 to 1510547). Briefly, target regions were amplified using differentially labeled forward and reverse primers to produce amplicons with different fluorophores on each end of the amplicon. The amplicons were heteroduplexed and incubated in the presence of CELI enzyme, which will cut at the 3' end of a single basepair missing the distinctive wild type banding patterns produced by the presence of pre-existing heterozygosity (Fig. 2-1). 1152 ENU mutants were screened per locus

Confirmation of LOH and induced SNPs

Loss of heterozygosity at the loci was confirmed by re-amplifying and sequencing the PCR amplicons from mutants that did not display the wild type fragment pattern. Primers for

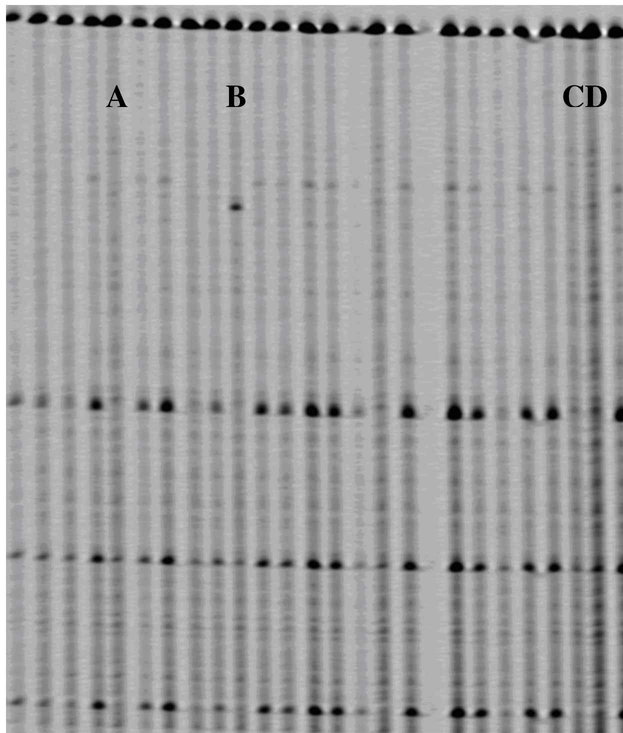

Figure 2-1. PAGE TILLING gel image of the locus 3 amplicons of ENU treated *P. capsici* mutants. The lanes marked as A, C, and D have lost all heterozygosity. Surrounding lanes with distinct bands indicate presence of wild type heterozygous alleles. The lane marked as B shows a mutant lacking heterozygosity, except for a single ENU induced point mutation, indicated by a single novel fragment.

amplification of these three loci from genomic DNA were designed using Primer3 (Rozen and Skaletzsky 2000). Primers were designed using the following criteria in Primer3 (http://fokker.wi.mit.edu/primer3/input.htm): Primer size: Min. 20, Opt. 24, Max. 27; Primer Tm: Min. 67, Opt. 70, Max 73. Amplicons were amplified using the PCR protocol from Lamour and Finley *et al*. (2006), and submitted to the University of Tennessee Molecular Biology Resource Center. Amplicons were sequenced in both directions.

Identification of LOH in non-mutagenized isolates

To test if LOH occurs in non-mutagenized *P. capsici* mitotic progeny, 192 single zoospore colonies were generated from *P. capsici* isolates CBS121656 and LT 1534. These isolates were screened for LOH at the three loci using TILLING. DNA was extracted using methods described in Lamour and Finley (2006). The TILLING gels were analyzed visually.

Length of genomic tracts exhibiting LOH in ENU mutants

Genomic regions up and or downstream of loci were sequenced to test if additional naturally occurring heterozygous sites converted to homozygosity in the LOH mutants. These regions were selected by aligning genomic 454 raw DNA sequence reads (http://capsici.ncgr.org/) and identifying regions with heterozygosity in LT1534 which is the strain that was used for genome sequencing. Putative polymorphic regions were re-sequenced in the wild type isolates to identify areas with pre-existing heterozygosity. Sequencing primers are listed in Table 2-1.

Genotyping LOH at locus 1 using DNA melting analysis

A DNA melting assay (DMA) was optimized to determine the directionality of conversion to homozygosity among ENU mutants exhibiting LOH at locus 3. The DMA was utilized to screen 192 *P. capsici* ENU mutants and differentiated either of the homozygote alleles from one another and from heterozygous ENU mutants. Locus 3 was earlier determined that

Table 2-1. Oligonucleotide primers used in this study. Forward and reverse primers are listed in 5′ to 3′ orientation. Sizes of loci are presented in number of base pairs.

Target Locus	Size in bp	Forward Primer	Reverse Primer
Locus 1	803	TGGCAATCTGGCGTCTGCCTCTAT	GGCACGAGGCACTTAGGGAGGAAT
Locus 2	721	TCATCGGCGACTGTCCGTTGAG	CGTTCAACGTTGTGGTCGCAGTG
Locus 3	917	AGTCGTTTCCGAGCCACGACTGAC	AGTTTTGGCCGCAGCTGTGAGTTC
Locus 3 DMA	56	GTTCTGGGCTTCCTTGTG	GCGTCTGGAGTCTGAAA
Locus 1 scaffold	823	CACGTGCAGACAGCAGCCACAGTA	CTGACGGGTCGTTACTCAGCGACA
Locus 2 scaffold	637	TCGAAACTCAACGCCCGGACA	CGGCTCAACAGCGTCCAAACA
Locus 3 scaffold	779	CTTGAGGACCATTGGCATCGCACT	AGGTGCCACGGGTAAGTTTGAG

this locus underwent LOH in approximately 1 in 10 ENU mutants (Table 2-2). Primers were designed using the LightScanner primer design software (Idaho Technologies, Salt Lake City, UT) to amplify a 50 bp (base pair) amplicon that spanned a single heterozygous site in WT CBS121656 (See Table 2-1 for primer sequences). PCR reactions for DMA consisted of 4 µl Lightscanner Mastermix (Idaho Technologies, Salt Lake City, UT), 1 µl of genomic DNA at 50 to 150 ng/µl, and 1 µl of each primer at 2.5 µM conc. The PCR temperature protocol was as follows: initial denaturation at 95 C for 2 min., then 45 cycles of 95 C for 30 s and 64 C for 30 s, and then a final step of 95 C for 30 s followed by 28 C for 30 s. DMA was performed according to manufacturer's instructions using a 384 well format Lightscanner instrument (Idaho Technologies, Salt Lake City, UT). PCR reactions were replicated on the 384 plate to ensure reproducibility. Data analysis and normality parameters were adjusted using LightScanner 2.0 software. PCR amplicons from isolates displaying each of the DMA melt types were sequenced to confirm the SNP genotypes and for the homozygous individuals to visualize the surrounding haplotype

Results

Detection of LOH and SNPs in mutagenized and non-mutagenized P. capsici

Isolate LT1534 has five heterozygous sites in locus 1 and is homozygous at loci 2 and 3.

For isolate CBS121656, locus 1 is homozygous, locus 2 has seven heterozygous sites, and locus 3 contains eleven heterozygous sites. Both the TILLING analyses and the DNA sequence data from ENU mutants displaying LOH indicate that for each loci all of the heterozygous sites co-converted to homozygosity within the amplified regions (Fig. 2-2). Conversion occurred to both of the underlying haplotypes at each loci (Table2-2). Of the 1152 ENU mutants screened, 10

Table 2-2. Summary of LOH events for three loci screened in LOH mutants.

Target	No. of heterozygous alleles	Number of LOH mutants (n=1152)	Ratios of homozygote genotypes among LOH mutants	Length of genomic conversion tracts surrounding target locus
Locus 1	5	10	5:5 (n=10)	≥3.4 kb (n=10)
Locus 2	7	9	5:4 (n=9)	≥4.6 kb (n=9)
Locus 3	11	99	2:22 (n=22)	≥1.6 kb (n=10)

mutants of LT 1534 exhibited LOH at locus 1, 9 mutants of CBS121656 displayed LOH at locus 2 , and 99 mutants of CBS121656 underwent LOH at locus 3 (Table 2-2).

In addition to the LOH, two induced point mutations were observed in LT 1534 at locus 1, three induced point mutations in CBS121656 at locus 2, and 10 ENU-induced point mutations at locus 3 in CBS121656. Three of the LOH mutants at locus 3 in CBS 121656 also had induced point mutations, as indicated by a single novel gel band within a LOH background (Fig. 2-1B). The co-occurrence of induced point mutations and LOH at locus 3 was confirmed for each of these isolates by re-sequencing.

No LOH was detected at the three loci screened in the 192 non-mutagenized *P. capsici* zoospore derived isolates of LT 1534 and CBS121656.

Length of LOH tracts in ENU mutants

For locus 1 an 823 bp region beginning 3.4 kb downstream was sequenced in LT 1534 and revealed 5 heterozygous sites. Re-sequencing of this region in the 10 LOH mutants confirmed that all 5 heterozygous sites at this location had also switched to homozygosity. For locus 2 a 637 bp amplicon which is 4.6 kb region downstream was sequenced in CBS121656 revealing 3 heterozygous alleles, all of which co-converted to homozygosity in the 9 LOH

At locus 3, a 779 bp region, beginning 1.6 kb upstream, was sequenced and found to contain 8 heterozygous alleles in CBS121656. Re-sequencing of this region in 10 LOH mutants indicated that all 8 of the sites co-converted to homozygosity (Table 2-1).

Genotyping mutants exhibiting LOH at locus 1 using DMA

The DMA assay of ENU mutants exhibiting LOH at the locus 3 allowed differentiation of the heterozygote and two homozygote genotypes (see Fig. 2-3). Of 192 mutants screened, twenty two displayed LOH at the locus 3. Of the twenty two mutants showing LOH at the locus 3,

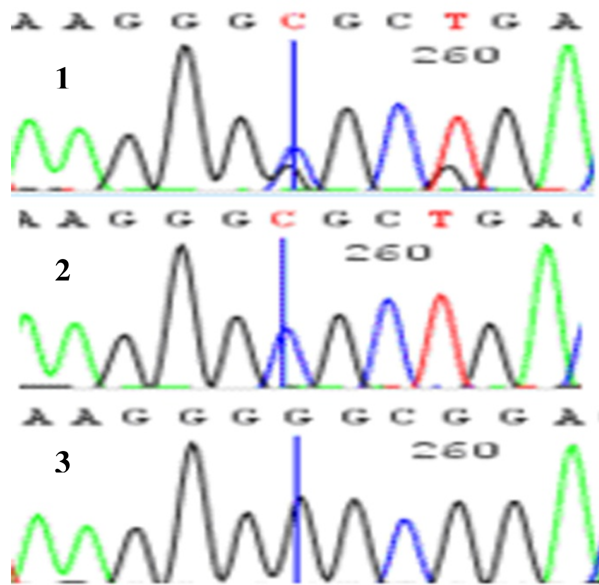

Figure 2-2. Electropherograms that display bidirectional switching to homozygosity at locus 3 in *P. capsici* ENU mutants. Trace 1 displays two heterozygote alleles, highlighted in red, from non-mutagenized *P. capsici*. Traces 2 and 3 display homozygote alleles of two ENU mutants exhibiting LOH. Each wild type haplotype is now present in the homozygous condition.

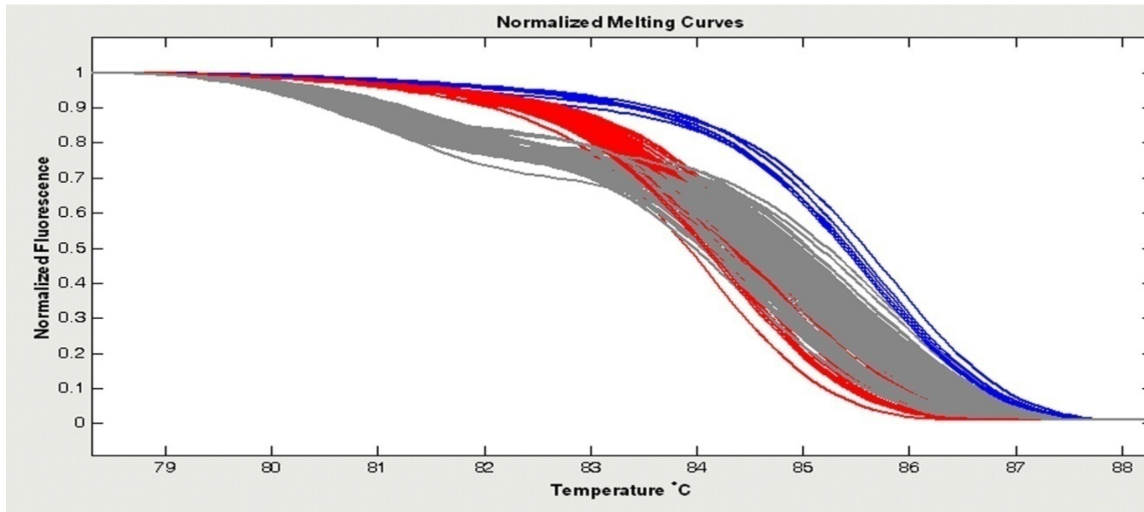

Figure 2-3. DNA melt curves of locus 3 PCR amplicons from 192 *P. capsici* ENU mutants. The curves marked A are from ENU mutants heterozygous at locus 3, and curves marked B and C are from ENU mutants having undergone LOH (homozygotes) at locus 3. All melting reactions were performed in duplicate.

twenty converted to one of the two homozygote genotypes. These data indicated the LOH events at locus 3 occur preferentially in one direction, favoring the conversion of one haplotype to homozygosity.

Discussion

In the process of developing reverse genetic resources for *P. capsici*, three loci with pre-existing heterozygosity were screened for induced point mutations. The first two loci screened, loci 1 and 2, had a low frequency of LOH (approximately one per 100 ENU mutants) and thus initially, LOH at these loci went unnoticed. Only when locus 3 was screened did it become obvious that a large proportion of the mutants had lost the pre-existing heterozygosity (approximately 1 in 10 mutants). For each of the loci tested, all of the heterozygous alleles present within an amplicon co-converted to homozygosity to one or the other haplotype. For loci 1 and 2 there did not appear to be any bias towards a particular haplotype, but for locus 3, 20 of the 22 LOH mutants tested converted to a single haplotype. These data suggest a bias towards one of the haplotypes, possibly resulting from recessive deleterious mutation(s) linked to one haplotype. Our results indicate that the LOH extends beyond the length of the loci and in the case of locus 2 extends 4.6 kb. These LOH events may span much longer stretches of the *P. capsici* genome, and are also likely occurring at multiple locations in the genome. Genomic instability (as evidenced by an increased rate in spontaneous LOH) was observed in mutants of *Saccharomyces cerevisiae* possessing mutations retarding the recombination and repair machinery (Andersen et al. 2008), and such mutations may also play a role in observed rates of LOH in *P. capsici* ENU mutants.

Three mutants of CBS 121656 were found to have undergone LOH at locus 3 and to

contain a post-LOH point mutation (Fig. 2-1B). The presence of the point mutation in the heterozygous condition indicates that the locus, post-LOH, is present as a diploid. However, aneuploidy may be responsible for some of the observed LOH as ENU is known to induce aneuploidy in not only mammals, but wheat as well (Jagtar and Shabeg 1992). LOH was not detected in non-mutagenized zoospore isolates and this is not surprising, as spontaneous mitotic recombination and LOH in other diploid eukaryotes organisms is very rare.

Our findings highlight a significant problem for ENU-based reverse genetics for *Phytophthora capsici*. The rate of LOH was ten times that of ENU induced point mutation at locus 3 and was twice as high at locus 1 and 2. Linking phenotype to mutation during reverse genetic screening may be confounded by the loss of allelic variation that occurs in *Phytophthora capsici* in response to ENU mutagenesis.

In light of these data, it is reasonable to suspect that LOH events may occur in response to other mutagenizing agents in *Phytophthora*. *Phytophthora spp*., as well as all other described oomycetes, lack pigmented cell walls, and ambient UV B and C irradiation may cause DNA damage. Previously, UV irradiation was used to induce nutritional auxotrophies in *P. capsici* and *P. dreschleri* (Castro et al. 1971). UV mutagenesis experiments with diploid industrial strains of *Saccharomyces cerevisiae* indicate that auxotrophic mutations induced by UV occur as a result of LOH (Shinji et al. 2005). *Phytophthora capsici* produces massive amounts of hyaline sporangia on fruits of host plants such as pumpkin and squash which are typically grown in full sunlight and novel allelic variation may arise due to UV-induced LOH. Future experiments exploring the incidence and impact of UV induced LOH in *Phytophthora* and other oomycetes is warranted.

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Chapter Three

Co-occurrence and genotypic distribution of *Phytophthora* species recovered from watersheds and plant nurseries of East Tennessee

This chapter is modified from a paper published in Mycologia:

Hulvey, J., Gobena, D., Finley, L., K. Lamour. 2010. Co-occurrence and genotypic distribution of *Phytophthora* species recovered from watersheds and plant nurseries of East Tennessee. (In press, Mycologia)**.**

My primary contributions to it were all of the writing, all of the culturing, most of the DNA sequencing, and all of the DNA melting analysis, and all of the AFLP analysis.

Abstract

From 2007 to 2008, state-wide surveys of symptomatic foliage of nursery plants from Tennessee resulted in isolation of 43 isolates of *Phytophthora spp*. This sample set includes four described species (*P. citrophthora*, *P. citricola*, *P. nicotianae*, *P. syringae*), and a provisional species of *Phytophthora* ('*P. hydropathica*'). During the same time interval, a stream baiting survey was initiated to recover *Phytophthora* from eight watersheds in East Tennessee, some of which are in close proximity to nurseries sampled. Healthy *Rhododendron* leaves were submerged for approximately one week and *Phytophthora spp*. mycelium was isolated from leaf lesions onto an amended agar medium. Six baiting periods were completed, and in total, 98 *Phytophthora* isolates, and 45 isolates of *Pythium spp*. were recovered. Three described species (*P. citrophthora, P. citricola*, *P. irrigata*) and the provisional species '*P. hydropathica*' were obtained, as well as three undescribed *Phytophthora* taxa, and *Pythium litorale*. Isolates from both surveys were identified to species using morphology and the internal transcribed spacer (ITS) sequence. Isolates from species co-occurring in streams and nurseries (*P. citricola*, *P. citrophthora*, and '*P. hydropathica*') were further characterized using amplified fragment length polymorphism (AFLP) analyses and mefenoxam tolerance assays. Isolates representing a

putative clonal genotype of *P. citricola* were obtained from both environmental and nursery sample sets.

Introduction

Species of the oomycete genus *Phytophthora* infect a diverse array of plant species causing significant loss to commercial plant production and to native plants (Erwin and Ribeiro 1996, Blair et al. 2008). *Phytophthora* infestations require moist or wet conditions and can quickly spread as deciduous sporangia and swimming zoospores via irrigation drainage water, irrigation reservoirs and waterways (Ghimire et al. 2009). *Phytophthora* is also spread via infected plant material in the nursery trade and *Phytophthora ramorum*, causative agent of Sudden Oak Death (SOD), is a notable *Phytophthora* pathogen dispersed in this manner (Rizzo 2002). *Phytophthora ramorum* has been disseminated within the U.S. and Europe through the plant nursery trade (Stokstad 2004, Werres and Merlier 2003), and forest soils and leaf litter have also been shown to serve as reservoirs for this pathogen in California (Fichtner 2007). SOD causes forest decline of tan oak and coast live oak stands in northern California, and infects other native woody shrubs and trees of northern California and Oregon (Frankel 2008).

Two other *Phytophthora* species, *P. cinnamomi* and *P. lateralis*, are thought to have been introduced into forests of North America (Hansen 2008). *Phytophthora cinnamomi* may have played a significant role in the decimation of the Eastern chestnut tree, and more recently, *P. lateralis* has been devastating Port-Orford-Cedar stands in the western US (Erwin and Ribeiro 1996, Hansen 2000).

In recent years, the importance of early screening and monitoring for *Phytophthora* diseases from plant nurseries, as well as environmental sampling to detect *Phytophthora spp.*

from native watersheds has been recognized (Cook et al. 2007). In an effort to track and limit the movement of *P. ramorum*, the USDA surveys foliage of woody ornamentals from plant nurseries across the U.S. for *P. ramorum* (Stokstad 2004). Additionally, the USDA also conducts surveys of *Phytophthora spp.* from soil and waterways across the US to determine if *P. ramorum* has escaped into native watersheds (Frankel 2008). *Phytophthora ramorum* has been detected in riparian waterways in Oregon, Washington, and California using leaf baiting and/or PCR detection (Fichtner et al. 2007, Rizzo et al. 2005). Plant nursery surveys for *P. ramorum* have been carried out in East Tennessee, resulting in the discovery of a new species of *Phytophthora*, *P. foliroum*, from infected nursery material of *Pieris japonica*. This species if of particular interest, because it can cross react to produce a false positive for *P. ramorum* in a previous PCR based molecular detection assay (Donahoo et al. 2006). *Phytophthora spp*. obtained from nursery surveys for *P. ramorum* in Tennessee have been genetically characterized, and shown to comprise several species (*P. foliorum*, *P. citricola*, *P. citrophthora*, *P. nicotianae*, *P. drecshleri*), of which, *P. citricola* and *P. citrophthora* contain clonal genotypes which co-occur in geographically disparate nurseries (Donahoo et al. 2008). Our primary goal was to determine whether *Phytophthora* species and genotypes common to the nursery trade are also co-occurring in nearby forest watersheds, as has been shown for *P. ramorum* and other invasive *Phytophthora* species.

A survey of *Phytophthora spp*. from symptomatic foliage of woody perennial plants was conducted from the fall of 2007 through the spring of 2008 from twelve plant nurseries. During the same time period eight watersheds in East Tennessee were surveyed for *Phytophthora* using rhododendron leaf baiting. These surveys did not yield *P. ramorum*, but did yield a large number of *Phytophthora* isolates. Here we report on the species diversity, distribution, and co-occurrence

of unique genotypes among plant nurseries and watersheds in East Tennessee.

Materials and Methods

Sampling for and culturing of Phytophthora spp. isolates used in this study

Phytophthora isolates were recovered from infected leaves of nursery plants from Tennessee as part of the USDA SOD Survey from May until November 2008 (Table 3-1). Infected material was obtained after leaves that tested positive using a *Phytophthora* specific Enzyme Linked ImmunoSorbent Assay (ELISA) test. Leaf samples originated from twelve nurseries in East Tennessee designated N1 – N12 (Table 3-1). Leaves were processed 24 to 48 hours later in the following manner: 10 mm leaf discs were aseptically removed from symptomatic leaf material, and plated onto dilute V8 PARP+H agar (40 ml V8 juice, 3 g CaCO3, 16 g Bacto agar and 960 ml water amended with 25 ppm pimaricin, 100 ppm ampicillin, 25 ppm rifampicin, 25 ppm pentachloronitrobenzene, and 25 ppm hymexazol). *Phytophthora* mycelium was observed growing from leaf discs into agar after 1 to 3 days and was aseptically transferred to water agar plates, resulting in diffuse colonies. Single hyphal tips were transferred to Dilute V8 PARP agar Petri plates and these cultures were utilized for genetic and morphological characterization.

Isolates of *Phytophthora* were baited from rivers or streams of eight east Tennessee watersheds using Rhododendron leaves as part of a national survey for the Sudden Oak Death (SOD) pathogen, *P. ramorum* (Table 3-1). The specific locations (designated TN1 – TN8) are in Tennessee watersheds that contain nurseries receiving plants from the west coast of the US. Sites were sampled three times during the months May and November of 2008, and each site was sampled in duplicate (Fig. 3-1). Bait leaves were asymptomatic rhododendron leaves collected from Seveir Co. Tennessee, rinsed in tap water, and stored at 5° C for 1 week prior to each

baiting session. Leaves were placed in mesh bags, and the bags were anchored with a 2.5 lb weight. Bait bags were tethered to the shore by a 15 ft. nylon cord, and submerged from 15 to 50 cm deep for seven days. Upon retrieval, *Rhododendron* leaves were rinsed with tap water, placed in plastic bags, and kept at 5° C prior to processing. Leaves were processed in the same manner as leaf material from the nursery survey, with care taken to excise leaf discs adjacent to lesions.

AFLP Analyses

Mycelium for DNA extraction was grown in V8 PARP broth, lyophilized, and high molecular weight DNA was then extracted from the mycelium using the methods of Lamour and Finley (2006). Genomic DNA from all isolates was interrogated by Amplified Fragment Length Polymorphism (AFLP) analysis, performed with the aid of Eco RI, and MseI restriction enzymes, adapters, and polymerase chain reaction (PCR) amplification primers, in accordance with method of Vos et al. (1995). Selective PCR amplifications were performed with Eco-AC and Mse-CCC primer pairs. Amplicons were fluorescently labeled in separate reactions following the method of Habera et al. (2004), and the resultant fluorescently labeled amplicons were resolved by a Beckman-Coulter CEQ8000 capillary genetic analysis instrument. Fragment peaks were manually confirmed, and peaks between 100 and 600 base pairs in size were manually scored for presence or absence.

DNA Melting Analysis

A DNA melting analysis (DMA) was utilized to screen the ITS1 and ITS2 regions. The ITS1 and ITS2 regions of the rDNA ITS region were PCR-amplified separately in duplicate for each isolate. PCR reactions consisted of 4 ul LightScanner Mastermix (Idaho Technologies, Salt Lake City, UT), along with 1 ul of genomic DNA at 10 to 50 ng/ul, and 1 ul of each primer,

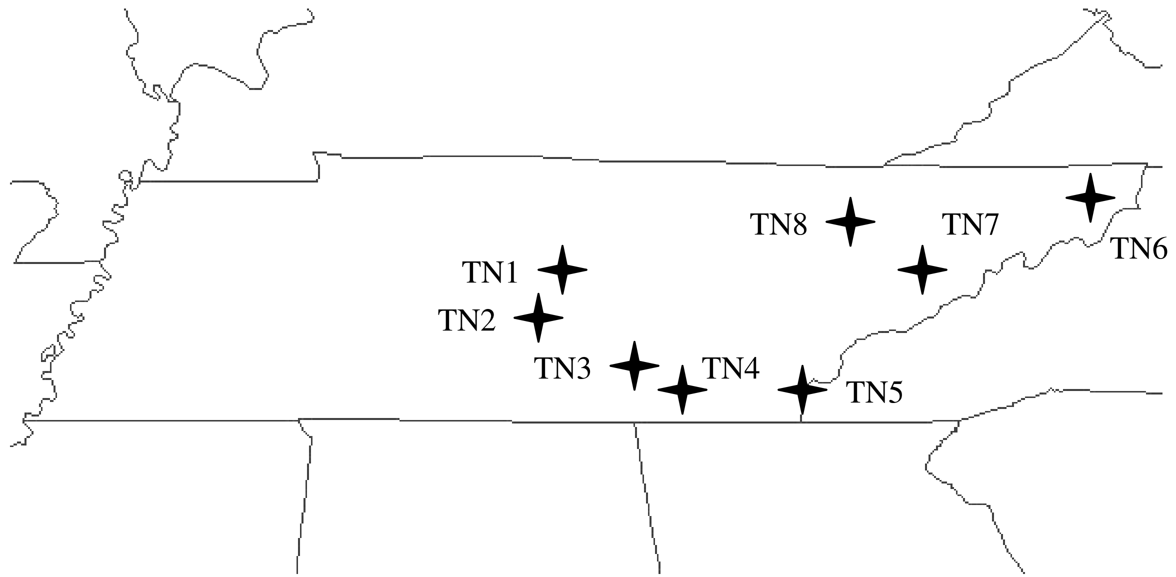

Figure 3-1. Tennessee state map with watershed sample sites TN1-TN8 indicated by stars.

ITS1, TCCGTAGGTGAACCTGCGG, and ITS2, GCTGCGTTCTTCATCGATGC, for the ITS1 region and ITS 3, GCATCGATGAAGAACGCAGC, and ITS4,TCCTCCGCTTATTGATATGC for the ITS2 region (Lee and Taylor 1992). All primers were added at 2.5 uM conc. The PCR temperature protocol was as follows: initial denaturation at 95° C for 2 min., then 45 cycles of 95° C for 30 s and 64° C for 30 s, and then a final step of 95° C for 30 s followed by 28° C for 30 s. DMA was performed according to manufacturers instructions using a 384 well format LightScanner instrument (Idaho Technologies, Salt Lake City, UT). PCR reactions were replicated on the 384 plate to ensure reproducibility. After PCR amplification and heteroduplex formation, PCR products were subjected to melting analysis using a light scanner instrument (Idaho Technologies, Salt Lake City, UT). Data analysis and normality parameters were adjusted using LightScanner 2.0 software, and isolates with identical ITS1 and ITS2 melt curves were identified. At least two isolates from each of the melt curve types from the ITS1 and ITS2 were chosen at random and the ITS region sequenced.

ITS Amplicon Sequencing and Isolate Identification

Genomic DNA from isolates was utilized for PCR amplification and DNA sequencing of the rDNA ITS region (comprising ITS1, 5.8s, and ITS2 sequences) for molecular identification of isolates (Cooke et al. 2000, Cook et al. 2007). The PCR primer combination ITS1 and ITS4, or the combination ITS6 and ITS4, were used in 5 uM concentration, along with 30 ng genomic DNA, 1 ul TAQ polymerase, and 38 ul 10X PCR buffer (White et al. 1990, Cook et al. 2000). The PCR temperature regime is as follows: an initial denaturation at 96° C for 2 min, followed by 35 cycles consisting of denaturation at 96° C for 1 min, annealing at 55° C for 1 min, and extension at 72° C for 2 min. A final extension step at 72° C for 10 minutes completed the reaction (Lee and Taylor 1992). The ITS DNA amplicons were sent to the University of

Tennessee Knoxville Molecular Biology Resource Center for sequencing. Forward and reverse sequence electropherograms were manually trimmed of poor sequence data, and assembled using the CodonCode Aligner 3.0.3 sequence alignment software (CodonCode, Dedham, MA). The resulting consensus sequences were searched against the NCBI Genbank non-redundant nucleotide database in August 2009 (Zhang et al. 2000). All of NCBI database entries that were compared to unknown isolates were also previously published in peer reviewed journal format. Unknown ITS sequences were also searched against the curated *Phytophthora* DNA sequence database, *Phytophthora* Database (http://www.phytophthoradb.org/) (Park et al. 2008).

Additionally, gross colony morphologies, colony growth at 35° C, and light microscopy of sporangia and gametangia from colonies grown on V8 PARP agar were examined to confirm species identifications.

Mating type and mefenoxam sensitivity of isolates

Mating type determination was attempted using *P. capsici* A1 and A2 tester isolates (CBS 121656 and CBS 121657, respectively). Seven millimeter agar discs from the leading edge of 10 day old colonies of tester and query isolates were plated together onto dilute V8 agar Petri plates, and the colony intersection was excised, slide mounted and observed microscopically at 7 to 14 days post inoculation. The presence or absence of oospores was then determined by light microscopy observation at 400X magnification. Mefenoxam sensitivity was assessed in isolates of species exhibiting overlap in nursery and watershed sample sets. A seven mm agar disc of a 10 day old culture was placed onto a 15 cm Petri plate of V8 agar medium amended with 100 ppm mefenoxam (Ridomil Gold EC), as well as a 15 cm plate of unamended V8 agar, as a negative control (Donahoo et al. 2008). Three replicates were performed for each mefenoxam-amended and control sample per isolate. The colony diameters were measured at three days post

inoculation, and data values were averaged. Mefenoxam sensitivity was estimated by comparison of mean growth on mefenoxam amended V8 agar to non-amended V8 agar controls and isolates are considered insensitive if able to grow >90% of control. Complete sensitivity was indicated by no growth on mefenoxam amended plates at 3 days post inoculation.

Results

Phytophthora isolates recovered from nurseries and watersheds of East Tennessee

A total of 186 isolates were obtained and characterized from nursery sampling and watershed baiting sites of East Tennessee, with *Phytophthora* isolates recovered from all collection sites of both surveys. The DNA melting analyses of ITS1 and ITS2 regions of all isolates obtained from both surveys revealed ten melt curve groups (Fig. 3-2). Sequenced amplicons of the ITS rDNA regions from isolates representing each melt curve grouping were from 697 to 798 base pairs in length and displayed 99 to 100% query coverage and 99 to 100% maximum sequence identity with GenBank sequence entries from the non-redundant database at NCBI (except '*P. hydropathica*', which displayed 96% query coverage). Of these entries, the complete ITS1, 5.8S and partial ITS2 sequences of *P. citricola* (AF266788), *P. citrophthora* (AF266785), *P. nicotianae* (AF266776), and *P. syringae* (AF266803) have been used as representatives of these species in previous literature on phylogeny of the genus *Phytophthora* (Cook et al. 2000). Identifications of these aforementioned species in our collections from their.

rDNA ITS sequences was also confirmed by the top five BLAST search results from the *Phytophthora* Database with 99% maximum identity and query coverage. Isolates were also identified as *P. irrigata*, '*P. hydropathica*', *Pythium litorale*, and *Phytophthora* unk1 (a taxon resembling but distinct from *P. gonapodyides*) by equally stringent similarity values to NCBI

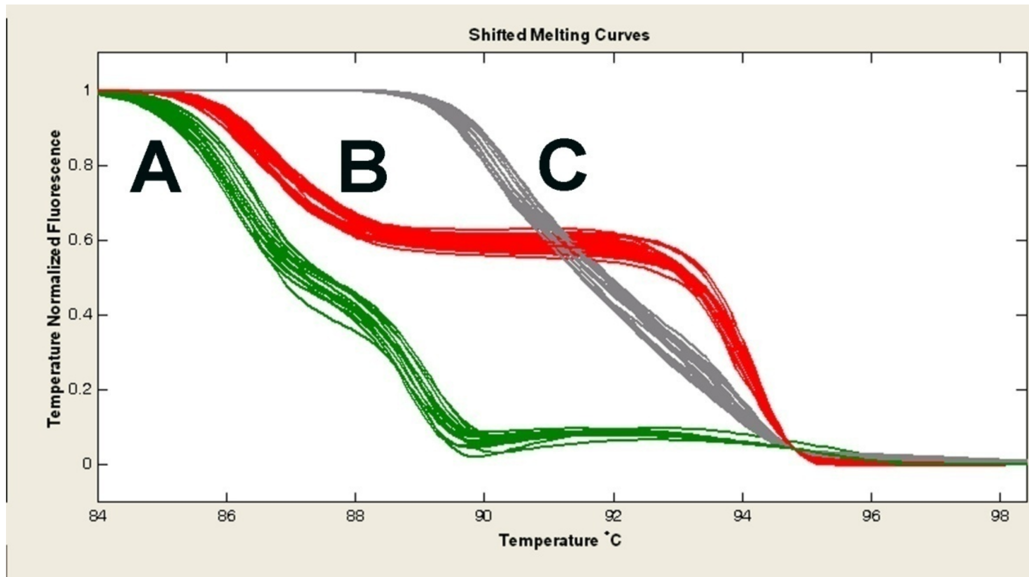

Figure 3-2. DNA melting analysis curves of ITS1 amplicons from three frequently encountered oomycetes in this survey. Curve A represents *Pythium litorale*, curve B, *Phytophthora* unk1, and curve C, the provisional species '*Phytophthora hydropathica*'

sequence entries from individual publications on these species (FJ196758, EU583793, DQ144637, and AF541900 respectively) (Hong et al. 2008B, Hong et al. 2008A, Nechwatal and Mendgen 2006, Brasier et al. 2003). Top hits for *Phytophthora* unk2 and *Phytophthora* unk3 were GenBank accessions EU644718 and EU644724, respectively, and matched with 99% query coverage and 96 and 97% maximum identity. These top hits are sequences originating from environmental *Phytophthora* isolates obtained from stream-baiting in West Virginia.

Of these 186 isolates, 43 originated from nursery samples and were identified as *P. syringae*, *P. citricola*, *P. citrophthora*, '*P. hydropathica*', and *P. nicotianae.* From nursery sampling, *P. citrophthora* was most frequently cultured, with 22 isolates obtained from eight of twelve nurseries sampled. These isolates were obtained from the following plant hosts: *Pieris japonica*, *Kalmia latifolia*, *Prunus laurocerasus*, and *Azalea sp*. Nine isolates of *P. citricola* were cultured from four nursery sites and from the following plant hosts: *Pieris japonica*, *Rhododendron sp.*, and *Kalmia latifolia*. Two isolates each of *P. syringae* and *P. nicotianae* were obtained from two sampled nurseries respectively. Two isolates of '*P. hydropathica*' were obtained from a single nursery. Material from four nursery sites were found to harbor *P. citricola* and *P. citrophthora*, with the remaining nine nursery sites yielding only a single *Phytophthora* species each (Table 3-1). The remaining 143 isolates, which originated from watersheds of East Tennessee, were identified as the following taxa: *P. citricola*, *P. citrophthora*, '*P. hydropathica*', *P. irrigata*, *Phytophthora* unk1, *Phytophthora* unk2, *Phytophthora* unk3 and *Pythium litorale*. From watershed sampling, isolates of *Pythium litorale* and *Phytophthora* unk1 were obtained from all eight sampled sites. Isolates belonging to *Phytophthora* unk1 were the most numerous, with a total of 61 isolates, followed by *Pythium litorale* with a total of 45 isolates recovered. *P. irrigata* and '*P. hydropathica*' were both recovered from six watershed sites, with eight and

Table 3-1. Summary results for stream baiting and nursery survey. Nursery sites are designated N1-N12 and watershed sites by TN1-TN8.

Site	County	Species recovered
N1	Bradley	*P. citrophthora*
N2	Bradley	*P. citrophthora, P. citricola*
N3	Bradley	*P. citrophthora, P. citricola*
N4	Hamilton	*P. citrophthora, P. citricola*
N5	Hamilton	*P. syringae*
N6	Williamson	*P. syringae*
N7	Knox	*P. citrophthora, P. citricola, 'P. hydropathica'*
N8	Sumner	*P. citrophthora*
N9	Fayette	*P. nicotianae*
N10	Gibson	*P. nicotianae*
N11	Henry	*P. citrophthora*
N12	Coffee	*P. citrophthora*
TN1	Warren	*Phytophthora* unk1, *Pythium litorale*, *P. citricola*, '*P. hydropathica*', *P. irrigata*
TN2	Warren	*Phytophthora* unk1, *Pythium litorale*, '*P. hydropathica*', *P. irrigata*
TN3	Bradley	*Phytophthora* unk1, *Pythium litorale*, '*P. hydropathica*', *Phytophthora* unk2
TN4	Hamilton	*Phytophthora* unk1, *Pythium litorale*, *P. citricola*, '*P. hydropathica*', *P. irrigata*, *Phytophthora* unk2, *Phytophthora* unk3
TN5	Polk	*Phytophthora* unk1, *Pythium litorale*, *P. citricola*, *P. irrigata*, *Phytophthora* unk2, *Phytophthora* unk3
TN6	Sullivan	*Phytophthora* unk1, *Pythium litorale*, *P. irrigata*, *Phytophthora* unk2, *Phytophthora* unk3
TN7	Sevier	*Phytophthora* unk1, *Pythium litorale*, *P. citrophthora*, '*P. hydropathica*', *P. irrigata*, *Phytophthora* unk3
TN8	Knox	*Phytophthora* unk1, *Pythium litorale*, '*P. hydropathica*'

twelve isolates obtained respectively. Three isolates of *P. citricola* were obtained from three watershed sites, and two isolates of *P. citrophthora* were recovered from one watershed site. Five isolates each of *Phytophthora* unk2 and *Phytophthora* unk3 were recovered from 4 watershed sites (Table 3-1). Three species, *P. citrophthora*, *P. citricola*, and the provisional species '*P. hydropathica*' were found to occur in both watershed sites and nurseries and East Tennessee (Table 3-1).

Genotypic diversity of Phytophthora species exhibiting overlap between streams and nurseries

Phytophthora citrophthora and '*P. hydropathica*' isolates were found to belong to 8 genotypes, designated C1-C8 and H1-H8 respectively, based on AFLP fingerprint analyses (Table 3-2). AFLP genotypes of *P. citrophthora* were defined by eight to twelve AFLP markers, and all of these isolates shared six AFLP peaks. Each of the genotypes was found to occur from only one or two locations, except for C7 which was recovered from 3 different nurseries. Only *P. citrophthora* isolates belonging to the C4 genotype exhibited insensitivity to mefenoxam. '*P. hydropathica*' genotypes were defined by four to nine AFLP markers, and these isolates shared 4 peaks. Of the eight '*P. hydropathica*' genotypes only one, H2, was sampled from multiple locations (TN2 and TN4). None of these isolates exhibited mefenoxam insensitivity. *P. citricola* isolates were comprised of 7 AFLP genotypes (CC1-CC7), with these genotypes defined by 4 to 11 AFLP markers (Table 3-2). All of these isolates shared five AFLP peaks. Of all species genotypes sampled from streams and nurseries of East Tennessee, only *P. citricola* genotype CC5 was found to co-occur in both the stream and nursery setting. The CC5 genotype was cultured from N3, N7, and TN5, and is characterized by eight AFLP peaks.

Table 3-2. Isolates from *Phytophthora* species exhibiting overlap among watershed and nursery sites. Genotype and the number of AFLP peaks (# AFLP peaks) that constitute genotypes are also presented. Asterisks indicate mefenoxam resistant genotypes.

Species/ Isolate	Location	Host	Genotype	# AFLP peaks
P. citrophthora				
LT5576	N3	*Pieris japonica*	C1	11
LT5592	N1	*Syringa patula*	C2	12
LT5590	N4	*Kalmia latifolia*	C3	11
LT5591	N4	*Kalmia latifolia*	C3	11
LT5580	N3	*Pieris japonica*	C3	11
LT5582	N3	*Pieris japonica*	C4	12
LT5600	N12	*Pieris japonica*	C4	12
LT5599	N12	*Pieris japonica*	C4	12
LT5602	N12	*Pieris japonica*	C5	12
LT5555	TN7	NA	C6	10
LT5557	TN7	NA	C6	10
LT5601	N12	*Pieris japonica*	C7	11
LT5609	N11	*Prunus laurocerasis*	C7	11
LT5608	N11	*Prunus laurocerasis*	C7	11
LT5607	N8	*Prunus laurocerasis*	C7	11
LT5573*	N3	*Pieris japonica*	C8	8
LT5574*	N3	*Pieris japonica*	C8	8
LT5579*	N3	*Pieris japonica*	C8	8
P. citricola				
LT5578	N3	*Rhododendron sp.*	CC1	9
LT5594	N5	*Rhododendron sp.*	CC2	11
LT5575	N3	*Pieris japonica*	CC3	10
LT5593	N4	*Rhododendron sp.*	CC3	10
LT5585	N3	*Pieris japonica*	CC3	10
LT5581	N3	*Pieris japonica*	CC4	9
LT5577*	N3	*Pieris japonica*	CC5	8
LT5606*	N7	*Pieris japonica*	CC5	8
LT5550*	TN5	NA	CC5	8
LT5589	N2	*Pieris japonica*	CC6	7
LT5572	N3	*Pieris japonica*	CC6	7
LT5484	TN4	NA	CC7	4
LT5509	TN1	NA	CC7	4
'P. hydropathica'				
LT5604	N7	*Pieris japonica*	H1	7
LT5605	N7	*Pieris japonica*	H1	7
LT6250	TN4	NA	H2	6

Table 3-2 continued.

LT6175	TN2	NA	H2	6
LT6239	TN1	NA	H4	6
LT6203	TN8	NA	H5	6
LT6230	TN7	NA	H6	5
LT6233	TN8	NA	H7	7
LT6238	TN1	NA	H8	9

Mefenoxam sensitivity and mating type

All isolates of '*P. hydropathica'* displayed mefenoxam sensitivity with maximum growth on mefenoxam less than 20% than that of the unamended controls. All but three isolates of *P. citrophthora* and *P. citricola* were sensitive to mefenoxam. *Phytophthora citricola* isolates belonging to genotype CC5 (LT 5550, LT5577, LT5606) grew as well on mefenoxam amended media as on the control. *Phytophthora citrophthora* isolates belonging to genotype CC5 (LT 5573, LT5574, LT5579) also displayed equal growth diameters on nonamended versus mefenoxam amended dilute V8 PARP plates.

Mating type determination was not successful for any of the isolates screened with the *P. capsici* tester isolates. The only isolates that displayed gametangial morphological features were *P. citricola* isolates, which is a homothallic species. All *P. citricola* isolates obtained produced oogonia and antheridia in culture.

Discussion

Tennessee is renowned for the production of hardwood, ranking second in the nation for hardwood lumber production. Employing over 180,000 Tennesseans, the forests products industry accounted for almost seven percent of the state's economy in 2000, and was worth over 21 billion dollars in 1997 (English et al. 2003). Thus, the health of Tennessee forest ecosystems is vital to the Tennessee economy (Young et al. 2007). The productivity of Tennessee hardwood forests could be threatened by the presence of an invasive *Phytophthora* species, such as *P. ramorum*, which is known to infect a broad range of perennial shrub and tree species. Therefore, monitoring the distribution and co-occurrence of *Phytophthora* and other plant pathogens from the nursery trade and watersheds is of utmost concern.

The most frequently recovered *Phytophthora* taxon in this study, *Phytophthora* unk1, was cultured exclusively from watershed sites and has also been reported from leaf baiting surveys of streams of the southeastern US (Hong et al. 2008A). *Phytophthora* unk1 has been reported from declining forests of *Austrocedrus chilensis* in Argentina but there are no reports definitively showing this *Phytophthora* taxon to be the cause of any forest disease (Greseleben et al. 2005). These reports mention the aberrant mating of these isolates which appear completely sterile in many cases, consistent with our observations. *Phytophthora* unk1 and *Pythium litorale* were recovered from all collections sites and may be important decomposers of allochthonous leaf litter in riparian ecosystems of Tennessee.

Another *Phytophthora* species frequently encountered exclusively from the watershed sites was *P. irrigata*. This species is reported by Hong et al. (2008A) to be common in irrigation reservoirs in Virginia, as well as rivers in Virginia and Pennsylvania (Hong et al. 2008B). This species was found to be pathogenic to some test plants in an experimental setting but whether it infects commercially grown plants is currently unknown (Hong et al. 2008B).

Phytophthora citrophthora, *P. citricola*, and '*P. hydropathica*' all co-occurred in watersheds and nurseries of East Tennessee. The provisional species '*P. hydropathica*' of Hong et al. 2008A is reported to occur in irrigation reservoir water from plant nurseries in Virginia. The pathogenicity of this provisional species has been demonstrated on several test plants. In light of these findings, '*P. hydropathica*' may impact nursery production in Tennessee due to its widespread geographic distribution, and presence in nurseries and streams of east Tennessee. AFLP fingerprinting does not indicate a clonal lineage, but does suggest very closely related genotypes, as they differ by a single AFLP peak among the 6 remaining shared peaks. Of the eleven isolates of *P. 'hydropathica'* recovered, seven had unique genotypes, which is not

surprising as '*P. hydropathica*' is a heterothallic species reported to be widely distributed in West Virginia and now east Tennessee (Hong et al. 2008A).

Our results support previous findings that *P. citricola* and *P. citrophthora* from plant nurseries of east Tennessee are comprised of considerable genotypic diversity, as well as widely dispersed clonal lineages (Donahoo et al. 2008). Isolates of the *P. citricola* putative clonal genotype CC5 genotype were found to occur at watershed site TN5, and nursery sites N3 and N7, and also exhibited mefenoxam insensitivity. The three isolates of the CC5 genotype all originated from different counties of east Tennessee, indicating that this genotype is distributed widely across Tennessee. Environmental isolates of *P. citricola* genotype CC7 were only recovered from watershed sites TN1 and TN4, and these isolates displayed complete sensitivity to mefenoxam. Mefenoxam insensitivity has been reported previously in isolates of *P. citricola* recovered from nurseries of east Tennessee (Donahoo et al. 2008). The origin of the CC5 genotype is most likely the plant nursery due to the mefenoxam selection pressure that *Phytophthora* would encounter there (Shew 1985, Joseph and Coffey 1984). *Phytophthora citricola* is a known pathogen to a diverse range of plant species, including economically important woody ornamentals, fruit trees, hardwood and evergreen trees (Erwin and Ribeiro 1996).

This is the first comparative survey of *Phytophthora* species and genotypic diversity from watersheds and plant nurseries and also the first report of a putative clonal genotype of *P. citricola* distributed among a plant nurseries and a natural watershed. This observation wasn't unexpected, as nursery irrigation waterways have been shown to harbor several *Phytophthora* species, and could easily allow clonal genotypes to spread in native habitats (Ghimire et al. 2009). Additional sampling may provide a clearer picture of movement and dispersal between

watersheds and plant nurseries, and a greater understanding of the movement of these pathogens between human influenced and natural habitats in turn may aid in the prevention of forest decline witnessed as a result of the encroachment of invasive *Phytophthora spp.* such as *P. ramorum*.

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Chapter 4

A clonal genotype of the pepper pathogen *Phytophthora capsici* is distributed throughout Peru

This chapter is adapted from a manuscript in preparation for submission:

Hulvey, J., Gobena, D., Story, D., Finley, L., K. Lamour. 2010. A clonal genotype of the pepper pathogen *Phytophthora capsici* dominates pepper pathosystems in Peru across the Andes Mountains.

My contributions to this work are all of the writing, AFLP analyses, and all of the DNA sequencing.

Abstract

Phytophthora capsici is an important oomycete pathogen of *Capsicum* peppers worldwide. From 2004-2007, populations of *P. capsici* infecting peppers along the coastal region of Peru were reported to be composed primarily of a single clonal genotype (PcPE-1). During 2008, 219 isolates of *P. capsici* were collected from *Capsicum pubescens* (Rocoto), *C. annum* (pimento), and *C. baccatum* (Aji) at ten locations in the Amazonian high jungle in the areas surrounding Oxapampa, and one coastal location, Carabayllo. Two isolates of *P. capsici* were also recovered from *Cyclanthera pedata* (Caigua fruit) near one field. A subset of 48 isolates were characterized using amplified fragment length polymorphisms (AFLP), and all isolates were characterized using eight single nucleotide polymorphisms (SNPs) markers that are fixed for heterozygosity in the coastal clonal lineage PcPE-1. Genotype designation made via SNPs and AFLP were concordant. Nine discreet genotypes were identified and the clonal A2 genotype previously identified from the coast was recovered from all of the fields. The implications of the genotypic diversity and distribution identified in this study are discussed.

Introduction

Phytophthora capsici is an important pathogen of vegetable crops such as tomato, pepper, cucumber and squash and more recently snap bean (Davidson et al. 2002, Erwin and Ribeiro 1996). Infestations of *Phytophthora capsici* on annual crops typically require warm and wetter than average conditions, and can spread rapidly due to the production of asexually produced deciduous sporangia and motile zoospores (Lamour 2009). In North America, sexual recombination and the production of the thick walled oospore is common and dormant oospores may persist in the soil for years. For North American populations, the combination of asexual and sexual reproduction affords *P. capsici* the benefits of both explosive clonal reproduction and the high genetic variation generated by sexual recombination (Lamour 2009). Characterizing genotypic diversity plays an important role in determining whether the asexual or sexual portion of the life cycle of a *Phytophthora* pathogen is driving the epidemiology of the pathogen. A number of techniques have been utilized to assess genotypic diversity in *Phytophthora* (e.g. amplified fragment length polymorphism (AFLP), microsatellite (SSR), and isozyme markers) and more recently, single nucleotide polymorphisms (SNPs) have provided useful markers for characterizing field isolates (Niepold 2005).

The population structure of *P. capsici* from fields in the US includes considerable genotypic diversity, along with the presence of both mating types (Lamour and Hausbeck 2001A, 2001B). It appears that the winter (or fallow) season imposes an effective selection pressure favoring the oospore for survival of the pathogen. Populations of *P. capsici* from pepper fields in Peru display a much different genotypic makeup, with only three genotypes documented and a single clonal lineage of the A2 mating type (PcPE-1) dominating pepper and tomato fields in coastal Peru (Hurtado-Gonzales et al. 2008). In some cases, cropping in the coastal area of Peru

includes pepper production year round as well as irrigation from common river systems. In these areas, the irrigation strategy and the availability of host material may explain the widespread occurrence of PcPE-1, as surface waters have been shown to harbor abundant populations of *P. capsici* (Gevens et al. 2007).

Our objective was to determine if epidemic populations of *P. capsici* from locations further inland across the Andes Mountains and into the Amazon rainforest harbored the PcPE-1 clonal A2 genotype, or if evidence of a more heterogeneous population structure characteristic of North American *P. capsici* populations could be detected.

Materials and Methods

Collection and Culturing

Isolates were collected from eleven total pepper fields in Peru during May 2008. These include nine fields surrounding Oxapampa, Peru, one field in Azucazu, just north of Oxapampa, and one field located on the coast in Carabayllo (Fig. 4-1). The nine fields surrounding Oxapampa span a total of approximately 200 km^2, and all fields in total span a distance of 400 km, from Azucazu to Carabayello. Isolates were obtained by plating small sections of infected fruit, crown, or root material of *Capsicum spp*. on V8-PARP agar medium (40 ml V8 juice, 3 g CaCO3, 16 g Bacto agar and 960 ml water amended with 25 ppm pimaricin, 100 ppm ampicillin, 25 ppm rifampicin, and 25 ppm pentachloronitrobenzene). Plates were observed daily and single hyphal tips recovered from expanding colonies. A single isolate was recovered per plant and used in the subsequent analyses.

Mating type and DNA extraction

Mating type determination was accomplished using *P. capsici* A1 and A2 tester isolates (CBS

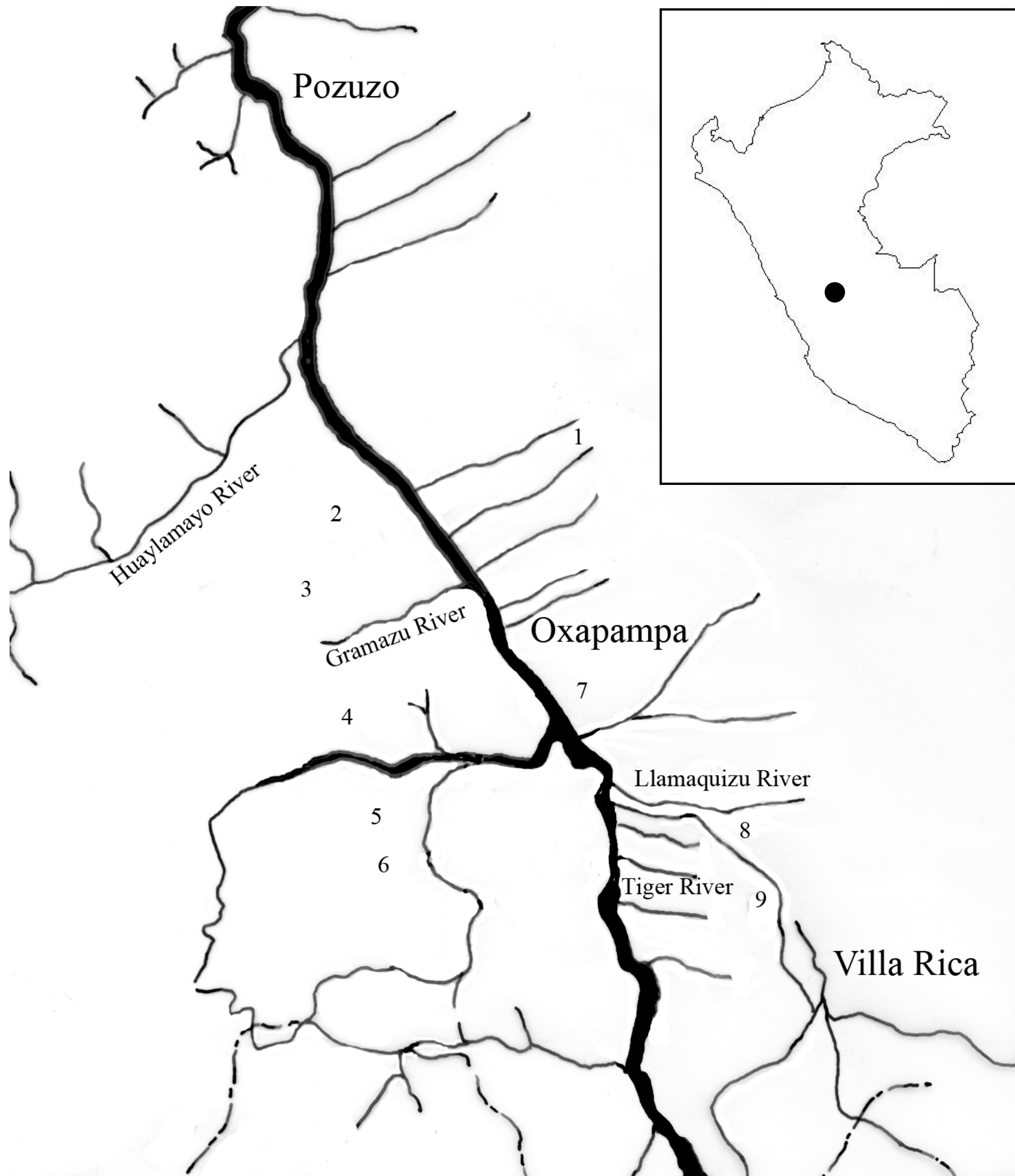

Figure 4-1. Map of collection sites surrounding Oxapampa. Sites are numbered 1 through 9. Inset is Peru map, with Oxapampa marked with a black dot, and Azucazu and Carabayllo marked with open circles. Carabayllo is coastal location.

121656 and CBS 121657, respectively). Seven millimeter agar discs from the leading edge of 10day old colonies of tester and query isolates were plated together onto dilute V8-PARP plates, and the colony intersection was excised, slide mounted and observed microscopically at 7 to 14 days post inoculation. The presence or absence of oospores was then determined by light microscopy observation at 400X magnification. DNA was produced by (i) growing mycelium in V8 PARP broth for 7 days, (ii) lyophilizing the harvested mycelium, and (iii) extracting high molecular weight DNA from the pulverized dried mycelium according to the methods outlined in Lamour and Finley (2006).

SNP Genotyping using DNA melting analysis

Eight high resolution DNA melting analysis (HR-DMA) assays were optimized to resolve SNP genotypes at loci heterozygous within single genes of the PcPE-1 clonal lineage. The assays were designed to differentiate homozygote and heterozygote alleles. Marker loci were chosen from an expressed sequence tag (EST) library of the *P. capsici* genome, and these genes were re-sequenced in a PcPE-1 representative isolate (LT2135). A single heterozygous SNP was targeted from each of the eight genes. Primers were designed using the LightScanner primer design software (Idaho Technologies, Salt Lake City, UT) to amplify a 45-65 bp amplicon that spanned a single heterozygous site (See Table 4-1 for primer sequences). PCR reactions for DMA consisted of 4 µl Lightscanner Mastermix (Idaho Technologies, Salt Lake City, UT), 1 µl of genomic DNA denaturation at 95 C for 2 min., then 45 cycles of 95 C for 30 s and 64 C for 30 s, at 10 to 20 ng/µl, and 1 µl of each primer at 2.5 µM conc. The PCR temperature protocol was as follows: initial denaturation at 95 C for 2 min., then 45 cycles of 95 C for 30 s and 64 C for 30 s, and then a final step of 95 C for 30 s followed by 28 C for 30 s. HR-DMA was performed according to manufacturer's instructions using a 384 well format Lightscanner instrument (Idaho

Table 4-1. Summary information for high resolution DNA melting analysis (HR-DMA) markers used to genotype isolates of *Phytophthora capsici*.

Locus ID[a]	Genbank accession	Base pair of SNP[b]	SNP	Forward Primer/ Reverse Primer
Flc3	BTO32098	2010	T/C	GCCCAAGTAGCAAAGCTCA/ GTCCACAGCGATGGTCT
Flc12	BTO31712	2137	C/T	TATCCTCCACGTACTCGAAG/ AGGTTGCTCAGGTGATG
Flc18	BTO32197	3395	T/C	GCACCTCTTCTGTGCAG/ GTCGTCTGGTCTTCACTTG
Flc19	BTO31656	2831	C/T	CATCATGCACCATGAGTTTG/ CCTTCTTACCGTCTTCGT
Flc23	BTO31999	1048	C/T	TCTGACGATGCTGTCCC/ TTCGTTCCTTAACGCCG
Flc24	BTO32352	989	C/T	ATCCTGGACATGGACCC/ CAGGTACAGGTGCCTCA
Flc29	BTO31539	684	C/A	AATGACCCGAACGAAGT/ GAAATAGCTGAAGAAATGCTCC
Flc34	BTO31610	1773	G/T	CGCCCCTGTATCAGAAG/ CACGCGTCCTTGCTTAC

[a]Informal locus identifiers
[b]Base pair number of polymorphic site from the 5' end of the Genbank sequence.

Technologies, Salt Lake City, UT). All assays were repeated to ensure reproducibility. Data sequencing of an isolate representing each of the DMA melt curve types was performed to confirm genotypes.

AFLP Analyses

Amplified Fragment Length Polymorphism (AFLP) analysis was performed using *Eco RI*, and *MseI* restriction enzymes, adapters, and polymerase chain reaction (PCR) amplification primers following the method of Vos et al. (1995). Selective PCR amplifications were performed with Eco-CG and Mse-CG primer pairs. Amplicons were fluorescently labeled in separate reactions following the method of Habera et al. (2004), and the resultant fluorescently labeled amplicons were resolved by a Beckman-Coulter CEQ8000 capillary genetic analysis instrument. Fragment peaks were manually confirmed, and peaks between 100 and 600 base pairs in size were manually scored for presence or absence.

Results

A total of 219 isolates were recovered from infected plants at the eleven locations sampled (Fig 4-1, Table 4-2). Isolates of A2 mating type were recovered from all fields, and only 7% of the isolates were of the A1 mating type, originating from only two fields (Table 4-2). The majority of isolates (166) were recovered from Rocoto, with fewer isolates from Aji (37), heterozygosity at all 8 loci assayed. The PcPE-1 genotype was recovered from all eleven fields, and comprised 75% of all isolates collected, whereas, isolates of the remaining eight additional genotypes each comprised less than 10% of the total isolates (Table 4-3).

Phytophthora capsici recovered from large scale paprika production fields in coastal Peru fits the profile of an introduced pathogen; there is very low genotypic diversity and a single

Table 4-2. Isolate summary information.

Location[a]	Hosts	No. isolates	Mating Type
Field 1*	*C. pubescens*	60	A2
Field 2	*C. pubescens* *C. baccatum*	18	A2(8), A1(10)
Field 3	*C. pubescens* , *C. baccatum*	12	A2(6), A1(6)
Field 4*	*C. baccatum*	7	A2
Field 5*	*C. pubescens*	2	A2
Field 6*	*C. pubescens*	2	A2
Field 7*	*C. pubescens*	33	A2
Field 8	*C. pubescens*	1	A2
Field 9*	*C. pubescens* , *Cyclanthera pedata*	66	A2
Acuzazu	*C. pubescens*	8	A2
Carabayllo	*C. annum*	14	A2

[a]All isolates were of the PcPE-1 genotype.

Table 4-3. Summary of the distribution of *Phytophthora capsici* genotypes.

Genotype	Mating Type	Host(s)	Locations	Number of isolates (percent of total)
PcPE-1	A2	*C. pubescens, C. annum, C. baccatum, Cyclanthera sp.*	1-7, Azucazu, Carabayllo	134 (61.4%)
PcPE-2	A2	*C. pubescens*	1	9 (4.1%)
PcPE-3	A2	*C. pubescens*	7	20 (9.1%)
PcPE-4	A2	*C. pubescens*	3,7,9	2 (1%)
PcPE-5	A2	*C. pubescens, C. baccatum*	9	18 (8.1%)
PcPE-6	A2	*C. pubescens, C. baccatum*	9	12 (5%)
PcPE-7	A2	*C. pubescens, C. baccatum*	9	7 (3.2%)
PcPE-8	A1	*C. pubescens*	2,3	10 (4.5%)
PcPE-9	A1	*C. pubescens*	2,3	7 (3.2%)

clonal genotype (PcPE-1) is widely spread (Hurtado-Gonzales et al. 2008). This is dramatically different from the situation in North America where hundreds of unique genotypes are often isolated from single fields and both the A1 and A2 mating types are routinely recovered from single fields and individual plants (Lamour 2009). The apparent clonality of *P. capsici* in Peru is important because it may allow planting of select varieties or cultivars of pepper that are tolerant of, or resistant to, the PcPE-1 clonal genotype – a strategy that would be impossible to implement in N. America due to the high levels of genetic diversity. Our objective was to determine if the PcPE-1 clonal lineage was also present at sites on the eastern side of the Andes Mountains where peppers are often grown on smaller plots and the main crop produced is Rocoto (*C. pubescens*). Samples were collected in March of 2008 shortly after the rainy season had ended. At this time of the year the plants had mature fruit and the pepper harvest was ongoing. Although isolates of *P. capsici* were recovered from *C. baccatum*, *C. annum*, and what is known as the wild cucumber (*Cyclanthera pedata*), most of the isolates were recovered from infected fruit of *C. pubescens* (Rocoto) which is the main crop grown in this area. The Rocoto fruit have a thick waxy cuticle which becomes detached during the infection process and the disease is referred to as the "pealing pealing" disease in the areas around Oxapampa (Fig. 4-2). In contrast to some of the areas sampled along the coast (e.g. along the Supe River), none of the sites were irrigated by a common river source. All of the sites were located at relatively steep areas of the cloud forest where the forest and undergrowth had been cut and burned or manually cleared. The farmers indicated that individual Rocoto plants had often been productive for up to 5 years prior to an increase of the "pealing pealing" disease over the past 3 to 4 years. The current strategy to combat the disease is to clear new forest every 1 to 2 years due to an increase in the prevalence of the disease.

Table 4-4. Summary of SNP genotypes for nine clonal lineages of *Phytophthora capsici* recovered from Peru.

Genotype	FL3[a]	FL12	FL18	FL19	FL23	FL24	FL29	FL34
PcPE-1	G/A	C/T	A/C	G/A	G/A	C/T	G/T	A/C
PcPE-2	A/A	C/T	A/C	G/A	G/A	C/T	G/T	A/C
PcPE-3	G/A	C/T	A/C	G/A	G/A	C/C	G/G	A/C
PcPE-4	G/A	C/T	A/C	G/G	G/A	C/C	G/G	A/C
PcPE-5	G/A	C/T	C/C	G/A	A/A	C/C	G/G	A/C
PcPE-6	G/A	C/T	C/C	G/A	G/G	C/C	G/G	A/C
PcPE-7	G/A	C/T	C/C	G/A	G/G	C/C	G/G	A/A
PcPE-8	A/A	C/C	A/C	G/G	G/A	C/C	G/T	A/A
PcPE-9	G/G	C/C	A/C	G/G	G/A	C/C	G/T	A/A

[a] Marker details are listed in Table 1.

Our results indicate that the PcPE-1 clonal genotype dominates the population structure of *P. capsici* in the areas surrounding Oxapampa. The PcPE-1 lineage was recovered from all of the hosts sampled and was present at every location. Although PcPE-1 dominates, interestingly, we also recovered two clonal lineages of the A1 mating type (PcPE-8 and PcPE-9) which were present at two different locations. Tests are underway to determine the fecundity of crosses between these A1 lineages and the dominant PcPE-1 lineage. Preliminary observations indicate that crosses produce abundant normally formed oospores but recovery of progeny and genotypes has yet to be attempted. Clearly clonal reproduction is the main driver for population structure in the areas surrounding Oxapampa and due to the limited number of unique genotypes it is difficult to assess the importance of sexual recombination – although the allelic combinations resolved via the SNP typing indicate that sexual recombination may have played a role in generating at least some of the observed genotypic variation. Similar to some of the coastal areas where pepper is produced, there is susceptible host material (pepper and caigua) throughout the year and there is no selection pressure against the survival and spread of clonal lineages. How the PcPE-1 lineage has become so widespread is difficult to assess as there is very little genotypic diversity. It may be that the PcPE-1 clonal lineage has been dispersed throughout Peru by the exchange of infected plant material or seed, as is seen with many economically important *Phytophthora* pathogens, such as *P. ramorum*, *P. infestans*, and other oomycete pathogens (Fry et al. 2009, Grunwald and Goss 2009, Hansen 2008, Hansen et al. 2000, Stokstad 2007).

Although the genotypic diversity was higher in this region than the coastal areas, it is still much lower than findings for *P. capsici* in North America (Lamour and Hausbeck 2001A, 2001B, Ristaino 1990). The PcPE-1 genotype was also isolated from *Cyclanthera sp.* in the nearby jungle of one field, indicating this host may be an important reservoir for *P. capsici* in the

Figure 4-2. (A) Rocoto fruit infected with *Phytophthora capsici* and (B) Healthy Rocoto fruit.

surrounding Amazon. This is not surprising as *Phytophthora capsici* has also been reported to infect weedy plants such as American black nightshade and Purslane in the US near vegetable farms (French-Monar 2006). The finding of limited genotypic diversity throughout Peru indicates that the strategic deployment of tolerant or resistant pepper germplasm may be effective in reducing the overall significance of this important disease.

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Chapter Five

Pythiaceae of southeastern estuarine marsh grasses: Biodiversity, phylogeny, and degradative capabilities

This chapter is adapted from a manuscript in preparation for submission:

Hulvey, J., Gobena, D. and K. Lamour. 2010. Pythiaceae of southeastern estuarine marsh grasses: Biodiversity, phylogeny, and degradative capabilities.

My contributions to this work are all of the writing, collecting and culturing of material, microscopy and enzyme assays and most of the DNA sequencing.

Abstract

Oomycetes of the family Pythiaceae have been frequently documented from leaf litter of grasses, and several species are important pathogens of cereal grains. This survey is an investigation into the biodiversity of Pythiaceous oomycetes from marsh grasses of salt and brackish marshes of the southeastern GA coast. Isolates were obtained from leaf litter of seven coastal intertidal marsh sites, each dominated by *Spartina alterniflora*, *S. cynosuroides*, and/or *Zizaniopsis milacea*. Sequences were generated for the ITS rRNA region for all isolates, and these sequences were interrogated using BLAST searches and phylogenies built using the Neighbor Joining method. Isolates were also screened for the ability to degrade chitin, crystal methyl cellulose, xylan, starch, and esculin. Thirty isolates were characterized, representing eleven taxa within the genus *Pythium* and three taxa within the genus *Halophytophthora*. Over half of the *Pythium* taxa recovered had ITS sequences most similar to *Pythium spp*. that cause diseases of grasses.

Introduction

Marine oomycete species are few in number, but consist of several diverse lineages. They are found in both the basal oomycete assemblage, including holocarpic parasites of invertebrates and algae, and also in the crown of more recently diverging oomycetes, the Peronosporomycetes

(Beakes and Sekimoto 2009). It has been suggested that oomycetes may have originated in the marine environment and migrated to land along with their nematode hosts, and later adapted the capability to infect plants and other organisms (Beakes 2009, DeLay 2006). Several genera within the Peronosporomycetes have been documented from estuarine environment. These include members of the Saprolegniaceae, some of which are pathogens of marine fishes, and also, members of the Pythiaceae, such as *Pythium porphyrae*, an important pathogen of a brown alga (Padgett 1978, Park et al. 2003). An entire genus of the Pythiaceae, *Halophytophthora*, has been documented exclusively from marine and estuarine habitats, and may represent an ancient lineage of oomycete that never migrated to land (Nakagiri 2002).

Most marine Pythiaceae are suggested to be primarily decomposers of plant material. *Pythium* taxa have been reported, along with *Halophytophthora*, as members of salt marsh mycoflora in *Spartina alterniflora* marshes of the southeastern US (1992B, Newell and Porter 2000). It has been shown that a substantial amount of decomposition of marsh grass leaves occurs within attached leaves of the stalks, and much of the initial decomposition is performed by Ascomycete fungi (Newell 1992a, Newell and Porter 2000). The fate of leaf litter once it becomes detached and/or submerged is determined by a diverse assemblage of eukaryotic decomposers, and there may be substantial biodiversity of oomycetes occurring on leaf litter of estuarine marshes (Newell and Porter 2000).

ITS and rRNA sequences have been used previously to characterize diversity of microbial communities of ascomycete fungi and bacteria from salt marsh leaf litter, respectively (Buchan et al. 2003). No dedicated survey has been put forth to characterize the oomycete flora of leaf litter from marsh grasses of the southeastern US using ITS sequences. The goals of this investigation are threefold: one, to document the biodiversity of Pythiaceous oomycetes from salt

marshes of southeastern GA, to elucidate the phylogenetic position of the species encountered, and finally to evaluate the abilities of these oomycetes to degrade several components of plant detritus.

Materials and Methods

Culturing of isolates

Marsh grass leaf litter was collected from two salt marsh sites at St. Simons Island, GA, two sites on Jekyll Island, one Brackish site in Darien, GA and three salt marsh sites on Sapelo Island during the summer of 2009 (Table 5-1, Figure 5-1). Briefly, attached leaf litter and leaf fragments from the mud surface were collected, rinsed in ambient brackish water, and plated onto dilute V8 PARP+H seawater agar (40 ml V8 juice, 3 g CaCO3, 16 g Bacto agar and 960 ml seawater amended with 25 ppm pimaricin, 100 ppm ampicillin, 25 ppm rifampicin, 25 ppm pentachloronitrobenzene, and 25 ppm hymexazol). Mycelium was observed growing from leaf material into agar after 1 to 3 days and was aseptically transferred to water agar plates, resulting in diffuse colonies. Single hyphal tips were transferred to Dilute V8 PARP agar Petri plates and these cultures were utilized for genetic and morphological characterization.

Gene amplification and Sequencing and Isolate Identification

Genomic DNA from isolates was utilized for PCR amplification and sequencing of the rDNA ITS region (comprising ITS1, 5.8s, and ITS2 sequences) for molecular identification of isolates (Cooke et al. 2000, Cook et al. 2007). The PCR primer combination ITS6 and ITS4 were used in 5 uM concentration, along with 30 ng genomic DNA, 1 ul TAQ polymerase, and 38 ul 10X PCR buffer (White et al. 1990, Cook et al. 2000). The PCR temperature regime is as follows: an initial denaturation at 96° C for 2 min, followed by 35 cycles consisting of

denaturation at 96° C for 1 min, annealing at 55° C for 1 min, and extension at 72° C for 2 min. A final extension step at 72° C for 10 minutes completed the reaction (Lee and Taylor 1992). The ITS DNA amplicons were sent to the University of Tennessee Knoxville Molecular Biology Resource Center for sequencing. Forward and reverse sequence electropherograms were manually trimmed of poor sequence data, and assembled using the CodonCode Aligner 3.0.3 sequence alignment software (CodonCode, Dedham, MA). The resulting consensus sequences were searched against the NCBI Genbank non-redundant nucleotide database in January 2010 (Zhang et al. 2000). Only NCBI database entries that were previously published in peer reviewed journals were considered as top hits to unknown isolates. Additionally, gross colony morphologies and light microscopy of sporangia and gametangia from colonies grown on V8 PARP agar were examined to characterize unknown isolates.

Phylogenetic Analyses

All *Pythium* species ITS sequences used in this analysis are from GenBank accessions used by Levesque and DeCock 2004, with the addition of *P. kashmirense* (DQ778899). All *Pythium* species representing top BLAST hits of *Pythium* taxa recovered in this study are included in the analysis, along with several additional taxa. *Halophytophthora* ITS sequences originated from NCBI nucleotide database (*H. avicenniae* AY598668, *H. batemanensis* GU258916, and *H. polymorphica* DQ335636). A *Lagenidium myophilum* ITS sequence is also used in the analysis (AB285499). Sequences were aligned automatically using MAFFT multiple sequence aligner. The L-INS-i option was chosen (Katoh et al. 2005). Neighbor-joining (NJ) trees were computed using MEGA phylogenetic analysis software, with factory settings, except for Tamura Nei model of nucleotide substitution. Support for tree nodes was assessed by calculating bootstrap proportion values (Felsenstein 1985). Bootstrap values for the NJ tree were derived from 10000

Table 5-1. Summary of collection locations for isolates characterized in this study.

Site	Location	GPS Coord.	Salinity	Marsh grass species	Taxa recovered
1	Champney's Island, Darien, GA	N31.20146 W81.26575	2 ppt	*Z. milacea, S. cynosuroides*	*Pythium sp*. 1,2,3,4,5,11
2	Brackish marsh, Sapelo Island, GA	N31.42883, W81.27264	16 ppt	*S. cynosuroides*	*Pythium sp*. 2
3	Cabretta Island, Sapelo Island, GA	N31.43888, W81.23908	26 ppt	*S. alternifora*	*Halophytophthora sp*. 2
4	Teal Boardwalk, Sapelo Island, GA	N31.39509, W81.27936	26 ppt	*S. alterniflora*	*Halophytophthora sp*. 1
5	Clam Creek Park, Jekyll Island, GA	N31.65641, W81.24583	26 ppt	*S. alterniflora*	*Halophytophthora sp*. 1, *Pythium sp*. 7
6	Hampton River, St. Simon's Island, GA	N31.17439, W81.20399	26 ppt	*S. alterniflora*	Pythium sp. 4,7,8,9, *Halophytophthora sp.* 1
7	Clover Marsh Lane, St. Simon's Island, GA	N31.84473, W81.22308	26 ppt	*S. alterniflora*	*Halophytophthora sp.* 1,2,3

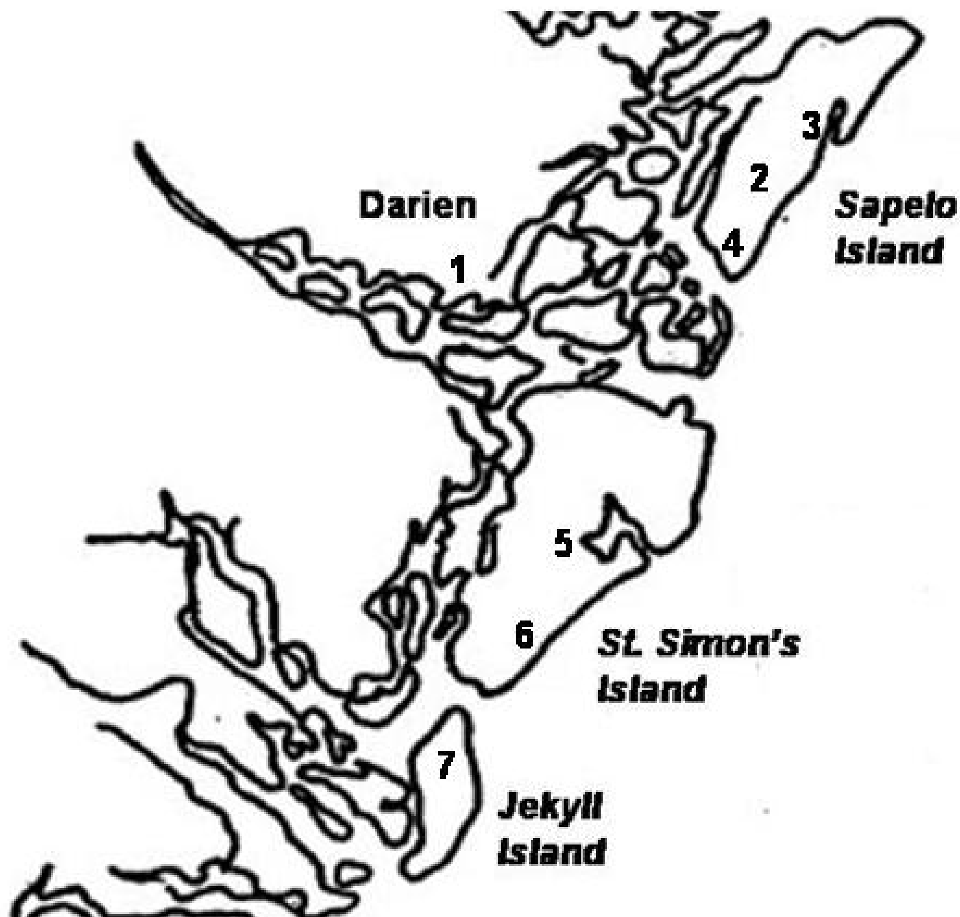

Figure 5-1. Map of collection sites one through seven, from Darien, GA and three barrier islands of coastal Georgia.

replicates. Maximum parsimony analysis was also carried out with factory settings. Trees were rooted with *Saprolegnia parasitica* (AB217688).

Enzyme assays

Enzyme assays are adapted from Simonis et al. 2008 and Pointing et al. 1998. Agar medium enzyme activity assays were be performed by first cultivating isolates 2% basal agar media amended with 0.02 g/L tryptone, 0.02 g/L yeast extract, and 0.2 g/L glucose. All media ingredients and substrates were obtained from Sigma. After 5 days growth, a 7 mm agar disc from the leading edge of each culture was used to inoculate plates of 2% basal medium agar amended with one of several substrates. All assays required incubation of plates at room temperature for five days and were performed in triplicate. For the detection of endoglucanase activity, plates with basal medium were amended with 0.2% crystal methyl cellulose (CMC). Five days post inoculation, plates were flooded with congo red dye for 10 minutes, which stains intact CMC red. Plates were next destained with 1 M NaCl for 5 minutes. A zone of clearing around the colony indicates CMC digestion, and endoglucanase activity. For detection of amylase activity, basal medium was amended with 2% starch, and plates were incubated at room temperature for five days. Plates were flooded with Lugol's iodine solution for five minutes. This stains the starch dark purple and a clearing zone around the colony indicates amylase activity. Hemicellulase activity was detected on 0.5% xylan plates, using the same Lugol staining procedure for amylase. Lugol stains hemicellulose brown, and a zone of clearing also indicates hemicellulose degradation. For chitinase activity, isolates were inoculated onto a layer of 0.005% chitan azure basal medium agar, and leaching of the dye into a lower layer of unamended basal medium indicated activity. A strain of *Achlya ambisexualis* isolated from leaf litter was used as a positive control in all assays (Steciow 1993).

Results

Eleven distinct *Pythium* taxa and *three Halophytophthora* taxa were recovered and included in the phylogenetic analysis of ITS rRNA sequences (Table 5-2). The ITS alignment included 34 taxa, the ten recovered here and 24 reference sequences of top BLAST hits and additional closely related species. This alignment was 1137 nucleotides, 735 of these sites were variable among the 34 taxa. There were 515 parsimony informative sites. Maximum parsimony tree topology was identical to the NJ tree (Figure 5-2).

Eight of the *Pythium* taxa recovered have top BLAST hits of *Pythium spp*. from Clades B1A, B1D, and B2 (Table. 5-2). Three of these taxa BLAST to *P. grandisporangium* of clade C (Levesque and De Cock 2004). The top BLAST hit for *Pythium sp*. 7 is *Lagenidium myophilum*, a pathogen of crustaceans (Nakamura et al. 1994). This *Lagenidium myophilum* was found to BLAST more closely with members of *Pythium* than with all other *Lagenidium* species for which sequence data has been deposited in NCBI. This species was described from infected coonstripe shrimp in Japan, and only asexual morphology was used to define this species (Nakamura 2004).

Only two of the *Pythium* taxa encountered were identified to species using morphology, and 99 to 100% ITS sequence similarity, *Pythium sp*.2 and *Pythium sp*. 10 (*P. catenulatum* of Clade B and *P. grandisporangium* of clade C). The remaining nine *Pythium* taxa encountered appear to be divergent enough in ITS sequence from known taxa to warrant their status as unknown taxa. Three of the *Pythium sp*. taxa (4,7,9) were recovered from multiple sites, while the remaining eight taxa were recovered from single sites only. All Pythium isolates were

Table 5-2. Summary of taxa isolated in this study.

Taxon (# isolates)	Best BLAST Hit	% Similarity/ % Query coverage	Pythium Clade	Site(s)	Substrate[a]
Pythium sp. 1 (2)	*P. catenulatum*, AY598675	99/100	B1a	1	*S. cynosuroides*
Pythium sp. 2 (1)	*P. catenulatum*, GU233294	98/100	B1a	1	*S. cynosuroides*
Pythium sp. 3 (2)	*P. angustatum*, AY598623	95/100	B1a	1	*S. cynosuroides*
Pythium sp. 4 (2)	*P. angustatum*, AY598623	95/100	B1a	1,6	*S. cynosuroides, S. alterniflora*
Pythium sp. 5 (2)	*P. inflatum*, AJ233446	89/99	B1d	1	*S. cynosuroides, Z. milacea*
Pythium sp. 6 (1)	*P. kashmirense*, DQ778899	96/100	B1d	2	*S. cynosuroides*
Pythium sp. 7 (4)	*Lagenidium myophilum*, AB285500	96/100	B2	5,6	*S. alterniflora*
Pythium sp. 8 (2)	*P. pachycaule*, DQ648141	98/100	B2	6	*S. alterniflora*
Pythium sp. 9 (2)	*P. grandisporangium*, AB444140	97/99	C	5,6	*S. alterniflora*
Pythium sp. 10 (2)	*P. grandisporangium*, AB444140	100/100	C	7	*S. alterniflora*
Pythium sp. 11 (1)	*P. grandisporangium*, AB444140	96/100	C	1	*S. alterniflora*
Halophy. 1 (5)	*Halophytophthora polymorphica*, AY598669	88/94	NA	5,6	*S. alterniflora*
Halophy. 2 (3)	*Halophytophthora polymorphica*, AY598669	87/96	NA	3,4,5,7	*S. alterniflora*
Halophy. 3 (1)	*Halophytophthora polymorphica*, AY598669	90/70	NA	7	*S. alterniflora*

[a]Indicates grass species from which leaf litter originated.

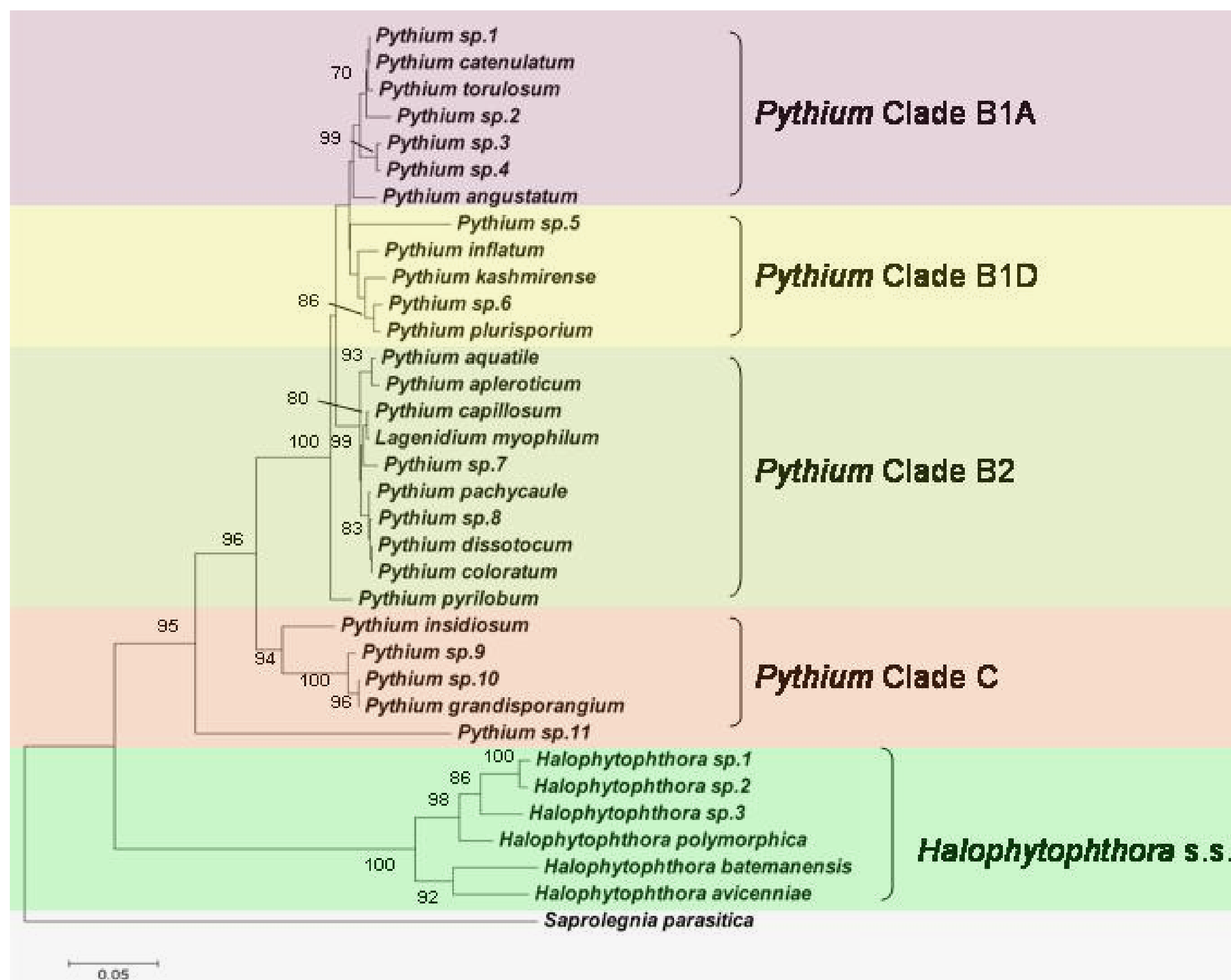

Figure 5-2. Neighbor joining phylogenetic tree of *Pythium* and *Halophytophthora* taxa and top BLAST hits. 10000 bootstrap replicates were performed. Bootstrap proportions 70 or greater are indicated. The tree is rooted with *Saprolegnia parasitica*. *Pythium* clades from Levesque and De Cock 2004, and *Halophytophthora* sensu stricto are indicated in brackets and color coding.

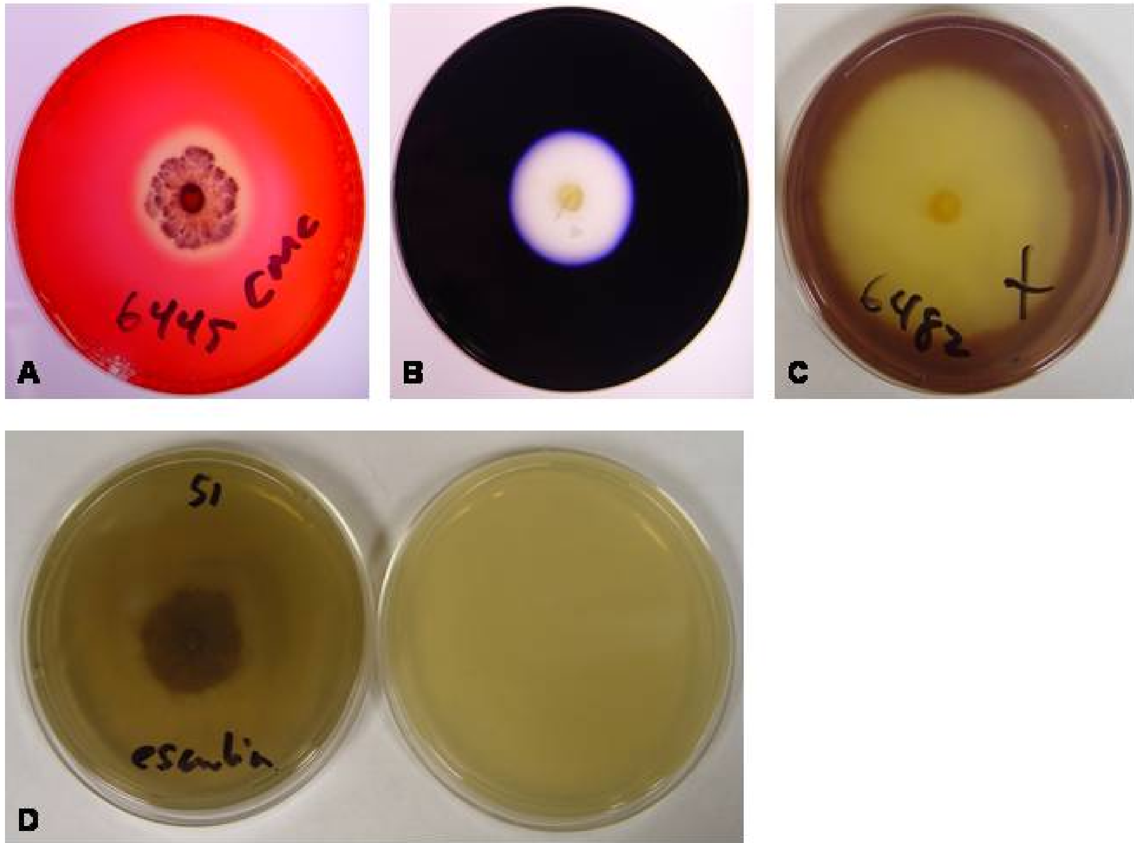

Figure 5-3. Agar plate assay plates after staining. A. Crystal methyl cellulose plate, B. Starch plate, C. Xylan plate, and D. Esculin plate.

observed to degrade CMC, esculin, xylan, and starch (Fig. 5-3). Only *Pythium sp*. 11 exhibited chitanases activity. Six taxa were recovered from *S. cynosuroides* and *S. alterniflora* each, and one was obtained from *Z. milacea*. Only two taxa were recovered from multiple grass species (Table 5-2).

The ITS sequence similarity of the two of the *Halophytophthora* taxa to *Halophytophthora polymorphica* was only 90%, likely due to the fact that there were only 4 *Halophytophthora* species represented in the NCBI database when searches were initiated (04/01/2010). Sporangia of *Halophytophthora sp*. 1 and 2 resemble those of *H. polymorphica*, *H. vesicula*, and *H. batemanensis*, in that they have a discharge vesicle that appears as an inverted cone at the apex of the sporangial papillum (Fig. 5-4). *Halophytophthora* 3 did not produce any sporangia. *Halophytophthora sp*. 2 was the most widespread of the Pythiaceae surveyed in this study, as it was recovered from four marsh sites. All three taxa were shown to degrade CMC, esculin, and xylan, but none were shown to degrade starch or chitin.

Discussion

Though there are no quantitative reports on the contribution of oomycetes to leaf litter decomposition, these results and previous studies have shown *Pythium* and *Halophytophthora* are significant players in the estuarine marshes (Newell 1992A, 1992B, Newell and Porter 2000). It was expected that all species of *Pythium* would be capable of degrading CMC, esculin, xylan, and starch. These substrates have previously been shown to be good indicators for enzyme activity of true fungi isolated from plant material, and like true fungi, *Pythium sp*. are readily adapted to degrade these materials (Simonis et al. 2008). Some of the enzymes utilized to degrade plant litter are suggested to likely have originated from fungi and other microbes, and

through lateral gene transfer, integrated into the genomes of oomycetes (Andersson 2006, Belhari 2008).

Halophytophthora species degraded CMC, xylan, and esculin but unlike *Pythium* didn't degrade starch. This discrepancy suggests that these two genera may occupy different niches during the microbial succession of marsh grass leaf litter. The decomposition of *S. alterniflora* and *S. cynosuroides* leaf litter is unique in that it begins while leaves are still attached to the stalk, and decomposition is initially dominated by true fungi of the Phylum Ascomycota (Newell 2003). Later stages of decomposition, once leaf litter has fallen and/or detached and becomes intermittently submerged with tidal inundation is likely a stage at which much of plant starches have been digested by fungal microbes and other primary decomposers. Thus, if *Halophytophthoras* are degrading fallen leaf litter that has already been degraded by fungi, they may have not adapted a need to use starch as a carbon source. Previous reports on *Halophytophthora* in mangrove and marsh ecosystems have demonstrated that these decomposers are able to quickly invade newly fallen leaf litter from oak and mangrove trees (Newell 1992B, Newell and Porter 2000Raghukumar et al. 1994). This would suggest that *Halophytophthora* may be able to degrade the pectin that surrounds the plant cell epidermis, though an assay to detect this has not been attempted yet.

Newell reports *H. vesicula* as a commonly encountered species from salt marshes of the southeastern US (1996). The ITS sequence data indicates that three distinct taxa of *Halophytophthora* were recovered here, but the sporangia all appear similar to *H. vesicula* (Figure 5-4). Taxonomy of this group relies on few asexual characters, and it appears that qualitative features of sporangia can't be used to separate taxa recovered (Ho and Jong 1990). Another species of *Halophytophthora*, *H. tartarea*, has also been reported from *Spartina* leaf

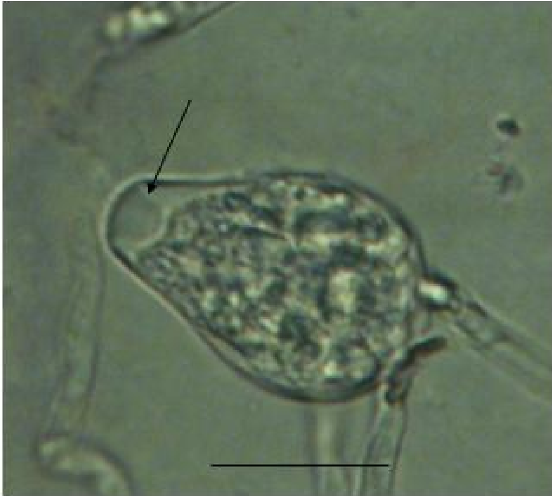

Figure 5-4. Sporangium of *Halophytophthora sp.*1 isolate. Bar=20 um. The arrow is pointing to an inverted cone located within the apex of the discharge papillum.

litter, though this species does not resemble the taxa characterized here (Nakagiri et al. 1994). Isolates *of Pythium sp*. 9 through 11 clustered with members of *Pythium* Clade C (Levesque and De Cock 2004). This clade is unusual in that it contains just two species of *Pythium*, and they exhibit very different morphologies and strikingly different ecologies: one is from marine leaf litter (*P. grandisporangium*) and the other, is the only known oomycete pathogen of mammals (*P. insidiosum*). *Pythium sp*. 10 is *P. grandisporangium*, but the other two taxa appear to be new species based on ITS sequence. Recovery of *P. grandisporangium* was to be expected, as it has been reported to exhibit 40% frequency of occurrence on *S. alternilfora* leaf litter (Newell 1992B). Interestingly, *Pythium sp*.11 was the only taxon shown to degrade chitin. *Pythium* species have been reported as mycopathogens, which would require chitanase activity. Thus, the pathogenicity of this taxon towards fungi needs to be assessed. One species of *Pythium* described from salt marshes in South Africa, *P. contiguanum*, is shown to be a mycoparasitic, but from BLAST searching (April 2010) the ITS1 sequence deposited in GenBank (AF203784), this species hits most closely to *P. dissimile*, which belongs to Clade B1C (Paul 2000).

Pythium sp.7 was found to BLAST most closely (96% identity) with *Lagenidium myophilum*, and appears nested within *Pythium* Clade B2. *Lagenidium myophilum* is a reported pathogen of coonstripe shrimp (Nakamura 1994). Thus, *L. myophilum* may be able to degrade chitin, but *Pythium sp*.7 did not display the ability to degrade chitin in our assay. Clearly, *L. myophilum* belongs in the genus *Pythium*, as it appears highly divergent from all other *Lagenidium* species in the NCBI non-redundant database (unpublished data). *Pythium sp*.7 is likely a new species, and was recovered as frequently as any of the other *Pythium* taxa (at two sites) but is found only in high salinity on *S. alterniflora*.

Pythium flora of marsh grass leaf litter appear to exhibit comparable biodiversity to

Pythium from a fresh water reed ecosystem (Nechwatal al. 2008). Nechwatal et al. 2008 noted that the majority of taxa encountered were unknown, and two new species have been described from isolates of the reed stands, one of which exhibits pathogenicity to reed (Nechwatal al. 2005, 2006). This pathogen, *Pythium phragmites*, was shown to exhibit ITS sequence similarity to the *Pythium* monocot diseases in Clade B1e (Nechwatal al. 2008, Levesque and De Cock 2004). No isolates characterized here cluster with members of *Pythium* Clade B1e in the phylogenetic analysis. However two taxa, *Pythium sp*. 5 and 6, were found to cluster with members of *Pythium* Clade B1d which does contain mostly grass pathogens. Further, isolates of *Pythium sp*.1 through 4 cluster with members of *Pythium* Clade 1Ba, and two of the four species previously assigned to this clade are also reported pathogenic to grasses (Levesque and De Cock 2004). *Pythium sp*. 1 exhibited 99% similarity and similar inflated sporangia to *P. catenulatum* of clade B1A. This species has been reported from salt marshes in Egypt, and isolates were shown to be pathogenic to seedlings of tomato and cucumber (Abdelzaher et al. 1997).

Thus, over half of the reported *Pythium sp*. taxa (*Pythium sp*. 1 through 6) bear ITS sequence affinity with lineages of *Pythium* that contain monocot pathogens (Clades B1a and B1d). *Pythium* species have been shown to play an important role in the dynamics of reed stand decline in Germany (Weilgoss 2009, Nechwatal et al. 2006, 2008). Future studies into the pathogenicity of these isolates towards marsh grasses should provide interesting results. A number of studies have sought to understand the dynamics of marsh die back zones in intertidal *Spartina* marshes, and none have sought to examine the possibility of oomycetes being involved. The exact causes of sudden dieback of salt marshes of the east coast is currently up to debate and the possible role of *Pythium spp*. warrants further investigation (Alber 2007).

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Vita

Jonathan Hulvey was born and raised in southeastern GA, and after graduating from Wayne County Highschool in 1998, he went on to get his undergraduate degree in Plant Biology at the University of Georgia in Fall of 2002. The following fall Jonathan went on to begin a Masters Degree in Marine Biology at the University of North Carolina at Wilmington. This degree was completed in 2005, and in the following year Jonathan began his Ph.D. in the Genome Science and Technology graduate program at the University of Tennessee.