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Investigation of Phytophthora species: Phytophthora colocasiae on Taro and Phytophthora Recovered from Streams in Eastern Tennessee

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To the Graduate Council:

I am submitting herewith a thesis written by Sandesh Kumar Shrestha entitled "Investigation of Phytophthora species: Phytophthora colocasiae on Taro and Phytophthora Recovered from Streams in Eastern Tennessee." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

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Investigation of *Phytophthora* species: *Phytophthora colocasiae* on Taro and *Phytophthora* Recovered from Streams in Eastern Tennessee

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Abstract

Oomycetes, also known as water molds, are morphologically similar to fungi. Unlike fungi, they are diploid and more closely related to plants. Several oomycetes are pathogenic to plants as well as aquatic animals. Members of the genus *Phytophthora* are a threat to many economically important crops and natural forest systems. The research presented in this thesis addresses intraspecific and interspecific variation in natural populations of *Phytophthora*. Chapter two summarizes genetic diversity and population structure for *Phytophthora colocasiae* attacking taro in Hawaii, Vietnam and Hainan Island, China based on novel single nucleotide polymorphism (SNP) markers. *Phytophthora colocasiae* causes Taro Leaf Blight, one of the most important diseases limiting this globally important source of food. Genetic analysis of *P. colocasiae* recovered from different locations showed that clonal lineages are widely distributed within populations and some clonal lineages are shared between the countries. Chapter three reports species diversity for *Phytophthora* and *Pythium* recovered from ten streams in eastern Tennessee in 2010 and 2011. This work is based on a program to monitor natural waterways for the sudden oak death pathogen, *P. ramorum*; a serious threat for many naturally occurring plants in east Tennessee. Molecular tools were used for identifying species recovered from the streams. During this survey, several known and unknown *Phytophthora* and *Pythium* species were identified along with a newly proposed genus *Phytopythium*.

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Chapter One

Introduction

Oomycetes are widely distributed eukaryotic plant pathogens that are morphologically similar to fungi (Thines and Kamoun 2010). Initially, the oomycetes were considered part of the Kingdom Fungi (Whittaker 1969). Shaffer suggested revising the five kingdom system and adding a new kingdom for oomycetes and slime molds because of their evolutionarily distinct characteristics (Shaffer 1975). The Kingdom Straminipila was proposed to describe oomycetes and related organisms (Dick 2001; Cavalier-Smith and Chao 2006). Oomycetes appear similar to fungi because of their similar nutritional habits and mycelial systems (Richards et al. 2006). However, there are several distinctly different characteristics. For oomycetes, the somatic hyphae and spores are diploid and in most cases, lack regular septa. This means that numerous nuclei are present in the hyphae, except in reproductive organs and older hyphae (Alexopoulos et al. 1996). The presence of 1,3-β-glucans, 1,6-β-glucans, and 1,4-β-glucans (cellulose) as the predominant structural component of the cell wall also makes this group distinct from true fungi where the cell walls are largely chitin (Latijnhouwers et al. 2003) . Contrary to true fungi, oomycetes also have tubular mitochondrial cristae and Golgi bodies with flattened cristae (Alexopoulos et al. 1996). The storage carbohydrate compound for oomycetes is mycolaminarin (Kamoun 2003) while fungi store energy as glycogen, similar to animals (Baldauf and Palmer 1993). Most members of Oomycota have motile stages known as zoospores in their life cycle (Alexopoulos et al. 1996). Swimming zoospores have a tinsel flagellum with tripartite hairs, a conserved feature indicating a shared ancestry with algae and diatoms (Thines and Kamoun 2010). In some species, there are two different motile stages known as primary and secondary zoospores, a condition

known as diplanetism (Dick 2001). Primary zoospores are pear shaped with two flagella attached at the anterior end. Secondary zoospores are kidney shaped with two flagella attached laterally; one tinsel type directed forward and a whiplash type facing backward (Alexopoulos et al. 1996). Some *Phytophthora* spp., like *P. ramorum*, produce identical primary and secondary zoospores, a condition known as monomorphic diplanetism (Moralejo and Descals 2010).

Oomycetes are also known as 'water molds' (Margulis and Schwartz 2000). Several species have marine habitats; however, 60% of species are pathogenic to terrestrial plants (Thines and Kamoun 2010). Among the pathogenic oomycetes, members of the genus *Phytophthora* are among the most devastating and attack a range of economic crop species including potato, pepper, tomato, soybean and alfalfa (Lamour et al. 2007). In 1845 and subsequent years, *Phytophthora infestans* caused epidemics on potato in Europe and was partially responsible for what is now known as the Irish potato famine; a period in Irish history where many migrated and more than 1.5 million people died (Austin Bourke 1964). This tragedy eventually led to the naming of *P. infestans* and the beginning of plant pathology.

Phytophthora produce thick-walled asexual chlamydospores and thick-walled sexual oospores for survival in unfavorable environmental conditions. In addition, *Phytophthora* produce asexual sporangia (Figure. 1.1A) that can be dispersed by water and wind (Alexopoulos et al. 1996). When the sporangia are exposed to high humidity or free water, they can germinate directly or indirectly (Alexopoulos et al. 1996). Direct germination is common under conditions of high humidity and occurs when the sporangium produces a germ tube that is able to cause infection (Alexopoulos et al. 1996). Indirect germination occurs in the presence of free water. The

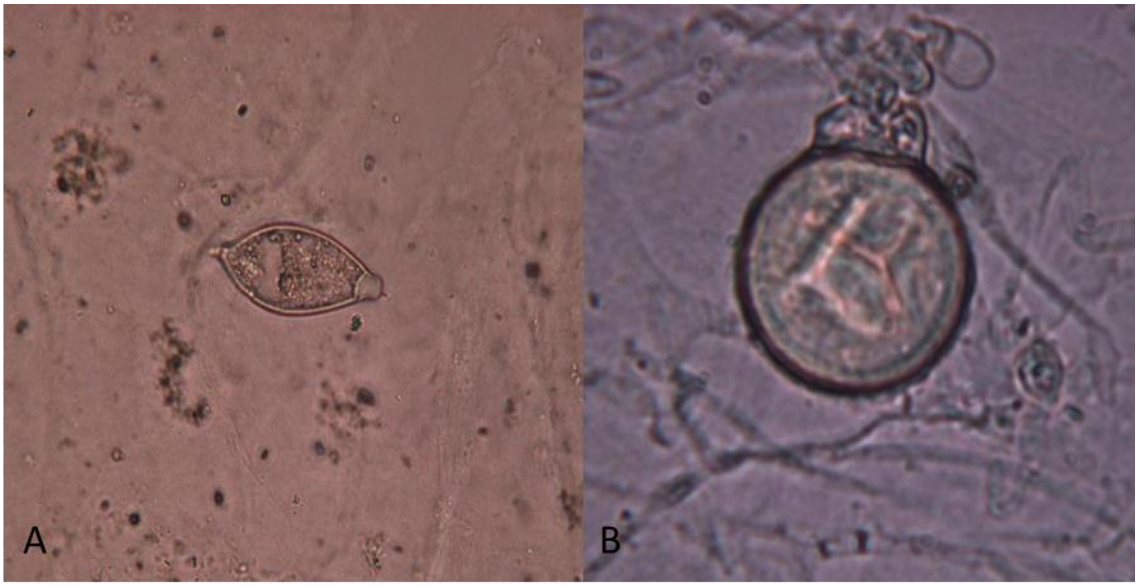

Figure 1.1 Asexual sporangium (A) and sexual oospore (B) produced by *Phytophthora colocasiae.*

cytoplasm inside the sporangium cleaves to produce biflagellate motile zoospores (Hohl and Hamamoto 1967). The motile zoospores are chemotactically attracted towards root exudes and after reaching the surface of a plant will shed their flagella and encyst before producing a germ tube and attempting infection (Alexopoulos et al. 1996). Sexual reproduction is accomplished two different ways. Some species are self-fertile and produce oospores in single culture. For other species, oospore production requires factors secreted from two different mating types; A1 and A2 (Alexopoulos et al. 1996). Both mating types can produce male (antheridia) and female (oogonia) gametes and hormonal regulation initiates sexual reproduction and production of oospores (Qi et al. 2005). The nuclei from an antheridium pass into the oogonium through a fertilization tube for fusion and oospore formation (Alexopoulos et al. 1996). Oospores (Figure 1.1B) are thick-walled resistant resting spores that can germinate directly to produce mycelium or sporangia (Alexopoulos et al. 1996). The dispersal of *Phytophthora* is accomplished via movement with soil, water splash, air, insect movements, and irrigation water (Ristaino and

Gumpertz 2000). Sexual spores can survive in soil for years, acting as long-lived structures to survive fallow, non-host, or winter periods (Erwin and Ribeiro 1996; Ristaino and Gumpertz 2000; Lamour and Hausbeck 2003).

Phytophthora is hemibiotrophic. During the initial stages, there is no obvious cell death or symptoms of infection. This is followed by a necrotrophic phase where cells are killed and infection is obvious (O'Connell and Panstruga 2006). Once inside the host, the mycelium produces bulged structures known as haustoria that serve to absorb host nutrients (Latijnhouwers et al. 2003).

Historically, morphological characteristics were used for species identification. These include the shape of the sporangia, dimensions and shape of the oospores, self-fertility, production of chlamydospores, and host range (Erwin and Ribeiro 1996). Species identification based solely on morphological characters or host range is difficult because they often vary tremendously within a species and some isolates may not produce spores under laboratory conditions (Erwin and Ribeiro 1996).

Phytophthora cause disease in a huge variety of dicotyledonous as well as monocotyledonous crops (Lamour et al. 2007). Examples include *P. capsici*, which is responsible for significant losses to vegetable crops including pepper, tomato, eggplant, cucumbers and summer squash (Hausbeck and Lamour 2004); *P. ramorum*, which causes sudden oak death (SOD) disease and is threatening natural forests of western USA, Canada and Europe (Davidson et al. 2003); *P. colocasiae* (Figure 1.2A and B), which causes taro leaf blight and limits taro corm production at

many locations worldwide (Bergquist 1972); and *P. sojae*, which is responsible for significant economic losses in soybean worldwide (Tyler 2007). For many species, very little is known about the population biology, species richness in natural settings, or overall dispersal. The following chapters outline the results from a study to characterize the genetic diversity of *P. colocasiae* at key locations in the U.S.A., Vietnam and China and the results from an ongoing survey of streams in eastern Tennessee to monitor for *Phytophthora* species diversity and to test for the SOD pathogen, *P. ramorum*.

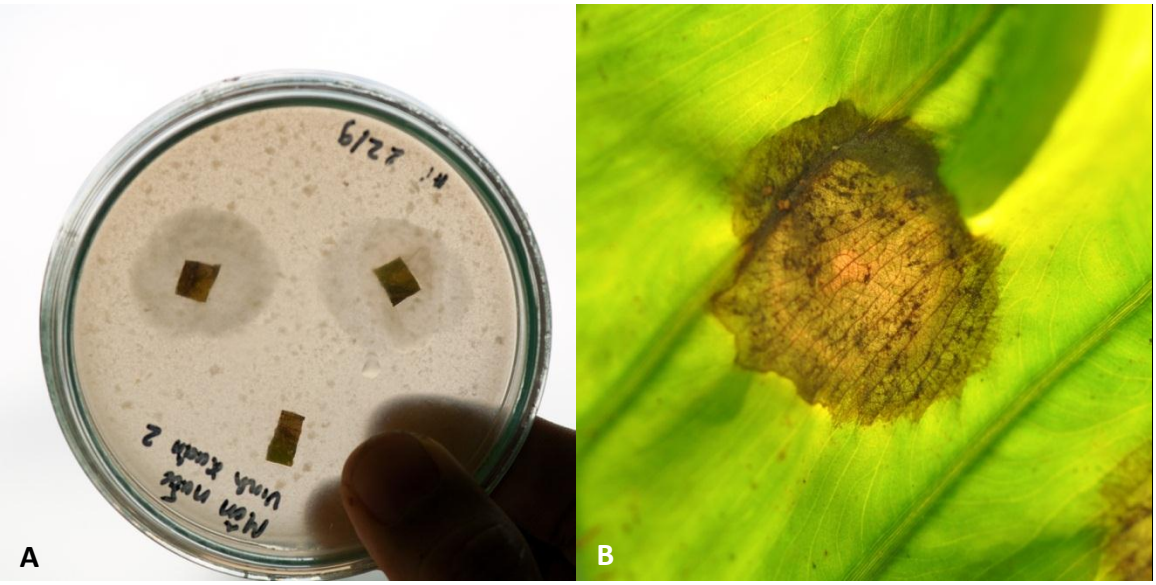

Figure 1.2 *Phytophthora colocasiae* mycelium growing out from sections of infected taro leaf (A) and typical necrotic lesion of Taro Leaf Blight (B).

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Chapter Two

Single nucleotide polymorphism diversity of *Phytophthora colocasiae* recovered from Vietnam, Hawaii, and Hainan Island, China

Abstract

Taro (*Colocasia esculenta*) is an important food crop to humans. Taro leaf blight, which is caused by *Phytophthora colocasiae*, can significantly impact production worldwide. Our objectives were to develop single nucleotide polymorphism (SNP) markers for *P. colocasiae* and characterize populations in Hawaii, USA (HI), Vietnam (VN), and Hainan Island, China (HIC). In total, 386 isolates were analyzed for mating type and multi-locus SNP profiles including 217 from HI, 98 from VN, and 71 from HIC. A total of 1152 single nucleotide variant (SNV) sites were identified via restriction site-associated DNA (RAD) sequencing of two field isolates. Genotyping with 12 SNV sites revealed a total of 41 unique genotypes with at least 10 markers informative in each country and estimated allele frequencies ranging from 6 to 45%. Isolates with identical multi-locus SNP profiles were considered clonal lineages, which varied from 2 to 196 isolates. Four clonal lineages were shared between countries. In addition, five SNP markers had a low incidence of loss of heterozygosity (LOH) during asexual laboratory growth. For HI and VN, >95% of the isolates were the A2 mating type. On HIC, A1, A2 and A0 (neuter) isolates occurred within single clonal lineages. The implications for the wide dispersal of clonal lineages are discussed.

Introduction

Phytophthora colocasiae Raciborski was first described on taro in Java in 1900 and is widely distributed in Asia, the Pacific region, and Africa (Raciborski 1900; CABI/EPPO 1997; Lebot et

al. 2003). *Phytophthora colocasiae* causes disease of taro known as taro leaf blight (TLB), although *P. colocasiae* also infects the stem and corm. Epidemics can be severe during warm, wet conditions and infected leaves often have distinctive foliar lesions. Leaf infections first become visible as water soaked spots that expand successively to produce sporangia on the newly colonized tissue. As the lesions grow, the older infected tissue becomes necrotic and under dry conditions will fall apart leaving variable sized holes (Trujillo 1967; Bergquist 1972).

Taro varieties have varying levels of resistance to TLB with yield reductions varying from 3 to 5% in resistant varieties, to 25 to 100% in more susceptible varieties (Vasquez 1990; Paiki 1996). When *P. colocasiae* was introduced to Samoa in 1993 the resulting epidemics were severe and destroyed almost all of the local varieties (Caillon et al. 2006). In 2005, Hawaii recorded the lowest Taro production since 1946 and TLB was one of the contributing factors for the reduced production (Hawaii Agricultural Statistics Service 2006).

Since taro is often grown in flooded conditions, the sporangia and swimming zoospores of *P. colocasiae* are likely an important factor in disseminating and spreading TLB. *Phytophthora colocasiae* is heterothallic, requiring the interaction of two different mating types (A1 and A2) for sexual reproduction and production of oospores (Ko 1979). At this point, there have been no reports of progeny produced in the laboratory although prior investigators suggest that the sexual stage is likely important in Southeast Asia (Zhang et al. 1994).

The earliest surveys of diversity in *P. colocasiae* report the presence of A1 and A2 mating types. An analysis of 114 isolates collected from Hawaii, Maui, and Kauai in the 1970's found only the

A1 mating type and concluded that *P. colocasiae* was likely an exotic invasive pathogen (Ko 1979). A decade later, analyses of 799 isolates collected in Taiwan revealed that all were the A2 mating type (Ann et al. 1986). In 1994, Zhang et al. identified the presence of three different mating types; A1, A2 and A0 (unable to produce oospores when paired with either an A1 or A2 tester isolate), for isolates collected from taro on the agriculturally important Hainan Island in China. The authors suggested that this region may be within the center of origin for *P. colocasiae* (Zhang et al. 1994). Previous investigators also postulated that Asia was the probable center of origin for *P. colocasiae* (Ko 1979) as this is the center of origin of many wild and cultivated species of taro (Vavilov 1951). A more recent survey of mating type for isolates from Asia and the Pacific region found only the A2 and A0 mating types, and on Taiwan there were seven isolates that appeared to be homothallic, capable of producing oospores in single culture (Tyson and Fullerton 2007). Subcultures derived from mitotic spores of these homothallic isolates produced isolates that were either strictly A1 or A2 and some that were still homothallic. All the isolates were able to cause disease (Lin and Ko 2008).

In 2003, Lebot et al. utilized isozyme profiles and random amplified polymorphic DNA (RAPD) markers to characterize a large collection of *P. colocasiae* from Indonesia; Papua New Guinea; the Philippines; Thailand; and Vietnam (Lebot et al. 2003). The isozyme profiles varied considerably between countries and even isolates with identical profiles from within the same country were found to be diverse based on the RAPD fingerprints (Lebot et al. 2003). None of the unique isozyme profiles or multi-locus RAPD genotypes was shared among the countries. A recent report of 14 isolates from India indicated that all had unique isozyme and RAPD profiles (Mishra et al. 2010).

Efforts to breed for tolerant or resistant varieties of taro are being conducted and our goal is to identify isolates suitable for screening promising germplasm. Here our objectives were to develop novel single nucleotide polymorphism (SNP) markers for *P. colocasiae* and to measure the genotypic diversity of *P. colocasiae* within three important taro growing countries.

Materials and Methods

Isolate collection and mating type analysis

Phytophthora colocasiae isolates were collected from locations in Vietnam in 2009 and 2010, Hainan Island, China in 2011 and four of the Hawaiian Islands in 2011. Samples were collected from foliar lesions as these were the easiest to see and the collections caused the least disruption to the producer's crop. The entire leaf and petiole was harvested and a small (approximately 1-cm^2) piece of the diseased leaf that included both healthy and necrotic tissue was excised and embedded into PARP-V8 agar (100 mL V8 juice, 3 g $CaCO_3$, 16 g Bacto agar and 900 mL water amended with 25 ppm pimaricin, 100 ppm ampicillin, 25 ppm rifampicin, and 25 ppm pentachloronitrobenzene (PCNB)) and incubated for 3 to 5 days at room temperature (Gobena et al. 2011). Isolates were sub-cultured onto water agar or dilute PARP-V8 (with a substitution of 40 mL V8 juice for 100 mL) to produce hyphal-tip cultures. Hyphal-tip cultures were assigned a unique identifier and the isolates stored long term at room temperature in 2-mL tubes containing three to four plugs of mycelium, three to four sterile hemp seeds, and 1 mL water.

Mating type was tested using *P. colocasiae* isolates LT7299 (A1) and LT7291 (A2). As no previously studied *P. colocasiae* isolates were readily available, the mating type for these isolates was determined using *P. capsici* tester isolates CBS 121656, which is an A1 mating type

and CBS 121657, which is an A2. To determine the mating type, plugs of mycelium from the query and tester isolates were co-inoculated onto dilute PARP-V8 agar and incubated at room temperature for 7 to 14 days. The intersection of the colonies was observed under a light microscope at 400× magnification to determine oospore production.

Genomic DNA

Mycelium production and DNA extraction were done according to the procedures outlined previously (Lamour and Finley 2006). Mycelium was produced in 24-well Uniplate microplates (Whatman Inc., Clifton, NJ) having a capacity of 10 mL per well. Two mLs of PARP-V8 broth was loaded in each well followed by inoculation with a small weft of mycelia from the surface of agar plates, with care taken to avoid transferring agar to the broth. The plate was sealed with breathable rayon tape and incubated for 6 days at room temperature. The mycelium was harvested and transferred to a 96-well 2-mL deepwell (DW) plate containing three 3-mm glass beads per well. The deepwell plate was covered by breathable rayon tape and frozen to -80°C for at least 1 h. Samples were then lyophilized for 48 h (24 h at -10° C followed by 24 h at 23°C) using a Labconco Stoppering Tray Drying System (STDS, Labconco Corp., Kansas City, MI). Immediately after removal of the sample from the chamber, a capmat was applied using a Capmat Applicator (CMA, Fischer Scientific) to avoid moisture absorption. The lyophilized sample was disrupted with a Mixer Mill 300 for 30 s at 30 revolutions per second (QIAGEN, Valencia, CA). Two biological replications (separately grown mycelial cultures) were included for each isolate.

For the DNA extraction, plates containing pulverized dried mycelium were centrifuged 5 min at 5000 rpm, the capmat removed, and 400 µL of lysis buffer added to each well (100 mM Tris of

pH 8.0, 50 mM EDTA, 500 mM NaCl, 1.33% SDS with 0.8 % Fighter F antifoaming agent (Loveland Industries, Greely, CO) and 0.2 mg per mL RNase A using an Apricot 96 channel pipette (Apricot Design, Inc., Monrovia, CA), followed by application of a capmat. The plate was shaken vigorously and placed in a 65°C oven for 30 min. The capmat was removed gently after centrifuging the plate for 2 min at 5000 rpm. Potassium acetate (150 µL) was added to each well and a new capmat was applied. The sample was kept at -20°C for 30 min. After centrifugation for 10 min at 5000 rpm, 400 µL of supernatant from the sample plate was transferred to new 2-mL 96-well DW plate preloaded with 600 µL guanidine hydrochloride (0.66 M) and ethanol solution (63.3%), followed by applying the capmat and shaking the mixture vigorously. One mL of the mixture was transferred to a Nunc spin column plate (Nalge Nunc Int., Rochester, NY) sitting on a 2-mL 96-well DW plate, centrifuged for 5 min at 5000 rpm and the flow through discarded. The spin column plate was washed further with 500 µL wash solution (10 mM Tris (pH 8.0), 1 mM EDTA, 50 mM NaCl, and 67% ethanol) and then 500 µL 95% ethanol followed by centrifugation for 5 min at 5000 rpm. The spin column plate was incubated at 65°C for 10 min to evaporate the ethanol. Two hundred µL of 10 mM Tris (pH 8.0) was added to each well and the plate was incubated for about 30 min at room temperature. The spin column plate was centrifuged onto a new 1-mL DW plate for 5 min at 5000 rpm to elute DNA. Plates with genomic DNA were sealed with aluminum foil and stored at -20°C.

SNP identification and selection

Initial SNP marker discovery was based on sequence data from two isolates of *P. colocasiae*, LT7299 and LT7291, recovered from taro in Phú Lương and Phú Hồ, Vietnam in 2010. The sequences were produced in conjunction with the company Floragenex (Floragenex, Inc., Portland, OR) using a technique known as restriction site associated DNA (RAD) sequencing

(Baird et al. 2008). The RAD technique focuses sequencing to the regions directly adjacent to a restriction enzyme cut site and includes cutting the genomic DNA with a restriction enzyme and modifying the restricted fragments to allow multiplexing and sequencing on the Illumina platform (Davey and Blaxter 2010). Any restriction enzyme can be used, although the amount of sequencing needed to cover the fragments will vary based on the frequency of the site in the genome. The restriction enzyme utilized was *SgrA1* (EC 3.1.21.4), an enzyme used recently with *P. capsici* (Lamour et al. 2012). The resulting sequences were aligned against the reference genome of *P. capsici* (available at http://genome.jgi-psf.org/Phyca11/Phyca11.home.html) and single nucleotide variant (SNV) sites were identified using CLC genomics workbench (CLC bio, Aarhus, Denmark). Reads were aligned using the following criteria: the reads must have at least 90% similarity to the reference and have an average quality of 20. Only those sites meeting these criteria with at least 10× coverage in both isolates were considered for SNV identification. Polymorphic sites were scored using the following criteria: loci with an alternate allele frequency between 35-65% were scored as heterozygous and loci with an alternate allele frequency <10% or >90% were scored as homozygous. Only loci where genotypes could be assigned for both isolates were considered for further marker development.

A subset of the SNV markers was selected to analyze the populations based on the following criteria: distribution of the markers among the 18 linkage groups recently described for the vegetable pathogen *P. capsici*, and if there were no other polymorphic sites within 50 bp on either side of the polymorphic site (Lamour et al. 2012).

SNP Genotyping

Genotypes were assessed using high resolution DNA melting analysis (HR-DMA) with a LightScanner device (Idaho Technology, Salt Lake City, UT). The device provides a sensitive measurement of the melting dynamics for short PCR amplicons (20 to 400 bp) with greatly increased sensitivity as the amplicon size decreases (Zhou et al. 2005). The device measures the loss of fluorescence that occurs when the LCGreen dye is released (and ceases to fluoresce) from double-stranded DNA during the melting. Primers were designed to produce amplicons between 45 and 55 bp containing a single SNP locus using the LightScanner primer design software 1.0 (Idaho Technology, Salt Lake City, UT). PCR was done in 384-well plates with a 5-µL final PCR reaction volume consisting of 10-20 ng genomic DNA, 0.5 µL 10× buffer, 0.2 µL 5 mM dNTPs, 0.05 µL 50 mM $MgCl_2$, 0.025 µL 100 µM forward and reverse primers, 0.1 units Taq Polymerase and 0.5 µL 10× LCGreen plus dye (Idaho Technology, Salt Lake City, UT). The PCR cycling parameters were: 2 min initial denaturation at 95ºC, 35 cycles of 95ºC for 30 s followed by 64ºC, and finally, 3 cycles of heating at 95ºC for 30 s and cooling to 25ºC for 30 s to produce heteroduplexes in amplicons containing heterozygous SNP loci. Resulting melt curves were analyzed using LightScanner 2.0 software (Idaho Technology, Salt Lake City, UT) according to the manufacturer's guidelines.

The LCGreen plus dye fluoresces when bound to double-stranded DNA. Amplicons containing a heterozygous SNP site will form heteroduplexes and melt differently (at a lower temperature) from either of the possible homoduplex genotypes (AA or aa) (Montgomery et al. 2007). In order to clearly differentiate the homozygous genotypes (AA from aa), a second step is performed where a defined homozygous amplicon is added to each reaction (following the initial melting)

and the reactions are re-melted (Gobena et al. 2011). If the known homozygous amplicon is identical to the unknown homozygote, the melt curves remain the same. If the SNP site has a different homozygous genotype, a heterozygote is produced which has a lower melting temperature and the melting curve shifts. The defined homozygous amplicons were produced in a separate PCR reaction using a synthetic template of the same length as the target. Synthetic templates were ordered from Integrated DNA Technologies, Inc. San Diego, CA. The PCR reaction includes 1 µL of 10 µM template synthetic oligonucleotides, 3 µL 10× buffer, 1.2 µL 5 mM dNTPs, 1 µL 10 µM forward and reverse primers, 0.3 units Taq polymerase and water in a 30-µL final reaction volume. The PCR protocol was 95ºC for 2 min for initial denaturation followed by 30 cycles of 95ºC for 30 s and 64ºC for 30 s. For the second melting, 2 µL of the defined homozygous amplicon was added and re-melted as above (Gobena et al. 2011).

Clonal lineages and allele frequencies

Only isolates with all 12 marker genotypes were included in the analyses. Isolates with identical genotypes are considered clonal lineages and were designated as CLX-Y(Z), where X is the clonal lineage identifier, Y is used to specify the country and Z is the number of isolates in the lineage. Once the clonal lineages were determined, allele frequencies were determined based on unique genotypes. Allele frequencies were estimated for the collection in total and for single countries. The chance of assigning an isolate to a clonal lineage was calculated by cross multiplying the probabilities for each of the individual SNP genotypes within a specific multi-locus genotype. Loci were tested for Hardy-Weinberg equilibrium using allele frequencies calculated within countries and for the total unique isolates using a chi-square test.

Results

Samples and mating type

A total of 217 isolates were recovered from Hawaii, 98 from Vietnam, and 71 from Hainan Island, China. Isolates were recovered from 12 different sites on four islands of Hawaii (Oahu, Kauai, Maui, and the Big Island) and eight sites each from Hainan Island, China and Vietnam (Figure 2.1, 2.2 and 2.3, respectively). Three of the isolates from Hainan Island did not produce oospores when crossed with our tester isolates or with a small collection of randomly selected A1 and A2 mating types and are referred to as neuter (data not shown). Hawaii had 215 A2 and two A1 mating types, Vietnam had 95 A2 and three A1 mating types, and Hainan Island had 47 A2 and 21 A1 mating types.

Sequencing and SNP identification

Illumina sequencing of the RAD libraries described above produced 3.7 million reads from LT7291 and 5.5 million reads from LT7299. Alignment to the *P. capsici* reference genome resulted in approximately 15% of the reads aligning. This provided coverage for a total of 389,269 nucleotide sites (nts) with 10× coverage in both isolates. Scoring these sites based on the above criteria resulted in 1152 polymorphic sites and 24,941 nts fixed sites for an alternate allele in both isolates compared to *P. capsici*. Of the polymorphic sites, 821 were heterozygous in LT7291 and 776 were heterozygous in LT7299.

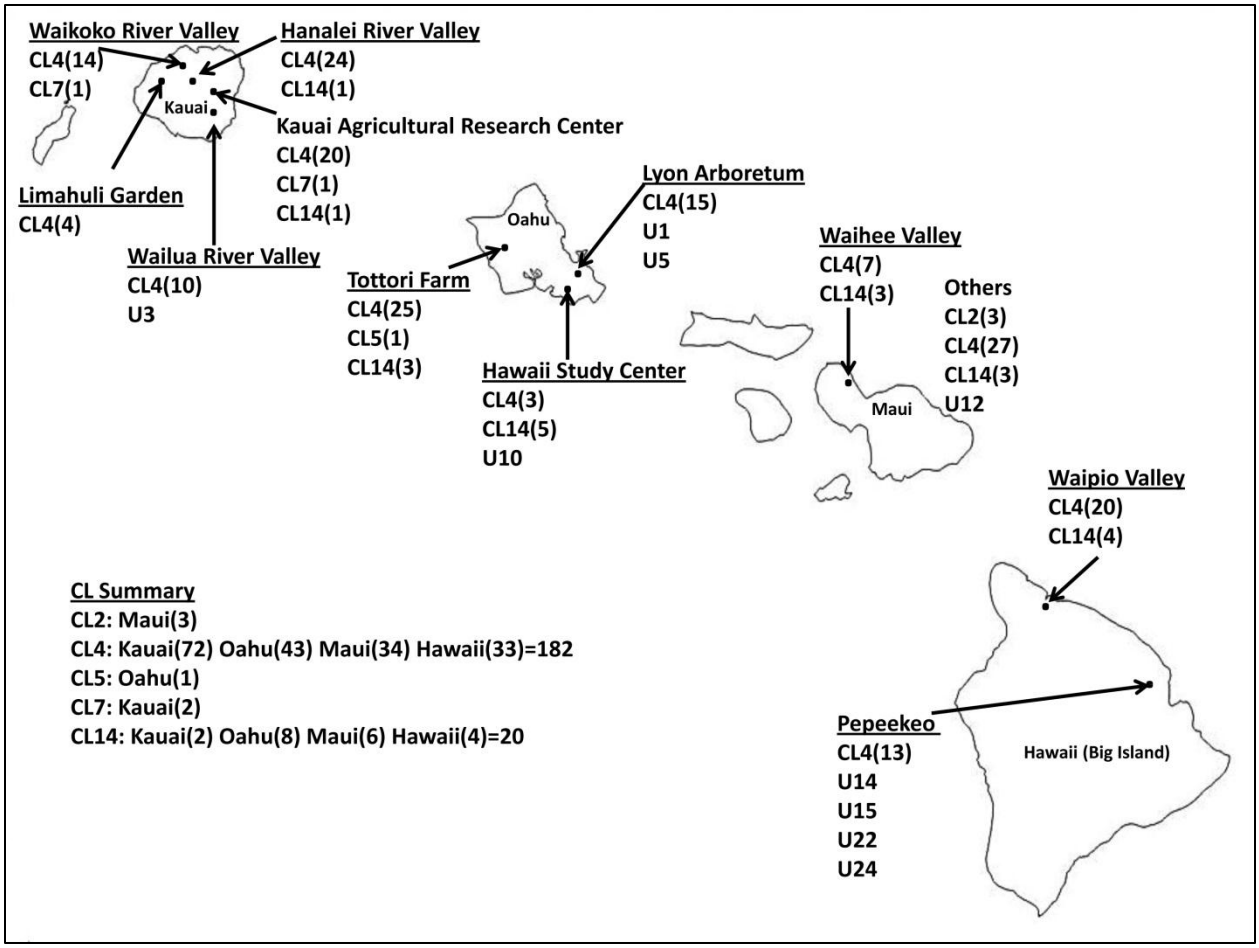

Figure 2.1 Distribution of unique and clonal genotypes in Hawaii. The number of isolates for the clonal lineages is in brackets.

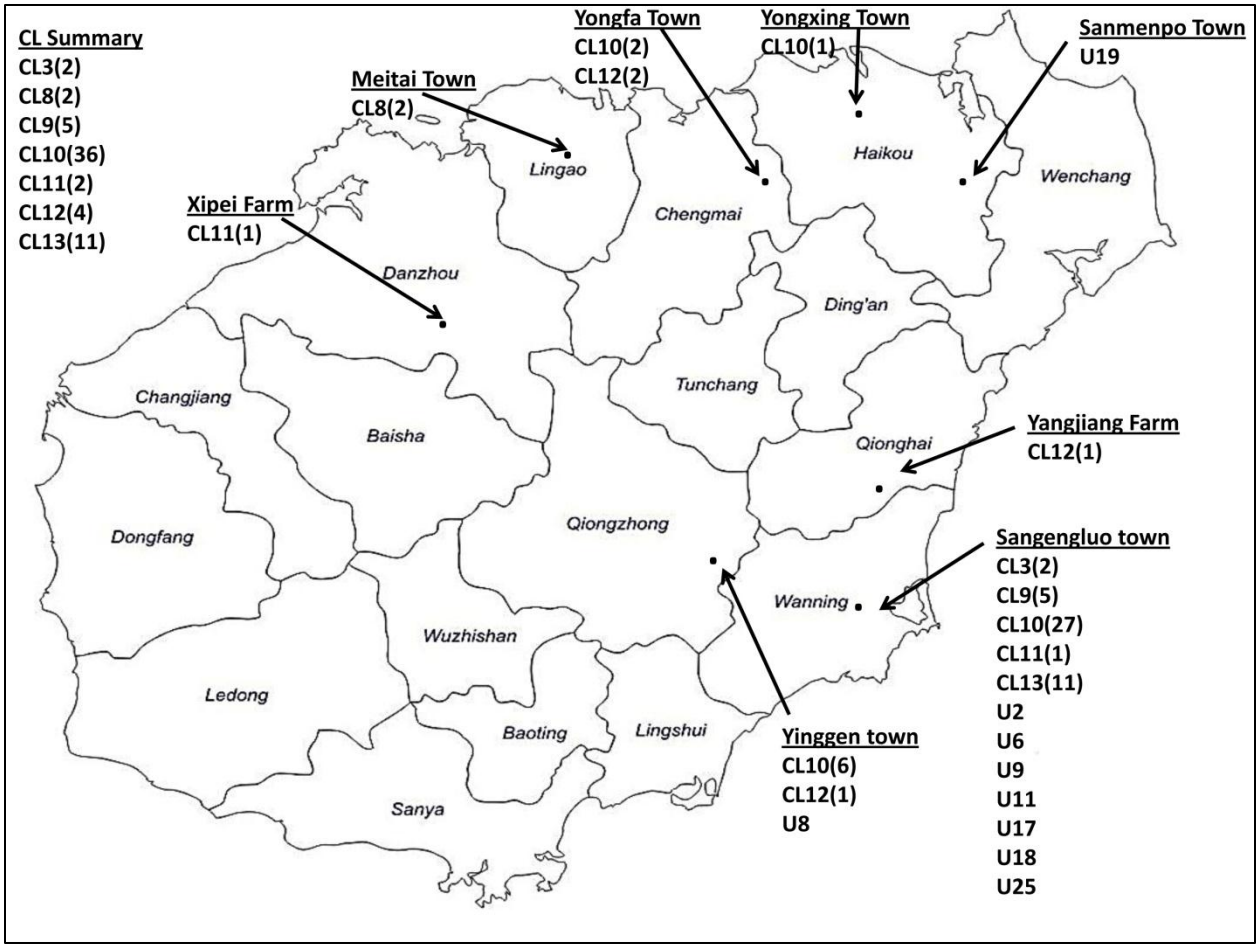

Figure 2.2 Distribution of unique and clonal genotypes on Hainan Island, China. The number of isolates for the clonal lineages is in brackets.

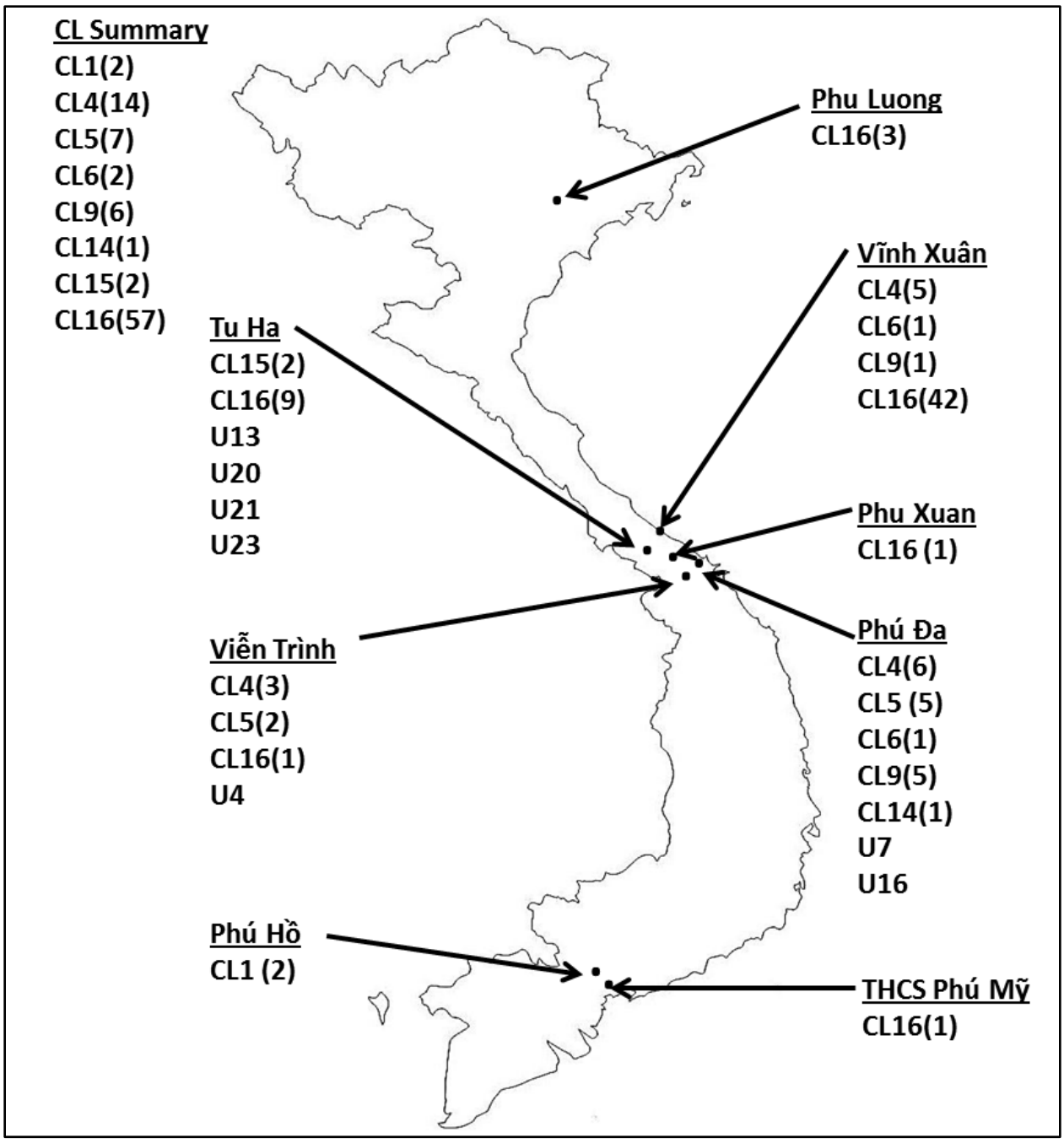

Figure 2.3 Distribution of unique and clonal genotypes in Vietnam. The number of isolates for the clonal lineages is in brackets.

Genotyping, clonal lineages, and allele frequencies

All isolates were successfully genotyped with all 12 SNP markers using HR-DMA (Table 2.1). Of the 4,632 genotypes assessed (9,264 separate genotyping reactions), there were five SNP markers that did not replicate perfectly in all isolates. Instead, these SNP markers showed clear heterozygous or homozygous genotypes for the biologically replicated samples. Further replication indicated that these were not genotyping errors. The same phenomenon was recently described as loss of heterozygosity (LOH) for *P. capsici* where heterozygous single nucleotide variant sites switched to homozygosity during asexual growth (Lamour et al. 2012). For *P. colocasiae*, LOH occurred on 18 occasions in a total of 16 different isolates. Of these, 13 instances occurred for SNP locus Sc26 _165308 (13 Hawaiian isolates), twice for locus Sc77_114487 (one Hawaiian and one Hainan isolate), and once for the loci Sc6_188972 (Hawaiian isolate), Sc11_181526 (Hawaiian isolate), and Sc47_379264 (Hainan isolate). Two isolates, one from Hawaii and one from Hainan had LOH occur on two different loci. Figure 2.4 shows representative curves for the first and second melting analyses for SNP locus Sc4_1087641. Although it is difficult (or impossible) to differentiate the two possible homozygous genotypes in the first melting, the homozygous and heterozygous curves are easily differentiated after adding a known homozygote and re-melting the sample.

Once all the isolates were genotyped, a total of 41 unique multi-locus SNP genotypes were identified. For genotyping purposes, the heterozygous genotype was retained for the five SNP loci undergoing LOH. The 41 unique genotypes were found as singletons (N = 25) or were part of 16 clonal lineages comprising between 2 and 196 members (Table 2.2). For all isolates combined, the allele frequencies of the least frequent allele varied from a low of 6% up to a high

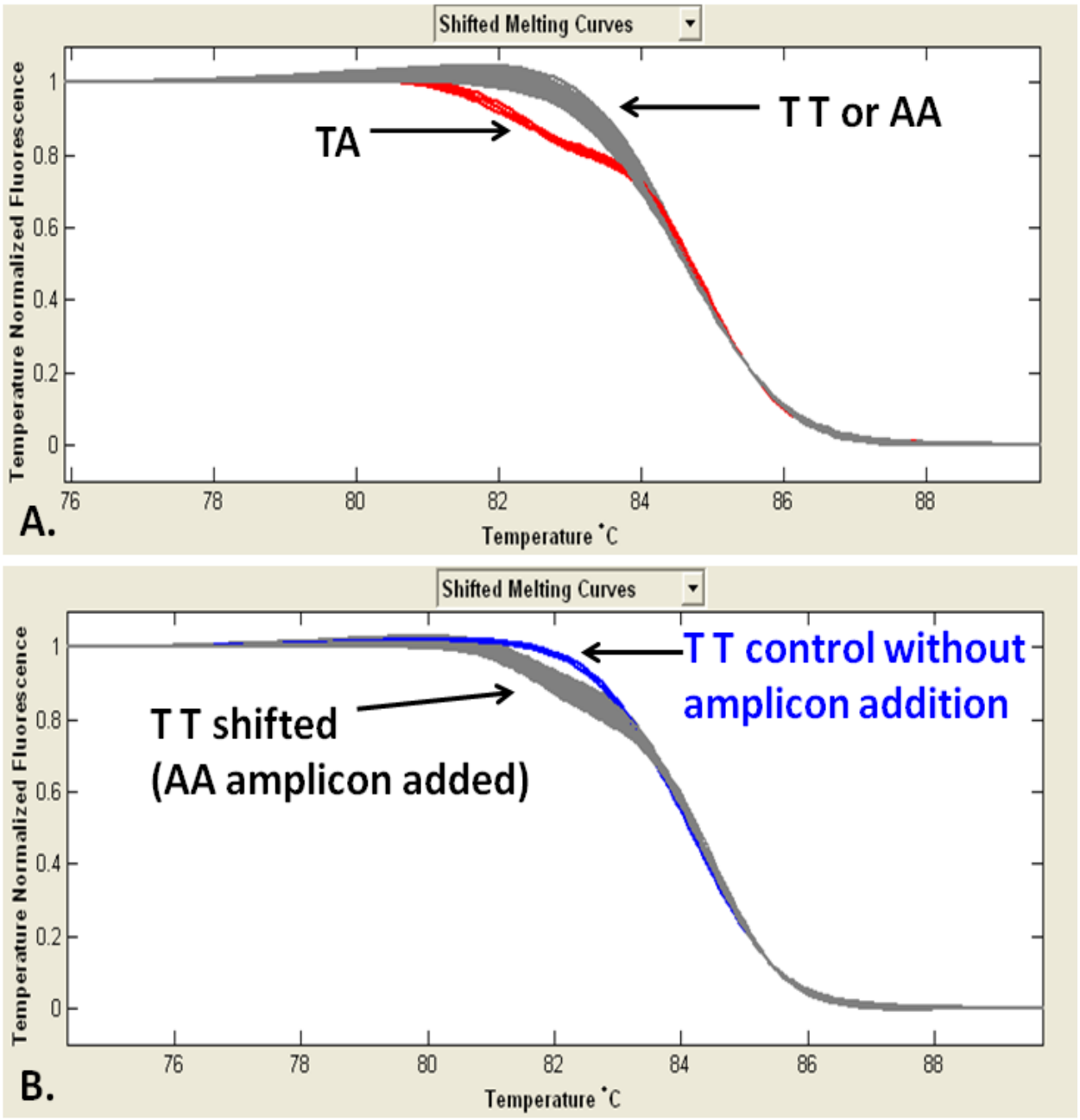

Figure 2.4 High resolution DNA melting analysis genotyping curves for SNP locus Sc4_1087641. Panel A is the normalized melting curve showing homozygous (TT or AA) and heterozygous TA (red) curves for 128 isolates from Hawaii. Panel B is the melt curve produced after the addition of the known homozygous amplicon containing AA. Note the shifting of the known TT (and many of the unknown isolates) to a typical heterozygous curve when the known PCR amplicon carrying the AA genotype was added to the reaction and re-melted.

of 44% (Table 2.3). Using only the unique genotypes from the individual countries, the allele frequencies varied from fixation (2 loci in Hawaii and 1 on Hainan) to >45% in each country. The chance of assigning an isolate to a clonal lineage was below 1% for each of the genotypes based on the combined and individual country allele frequencies. Tests for deviation from the expectations based on Hardy-Weinberg equilibrium showed that most of the loci with alternate allele frequencies between 30 and 70% deviate significantly from the expected under Hardy-Weinberg equilibrium in all scenarios.

Table 2.1 Summary information for SNP markers

SNP ID[a]	**Alleles**	**Forward primer**	**Reverse Primer**
Sc12 _713393	G/A	AGTTTTTGCACTGAGCAGTATC	CCAACACCTCCGGTCAG
Sc26 _165308	G/A	CTGGGGATTGAAGTGGCTA	GCCTCCATTTGGAAGCTTGATA
Sc4_637607	A/T	TCCCTCAAGTTCTCAACTTC	TGGTACGAGCCATGGTCT
Sc77_ 114487	T/C	AACGAAGAACGCACCAGTGT	CGTTGCGATAGTTCGGGT
Sc11_ 181526	C/T	GTCCATACTTCCGCGTG	TGCAGCACAACGGATCA
Sc47_ 379264	T/C	CGGCTCGACCTACGGCTGTC	ACCACGCGTTCCGTCGCCC
Sc2_ 462022	C/T	CACGAGAGACGCACTACA	CTGGCTACTTTGCCCTG
Sc4_1087641	T/A	TCGTAAAGCCCAGCACC	TAATGCAGAGGCAAGTGGAG
Sc17_55235	C/A	TACGTGAGCAACTCAGCAAA	GTGGTCGCGAATGCCAC
Sc6_188972	C/T	ACCGACCGAATTACTTCG	TGTCGCCATGAGCTACTT
Sc8_ 709842	C/T	ACGGAGGTCCGGCTTACCCT	GGGTGACATGGTGCTGGC
Sc4_ 622850	G/A	GTGGCTCTTAACTTGAT	GAGCCTTGGCGTACACAT

[a] Location in the reference genome of *P. capsici*, Sc = Scaffold followed by and underscore and the nucleotide location for the SNP locus.

For the 217 isolates recovered from the Hawaiian Islands, 14 different genotypes were identified (Figure 2.1) including five clonal lineages and an additional nine singleton genotypes. Clonal lineages ranged in size from 2 to 182 members. The islands were dominated by clonal lineages CL4-HI(182) and CL14-HI(20), which only differed at a single locus, Sc26 _165308.

CL4-HI(182) was recovered from all 12 collection sites on the Hawaiian Islands and both CL4-HI(182) and CL14-HI(20) were recovered from all four islands. Clonal lineages CL2-HI(3), CL5-HI(1), and CL7-HI(2) also differed from CL4-HI(182) by single loci (Sc47_ 379264, Sc8_ 709842, and Sc2_ 462022, respectively).

Table 2.2 Summary of clonal lineages (CL), unique genotypes (U), multi-locus genotypes, mating type, and distribution for *P. colocasiae*.

Clonal Lineage/Unique genotype	**Multi-locus SNP genotype [a]**	**Number of Isolates**	**Mating Type**	**Country [b]**
CL1	aaaaaaaaabab	2	A1	Vietnam
CL2	aabbbaaaabaa	3	A2	Hawaii
CL3	aabbbbaaaaaa	2	A1(1) A2(1)	Hainan Island
CL4	aabbbbaaabaa	196	A2	Hawaii (182) + Vietnam (14)
CL5	aabbbbaaabba	8	A2	Hawaii (1) + Vietnam(7)
CL6	aabbbbababaa	2	A2	Vietnam
CL7	aabbbbbaabaa	2	A2	Hawaii
CL8	abaaaaaaabab	2	A1	Hainan Island
CL9	abaabaaaabba	11	A1 (2) A2 (9)	Vietnam (6) + Hainan Island (3A2 and 2A1)
CL10	ababbaaaaaab	36	A1 (7) A2 (27) A0 (2)	Hainan Island
CL11	ababbaaaabab	2	A1	Hainan Island
CL12	ababbaaabaab	4	A1 (1) A2 (3)	Hainan Island
CL13	abbbbbaaaaaa	11	A1 (1) A2 (9) A0 (1)	Hainan Island
CL14	abbbbbaaabaa	21	A2	Hawaii (20)+ Vietnam (1)
CL15	bbbbbbbbaabb	2	A2	Vietnam
CL16	bbbbbbbbbabb	57	A2	Vietnam
U1	aaaaabaaaaaa	1	A1	Hawaii
U2	aaabbaaaaaab	1	A1	Hainan Island
U3	aabbbbaaabab	1	A2	Hawaii
U4	aabbbbacabaa	1	A2	Vietnam
U5	abaaabaaaaaa	1	A1	Hawaii
U6	abaabaaaaaba	1	A2	Hainan Island
U7	abaabaababaa	1	A2	Vietnam
U8	ababbbaaabab	1	A1	Hainan Island
U9	abbbbaaaaaaa	1	A2	Hainan Island
U10	abbbbbaaabbb	1	A2	Hawaii

U11	abbbbbaabaaa	1	A1	Hainan Island
U12	abbbbbbaabba	1	A2	Hawaii
U13	abbbbbbbbabb	1	A2	Vietnam
U14	acbbbbaaabaa	1	A2	Hawaii
U15	babbbbaaabaa	1	A2	Hawaii
U16	babbbbacabba	1	A2	Vietnam
U17	bbabbaaaaaab	1	A2	Hainan Island
U18	bbabbaaaabbb	1	A1	Hainan Island
U19	bbabbaabaaab	1	A1	Hainan Island
U20	bbbbbbabaabb	1	A2	Vietnam
U21	bbbbbbabbabb	1	A2	Vietnam
U22	bbbbbbbaabaa	1	A2	Hawaii
U23	bbbbbbbbaaab	1	A1	Vietnam
U24	bbbcabbaaaaa	1	A2	Hawaii
U25	cbabbaaaaaab	1	A2	Hainan Island

[a] The order is according to the order of SNP ID in Table 2.1 and a = homozygous for the most frequent allele, b = heterozygous, and c = homozygous for the alternative allele.
[b] Clonal lineages found in more than one country are in brackets next to the location designation.

For the 71 isolates recovered from eight locations on Hainan Island, China, a total of 16 distinct genotypes were identified (Figure 2.2). There were seven clonal lineages and an additional nine unique singleton genotypes. Clonal lineage CL10-HIC(36) was recovered from four of the eight collection sites and differed from clonal lineages CL11-HIC(2) and CL12-HIC(4) at single loci (Sc6_188972 and Sc17_55235, respectively). One collection site (Wanning County) had five clonal lineages and seven unique genotypes.

For the 98 isolates recovered from eight locations in Vietnam, a total of 15 multi-locus genotypes were identified (Figure 2.3). Eight were clonal lineages with seven additional singleton genotypes. Clonal lineage CL16-VN(57) was the most common with 57 members and was found at six locations. Clonal lineage CL4-VN(14), which is shared with Hawaii, was recovered from

three sites. CL16-VN(57) differed from CL4-VN(14) at eight of the twelve loci. Contrary to the previous finding by Lebot et al. (Lebot et al. 2003), our study suggested that clonally derived

Table 2.3 Summary of allele frequencies based on unique genotypes recovered from Hawaii, USA; Hainan Island, China; and Vietnam.

			Combined N = 41			Hawaii N = 14			Hainan N = 16			Vietnam N = 15		
SNP ID[a]	**Allele 1**	**Allele 2**	**%1**	**%2**	**χ2b**	**%1**	**%2**	**χ2**	**%1**	**%2**	**χ2**	**%1**	**%2**	**χ2**
Sc12 _713393	G	A	83	17	0.05	89	11	0.20	84	16	1.34	80	20	0.94
Sc26 _165308	G	A	67	33	8.64	71	29	0.04	56	44	9.68	70	30	2.76
Sc4_637607	A	T	70	30	7.89	57	43	7.88	87	13	0.33	60	40	6.67
Sc77_ 114487	T	C	60	40	17.72	54	46	4.70	59	41	7.49	60	40	6.67
Sc11_ 181526	C	T	56	44	25.11	61	39	5.86	53	47	12.5	53	47	11.48
Sc47_ 379264	T	C	68	32	8.84	54	46	10.52	87	13	0.33	60	40	6.67
Sc2_ 462022	C	T	90	10	0.50	86	14	0.39	100	0	-	87	13	0.36
Sc4_1087641	T	A	89	11	4.91	100	0	-	97	3	0.02	60	40	0.19
Sc17_55235	C	A	94	6	0.17	100	0	-	94	6	0.07	90	10	0.19
Sc6_188972	C	T	74	26	4.86	61	39	5.86	84	16	0.55	70	30	2.76
Sc8_ 709842	C	T	85	15	1.21	89	11	0.20	91	9	0.17	73	27	1.98
Sc4_ 622850	G	A	77	23	3.73	93	7	0.08	81	19	0.85	77	23	1.39

[a]SNP ID designates the scaffold and position of the markers on the *P. capsici* reference genome.

[b]Values >3.84 are significant at 5% and do not meet the expectations under Hardy-Weinberg equilibrium.

isolates may be shared between countries (Table 2.2). Clonal lineages CL4, CL5 and CL14 are shared between Hawaii and Vietnam and CL9 is shared between Vietnam and Hainan Island, China (Figure 2.5).

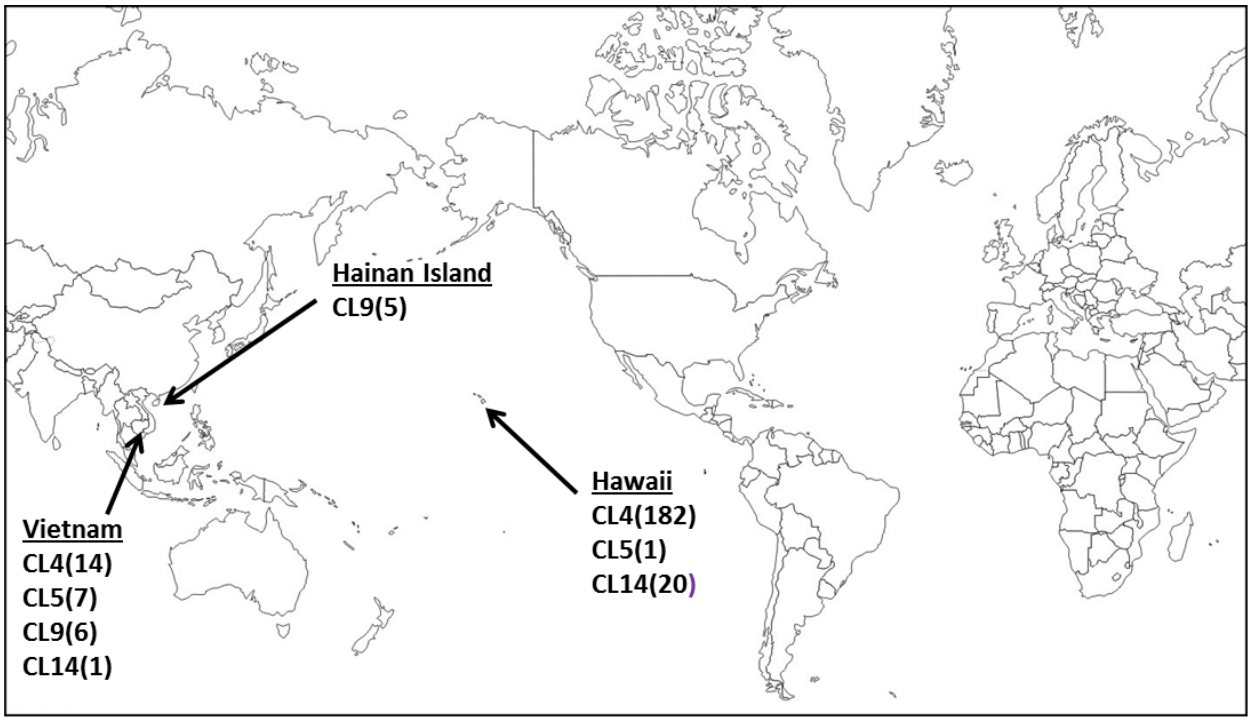

Figure 2.5 Clonal sharing between countries and number of isolates for the clonal lineages is in brackets.

Discussion

Taro is an important food crop in many areas of the world with production of 9 million tons in 2010 (FAO 2012). Taro is mainly grown for its starchy corms rich in carbohydrates, proteins, vitamins, and minerals, but the stem and leaves are also eaten (Sharma et al. 2009). *Phytophthora colocasiae*, which causes TLB, is one of the most important factors limiting taro crop production (Lebot et al. 2003; Sharma et al. 2009). Our objectives were to develop easily assayed co-dominant markers useful for characterizing diversity within and among populations of *P. colocasiae* and to apply these to populations in Hawaii, Vietnam, and Hainan Island. SNP markers have become the marker of choice in many biological systems as they are abundant,

often inherited as simple Mendelian characters, and there are many platforms that can be used for accurate genotyping. Previously, mating type and anonymous molecular markers (e.g. RAPDs and isozymes) were used to assess diversity in field populations of *P. colocasiae*. There are limitations with each of these approaches. The use of different mating type tester isolates may identify more or less neuter isolates, depending on compatibility of the isolates tested. For both isozymes and RAPD's, there are diverse methods to resolve polymorphisms, each of which can produce different results (size of the markers) making it difficult to share data. And finally, for any marker, it appears that LOH may alter our measure of the diversity present within the epidemic population by switching heterozygous autosomal DNA markers to homozygosity during the process of isolating and growing the isolates *in vitro* for genomic DNA production. Here, the overall impact of LOH appears to be slight but further investigation of this phenomenon is warranted.

Although there is nothing known about the *P. colocasiae* genome, the relatively close phylogenetic relationship of *P. colocasiae* to *P. capsici* proved useful to align the sequences and mine SNP sites. Despite only 15% of the *P. colocasiae* sequences aligning, it was possible to assess diversity across >300 Kbp of the *P. colocasiae* genome. Another way to analyze this sequence data is to compare the small contigs that were produced via RAD sequencing and to mine out polymorphic sites between the two isolates by a direct comparison of the homologous contigs. Although this would produce many more markers, the *P. capsici* genome and associated linkage map serve as a guide to the potential distribution of the sites. The 12 markers are found on 10 of the 18 *P. capsici* linkage groups and may be distributed similarly across the *P. colocasiae* genome. A further investigation of *P. colocasiae* testing markers for inheritance using

a laboratory cross would be useful to estimate linkage and to confirm them as simple Mendelian characters.

It is interesting and potentially significant that five different SNP markers showed evidence for loss of heterozygosity (LOH) in 16 of the 386 isolates analyzed. Although the overall frequency was low (less than 1%), the LOH occurred while the isolates were growing on agar plates or shortly after the small weft of mycelium was transferred to broth culture for mycelium production. Considering the massive numbers of asexual spores produced by *P. colocasiae* during sporulation on the leaf surface, even a low frequency may have important consequences. The LOH was more common with Hawaiian isolates; however, there were only two instances from Hainan and none observed for isolates from Vietnam. Also the frequency was clearly highest for locus Sc26_165308, suggesting the phenomenon may be more common in some areas of the *P. colocasiae* genome, at least in the Hawaiian population. LOH has recently been documented for *P. capsici* in clonally propagated isolates and was found to be more common in some areas of the genome (Lamour et al. 2012). Similar changes have been noted with other *Phytophthora* species during genetic marker assessment (Dobrowolski et al. 2002).

A 1970's study of *P. colocasiae* from Hawaii found only the A1 mating type (Ko 1979). Our study shows the A2 mating type is currently the most common on Hawaii and it is possible that the current epidemics are caused by a newly introduced strain that has replaced those active in the 1970's. It is also possible that the use of tester isolates from *P. capsici* may influence our findings. It is also possible that the use of tester isolates from *P. capsici* may influence our findings. As far as we know, the assignment of A1 and A2 mating types does not stem from an

archival source common to all species. A previous study of *P. colocasiae* on Hainan Island found three different mating types (A1, A2 and neuter) at locations across the island which is corroborated in our study (Zhang et al. 1994). The genotype results from Hainan Island are interesting as they include five different clonal lineages having A1, A2 and in two instances A0 isolates as part of the same clonal lineage (Table 2.2). A potentially related situation has recently been reported from Taiwan where self-fertile field isolates were able to be sub-cultured to A1, A2, and self-fertile asexual progeny (Lin and Ko 2008); illustrating that a switch in mating type can occur within a single culture during asexual growth. In light of the LOH documented for the various SNP loci, the mating type instability is likely the same phenomenon that has been documented for *P. capsici* where LOH at sites encompassing the mating type locus was associated with a mating type change (Lamour et al. 2012). We have also observed that isolates of *P. capsici* that contain both LOH and non-LOH loci across the mating type locus are self-fertile, producing abundant oospores in single culture (Kurt Lamour, unpublished data). Clearly, more work is needed to better understand the dynamics of LOH in the *P. colocasiae* genome and the stability of mating type. Our data, overall, suggest that mating type is a poor measure of diversity.

Although none of the sample sets contained sufficient unique genotypes to give a clear picture of the impact of sexual reproduction, the populations from Hainan and Vietnam had more loci meeting the expectations of Hardy-Weinberg equilibrium than Hawaii (Table 2.3). This is consistent with previous reports indicating that *P. colocasiae* was likely introduced to Hawaii and that the populations on Hainan Island may be much older and/or represent the center of origin for the species (Ko 1979; Zhang et al. 1994). The importance of clonal reproduction was

significant at all locations. This is not surprising as taro is present year round and is often continuously cropped using propagation material derived from the previous crop. A portion of the corm and leaf stem, known as a huli, is planted to start the new crop and may contain lesions from the previous epidemic. Although taro is produced on wet and dry land, most of the sampling here was from wet production sites, a situation highly conducive to asexual dispersal.

Genotyping indicates clonal lineages may spread beyond single islands or countries (Figure 2.5). At this point it is difficult to assess if this is due to the movement of plant material or if inoculum is dispersed via natural routes (e.g. carried via storms). Regardless of the route, it appears that lineages may be spread widely and this may be important in the context of breeding. Screening plants using a panel of isolates representing widely distributed clonal lineages may be useful to identify durable sources of resistance. In addition, since it is difficult to bring exotic strains of *P. colocasiae* into a breeding program (e.g. Hawaii), the dominant clonal lineages may be targeted for a genomics-based screening approach. For example, in the potato/*P. infestans* system, the R×LR effector genes are being cloned from virulent strains and the proteins expressed transiently in potato to track known resistance and to identify novel sources of resistance (Schornack et al. 2009). A similar approach may be feasible for the taro/*P. colocasiae* system where putative effector genes could be identified through more intensive sequencing, then cloned, and expressed transiently in taro to assist with the development of resistant lines.

The SNP markers described here can be assayed using a wide variety of approaches and the resulting genotypes are unambiguous. Although the genotypes are unambiguous, the stability of a heterozygous site is another matter. Although only a small proportion of the markers changed

due to LOH, it was only by chance that this was discovered. Clearly, LOH directly impacts genotyping and diversity studies and it may be difficult (or impossible) to determine if the LOH occurred naturally within the context of the epidemic or occurred after isolation and growth on nutrient media. It is possible that many of the unique genotypes on Hawaii are derived from a single clonal lineage. The only way to circumvent the effects of LOH may be to assess genotypes for *P. colocasiae* directly from individual lesions – skipping the isolation step altogether. This is currently being done to assess microsatellite markers in lesions of the late blight pathogen *P. infestans* (Li 2012).

In *P. capsici*, elevated levels of LOH were associated with loss of pathogenicity (Lamour et al. 2012) and this is another important factor to consider as it will obviously impact germplasm screening reliant on live isolates. Nonetheless, the SNP markers outlined here can be assessed using a wide variety of SNP-typing platforms and provide a starting point to characterize *P. colocasiae* at diverse locations, track the fate of clonal lineages over time, track the frequency of LOH during asexual growth, and may prove useful to better understand the dynamics of long range movement.

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Chapter Three

Phytophthora and *Pythium* baited from streams in east Tennessee in 2010 and 2011

Abstract

Ten streams in east Tennessee were surveyed for the sudden oak death pathogen *Phytophthora ramorum* in the spring and fall of 2010 and 2011. The surveys were conducted by immersing healthy *Rhododendron* leaves in streams for 7 to 10 days and dissecting resulting lesions onto selective media. A total of 137 isolates were recovered from a total of 12 baiting sessions (three in the spring and fall of each year). Sequence analysis of the ITS region identified 60 *Phytophthora* isolates, 76 *Pythium* isolates and one *Phytopythium* sp. These include five described *Phytophthora* species (*P. citricola, P. cryptogea, P. hydropathica, P. irrigata,* and *P. gonapodyides*), one undescribed taxon *Phytophthora* "Pgchlamydo", one undescribed *Phytophthora* species, seven described *Pythium* species (*P. helicoides, P. diclinum, P. litorale, P. senticosum, P. undulatum, P. vexans,* and *P. citrinum*), and three undescribed *Pythium* species. One newly described genus, *Phytopythium*, was also recovered in the survey. The biology and implications are discussed.

Introduction

Oomycetes thrive in wet environments and many of their evolutionary relatives (e.g. diatoms and brown algae) are aquatic (Baldauf et al. 2000; Garbelotto et al. 2001, Lamour and Kamoun 2009). Two important oomycete genera, *Pythium* and *Phytophthora*, contain species able to cause serious problems to plants within managed and wild settings. Organisms within each genera produce thick-walled asexually produced chlamydospores and sexually produced oospores for long-term survival, and asexually produced (thin-walled) sporangia which can cause infection when they directly germinate or cause infection indirectly by the release of swimming

zoospores (Erwin and Ribeiro 1996). Swimming zoospores survive for extended periods in free water; allowing spread via irrigation, drainage and natural waterways (Ghimire et al. 2009).

Identifying *Phytophthora* species is accomplished using various morphological and molecular approaches (Oudemans and Coffey 1991; Brasier et al. 1993a; Erwin and Ribeiro 1996). In many cases, identification is difficult based on morphological characters and the use of molecular genetic approaches is common (Cooke and Duncan 1997). The most frequently used genetic loci are based on the ribosomal RNA gene repeat (Lee and Taylor 1992; Cooke and Duncan 1997; Cooke et al. 2000). The highly repetitive ribosomal RNA gene contains non-translated sections known as internal transcribed spacers (ITS1 and ITS2) between the 18S and 28S genes. The 18S and 28S regions are highly conserved and are the basis for primers useful for amplifying the ITS spacers in most *Pythium* and *Phytophthora* species (Cooke et al. 2000; Paul 2001; André LéVesque and De Cock 2004).

Some common nursery hosts, such as *Rhododendron*, are susceptible to many *Phytophthora* species including *P. cinnamomi*, *P. cactorum*, *P. citricola*, *P. lateralis*, *P. cryptogea*, *P. citrophthora*, *P. gonapodyides*, *P. megasperma*, and *P. ramorum* and the leaves are used as bait materials to monitor natural areas. Baiting with *Rhododendron* leaves has proved useful to detect *P. ramorum* in streams in California, Oregon, and Washington State (Hoitink and Schmitthenner 1974; Werres et al. 2001; Oak et al. 2008). Surveys for SOD in the USA are ongoing at nurseries and natural areas considered potentially high risk, including Tennessee (Rizzo et al. 2002; Donahoo and Lamour 2008; Oak et al. 2008; Sutton et al. 2009; Hulvey et al. 2010). In Tennessee, surveys have been conducted since 2004 and *P. ramorum* was recovered from

nurseries in 2004 and 2005 (California Oak Mortality Task Force 2012). *Phytophthora ramorum* has also been isolated from nurseries in 20 additional states (California Oak Mortality Task Force 2012). In 2004-05, seven different species of *Phytophthora* including *P. cactorum, P. citricola, P. citrophthora, P. nicotianae, P. palmivora, P. tropicalis* and the new species *P. foliorum* were recovered from nurseries in Tennessee (Donahoo and Lamour 2008). Subsequent surveys identified five *Phytophthora* spp. (*P. citrophthora*, *P. citricola*, *P. nicotianae*, *P. syringae*, and *P. hydropathica*) from statewide nurseries and four species (*P. citrophthora*, *P. citricola*, *P. irrigata*, and *P. hydropathica*) from streams of eastern Tennessee (Hulvey et al. 2010).

Phytophthora species attack a wide range ornamental and tree hosts. The high density of plants, and in some cases the use of recycled irrigation water, makes tree and ornamental plant nurseries an ideal setting for epidemics (Ribeiro and Linderman 1991; Davidson et al. 2003). Several species of *Phytophthora*, including *P. cinnamomi*, *P. citricola*, *P. cryptogea*, *P. gonapodyides, P. quercina*, *P. cambivora,* and *P. cactorum,* have been found to be associated with the oak tree ecosystems (Vettraino et al. 2002; Balcì and Halmschlager 2003), with *P. quercina* (Vettraino et al. 2002) and *P. cinnamomi* (Brasier et al. 1993b) being the most significant. The broad host range pathogen *P. ramorum* was first identified in Germany and the Netherlands from *Rhododendron* and *Viburnum* (Werres et al. 2001). *Phytophthora ramorum* attacks plants in 12 families including Ericaceae and Fagaceae and some oak species (*Quercus* spp.) and tanoak (*Lithocarpus densiflora*) are highly susceptible (Davidson et al. 2003). The disease is known as 'Sudden Oak Death' (SOD) because some trees are killed within 2 to 4 weeks (Davidson et al. 2003). This disease was first observed in mid 1990s, affecting oak and tanoak of coastal forest in

California (Rizzo et al. 2005). *Phytophthora ramorum* is distributed in Europe, USA, and Canada (Davidson et al. 2003) and has caused extensive damage to forests in coastal areas of the Western USA (Rizzo and Garbelotto 2003). In addition, *P. ramorum* has been recovered from soil samples at forest sites with diseased trees (Fichtner et al. 2007).

Tennessee is one of the largest producers of oak trees and has significant forested natural areas important to tourism and the statewide economy (Forest product extension 2012). In 2009, forests contributed 4% to the Tennessee economy, which was valued at $21 billion (Menard et al. 2011). To date, *P. ramorum* has not been recovered from natural settings in Tennessee. Here we report species diversity for *Phytophthora* and *Pythium* recovered using *Rhododendron* leaf stream baiting at ten watersheds in eastern Tennessee in 2010 and 2011.

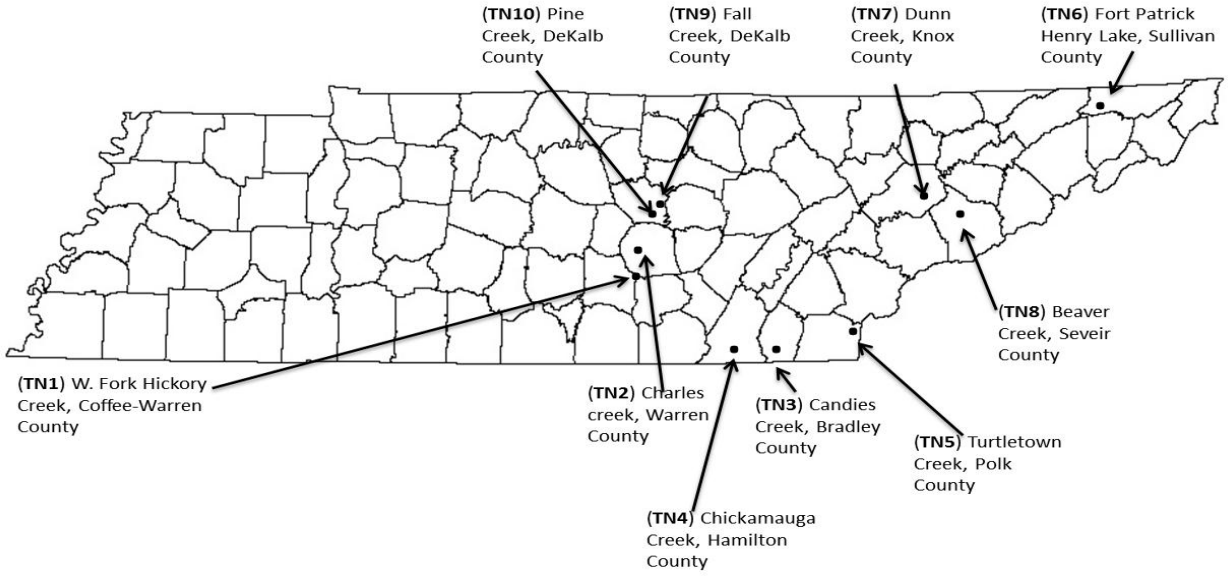

Figure 3.1 Ten east Tennessee locations surveyed for oomycetes using healthy *Rhododendron* leaves

Materials and Methods

Sampling and pathogen isolation from streams

Stream sites were selected at ten key watersheds (Figure 3.1). Baiting was conducted during the spring and fall of 2010 and 2011. Asymptomatic healthy *Rhododendron* leaves were collected from naturally occurring *Rhododendron* at the base of the Smoky Mountains. The leaves were stored at 5°C for one to three weeks before baiting sessions. Baits were deployed in a mesh bag and anchored by lines to the shore and a weight to hold the bags in place (Figure 3.2A and B). Two bags were placed at each location with four leaves in each bag. Each baiting session was 7 to 10 days and there were three baiting sessions in the spring and fall of each year. The leaves were collected and washed in tap water and four leaves were randomly selected for tissue isolation. The other four leaves were sent to the Plant Disease Diagnostic Lab at Pennsylvania Department of Agriculture for genetic testing for *P. ramorum*. The leaves processed in Tennessee were inspected for water soaked lesions and a 10-mm leaf disk containing both healthy tissue and necrotic lesions was removed aseptically and transferred to dilute V8-PARP + H agar (40 mL V8 juice, 3 g $CaCO_3$, 16 g Bacto agar and 960 mL water amended with 25 ppm pimaricin (Sigma Aldrich, St. Louis, MO), 100 ppm ampicillin (Fisher Scientific, Pittsburg, PA) 25 ppm rifampicin (Sigma Aldrich, St. Louis, MO), 25 ppm pentachloronitrobenzene (Fisher Scientific, Pittsburg, PA) and 71.4 mg/L Hymexazol (Bayer Corporation, Pittsburgh, PA) (Hulvey et al. 2010). After incubation for 1 to 3 days, mycelium was transferred onto water agar and single hyphal tip cultures were transferred to full strength V8-PARP agar (Figure 3.2C). Hyphal-tip cultures were then assigned a unique identifier.

Figure 3.2 (A) Deployment of *Rhododendron* leaves held in a mesh bag in a stream in east Tennessee. (B) Mesh bag with four compartments to hold four *Rhododendron* leaves and nylon rope for tethering the mesh bag. (C) Typical *Phytophthora* mycelial growth visible after 1-3 days. This plate shows the isolation of *P. ramorum* from leaf material from a nursery in Tennessee in 2005.

Long term storage, DNA extraction, and ITS amplification

For long-term storage, three to four plugs (7-mm diameter) of actively expanding mycelium were transferred into 2-mL screw-cap tubes preloaded with 1 mL sterilized water and three to four sterilized hemp seeds and stored at room temperature. Mycelium was produced and genomic DNA extracted according to the protocol described by Lamour and Finley (Lamour and Finley 2006).

The ITS region of putative *Phytophthora* isolates was PCR amplified using the universal primers ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC 3') (White et al. 1990). The PCR reaction had a 30-µL final volume consisting of 3 µL 10× buffer, 1.2 µL dNTPs at 5 mM, 1.5 µL DNA at 50-100 ng/µL, 1 µL TAQ polymerase, and 0.1 µL primers at 100 mM conc. The PCR cycling parameters were as follows: initial denaturation at 94°C for 2 min followed by 29 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 25 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products were treated to remove excess primers and dNTPs with ExoSAP-IT reagent (USB Corp, Cleveland, OH). The reagent was added at the rate of 2 µL per 5-µL PCR products and treated at 37°C for 15 min, followed by 80°C for 15 min (Bell, 2008; Dugan et al., 2002). Both ITS4 and ITS5 primers were used for sequencing at the University of Iowa DNA sequencing facility (Iowa City, IA). Sequences were assessed and assembled using CodonCode Aligner 3.7.1 software (CodonCode, Dedham, MA). Sequences were trimmed by removing any region with 2 bases below a PHRED score of 20 in a 40-bp window. High quality sequences were searched against National Center for Biotechnology Information (NCBI) Genbank non-redundant nucleotide database and the curated *Phytophthora* databases PhytophthoraDB

(http://www.phytophthoradb.org/) and Phytophthora-ID (http://www.phytophthora-id.org/) in July 2012.

Results

A total of 137 isolates were recovered from 10 different streams in 2010 and 2011. The sequence for the complete ITS1, 5.8s and ITS2 was searched against NCBI and *Phytophthora* database. The described and un-described species are listed in Table 3.1. In total, 12 different species with four unknowns were identified as: *Phytophthora citricola*, *Phytophthora cryptogea*, *Phytophthora hydropathica*, *Phytophthora irrigata*, *Phytophthora gonapodyides, Phytophthora* Unk, *Pythium helicoides*, *Pythium diclinum*, *Pythium litorale*, *Pythium senticosum, Pythium undulatum*, *Pythium vexans*, *P. citrinum*, *Pythium* Unk1, *Pythium* Unk2, and *Pythium* Unk3. Further, *Phytophthora* "Pgchlamydo" and the newly described genus *Phytopythium* were also identified during this survey. The following species, *P. citricola*, *P. cryptogea*, and *P. gonapodyides* were used previously in phylogenetic analyses of the genus *Phytophthora* spp. (Cooke et al. 2000). The other identified species were previously published in peer reviewed journals: *Phytophthora hydropathica* (Hong et al. 2010), *Phytophthora irrigata* (Hong et al. 2008a), *Pythium citrinum* (Paul 2004), *Pythium helicoides*, (Kageyama et al. 2002), *Pythium diclinum* (Abdelzaher 2004), *Pythium litorale* (Nechwatal and Mendgen 2006), *Pythium senticosum* (Senda et al. 2009), *Pythium undulatum* (Shafizadeh and Kavanagh 2005) and *Pythium vexans* (Zeng et al. 2005). The species recovered from 10 different sites of east Tennessee is presented in Table 3.1. There was a single unknown *Phytophthora* species identified in this study. Members of *Phytophthora* Unk were identical at both ITS1 and ITS2. This *Phytophthora* Unk has been previously recovered from forest soil and stream baiting in Northwestern Yunnan province, China (JQ755232.1). The nearest known species was

Phytophthora cryptogea with 100% coverage and 97% maximum identity. The search of this *Phytophthora* Unk in the curated *Phytophthora* database confirmed that this is unknown species. *Phytophthora* Unk had 98% (PD_01526_ITS) and 97% (AF541908.1) maximum identity with unknown *Phytophthora* sp. in PhytophthoraDB and Phytophthora-ID respectively. There were other three unknown *Pythium* species (*Pythium* Unk1, *Pythium* Unk2 and *Pythium* Unk3), which were not able to hit any of the known *Pythium* species. A search of the sequences deposited at the NCBI indicates *Pythium* Unk1, *Pythium* Unk2 and *Pythium* Unk3 had some level of similarity to *Pythium diclinum*, *P. helicoides* and *P. diclinum* with 94%, 89%, and 94% maximum identity, respectively.

Table 3.1 Summary data for *Phytophthora* and *Pythium* species identified from streams.

Name	**NCBI Taxonomy ID/Accession No.**	**Number of Isolates**	**Query Coverage**	**Maximum Identity**	**Locations**
***Phytophthora* species**					
P. citricola	53984	4	100	99	TN2, TN3, TN6, TN9
P. cryptogea	4786	36	100	100	TN1, TN2, TN4, TN5, TN6, TN7, TN8, TN9, TN10
P. hydropathica	565288	5	100	100	TN1, TN2, TN7
P. irrigata	565287	6	100	100	TN2, TN3, TN4, TN8, TN9
P. gonapodyides	78237	2	100	100	TN2, TN9
P. "Pgchlamydo"	479473	3	100	99	TN7, TN10
Phy Unk	JQ755232.1	4	100	99	TN2, TN9
***Pythium* species**					
P. citrinum	221743	1	100	98	TN5
P. diclinum	156628	5	100	99	TN2, TN3, TN6, TN7
P. helicoides	126844	2	99	99	TN1, TN4
P. litorale	340183	25	100	99	TN1, TN2, TN3, TN5, TN6, TN7, TN8, TN9, TN10
P. senticosum	643143	1	100	99	TN5
P. undulatum	127444	4	100	99	TN2, TN4, TN5

P. vexans	42099	1	99	99	TN7
Py Unk1	HQ261735.1	30	100	96	TN1, TN2, TN3, TN4, TN5, TN6, TN7, TN8, TN9, TN10
Py Unk2	DQ232768.1	1	100	100	TN9
Py Unk3	HQ261735.1	6	100	97	TN1, TN2, TN8, TN10
***Phytopythium* sp.**	795339	1	99	89	TN10

Discussion

Forests are an important part of the economy of Tennessee for tourism (e.g. Great Smoky Mountain National Park) as well as hardwood. In 2008, Tennessee produced 881 million board feet of hardwood lumber and is a top U.S. producer (Menard et al. 2011). *Phytophthora ramorum*, which causes SOD, has killed thousands of forest oak trees in California since it was first reported in 1995 (Rizzo et al. 2002). *Phytophthora ramorum* has been recovered from nurseries in many different states, including Tennessee, and has also been recovered from streams in California, Oregon, and Washington (California Oak Mortality Task Force 2012). The overall cost for survey work, quarantines, and destroyed nursery material is difficult to estimate, but is easily in the millions of U.S. dollars. Our objective was to monitor *Phytophthora* species in streams draining 10 different watersheds in east Tennessee. In the process, we also collected information on *Pythium* species recovered over the course of the study.

Rhododendron leaf baiting over a period of roughly 3 weeks in the spring and fall of 2010 and 2011 revealed multiple known and undescribed species of *Phytophthora* (N = 60) and *Pythium* (N = 76). In previous surveys, our laboratory selectively excluded putative *Pythium* species from further analyses (based on the rapid growth rate). In these surveys, any fungal-like isolate with coenocytic mycelium (typical for the oomycetes) was analyzed to better understand overall

oomycete diversity and to avoid missing any fast growing *Phytophthora* species. The most frequently occurring *Phytophthora* species was *P. cryptogea.* As in the previous survey done by Hulvey et al., *Phytophthora* Unk was recovered from streams in east Tennessee but the recent unknown were not identical to the previous one. Another survey of irrigation water reservoirs in Virginia also reported nine different species of *Phytophthora* and hundreds of isolates were identified as unknown taxa of this genus (Bush et al. 2006; Hong et al. 2008a). *Phytophthora cryptogea* was also reported in previous surveys in eastern Tennessee (Donahoo and Lamour 2008; Hulvey et al. 2010). *Phytophthora cryptogea* has also been isolated from the irrigation water survey in Virginia and water reservoirs in Germany (Themann et al. 2002; Bush et al. 2003). This study showed that *P. cryptogea* was present in nine out of 10 baiting locations in east Tennessee (Table 3.1). The occurrence of *P. cryptogea* was more common in fall compared to spring season in both years (Figure 3.3). *Phytophthora cryptogea* was present more in somewhat cooler environment in fall with average water temperature of 13°C compared to 18.5°C in spring. It seems that *Phytophthora cryptogea* is more dominant in cool weather conditions compared to warm spring conditions. *Phytophthora cryptogea* causes minor root rot of *Rhododendron* (Hoitink and Schmitthenner 1974) but the interaction with oak trees is unknown (Balcì and Halmschlager 2003).

In this study, another frequent species was *Phytophthora irrigata*, which was identified in six isolates from five locations. *Phytophthora irrigata* is a newly described species, which has been recovered from streams, rivers, and irrigation reservoirs of different places including Tennessee (Hong et al. 2008a; Hong et al. 2008b; Hulvey et al. 2010; Ghimire et al. 2011). Experimental inoculations indicate *P. irrigata* is pathogenic to azaleas and some vegetable crops including

pepper, tomato, and eggplant (Hong et al. 2008b); however, damage to these plants has not been reported under field conditions.

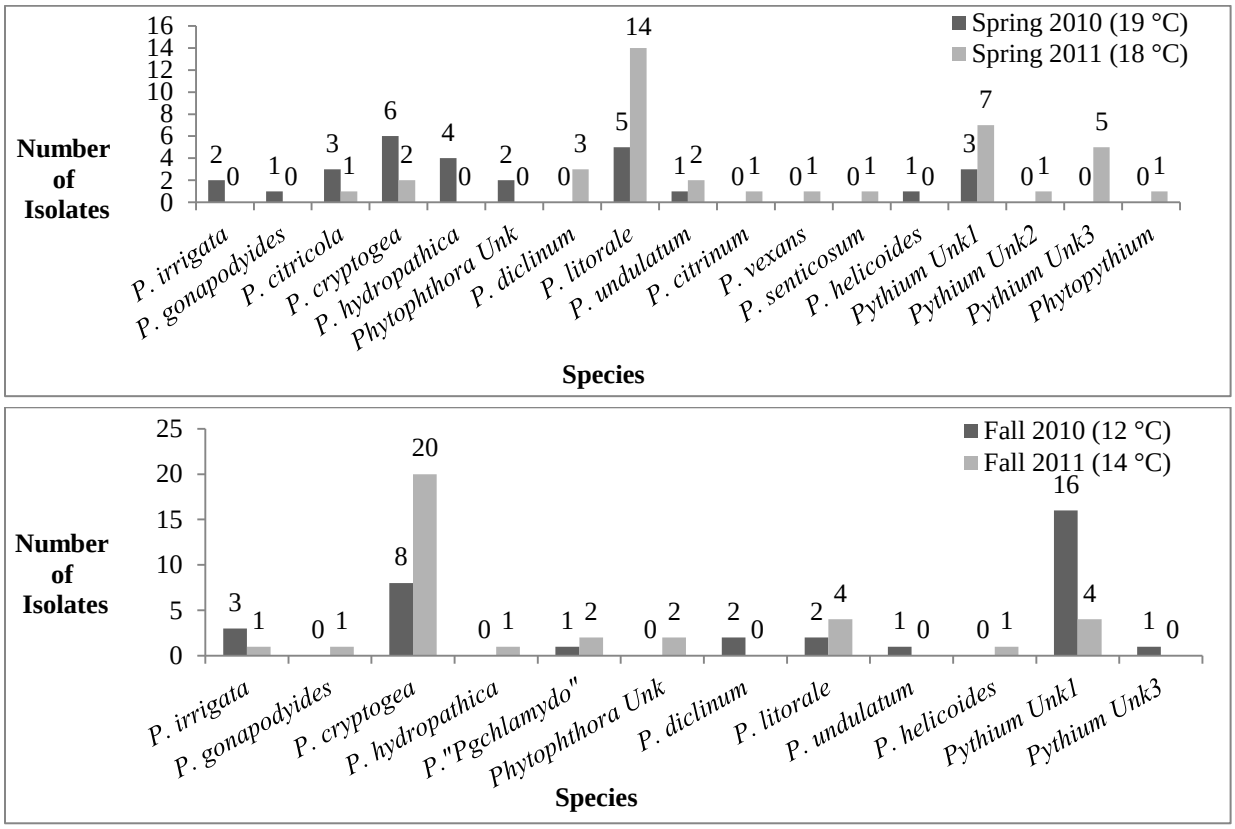

Figure 3.3 Species recovered during the spring and autumn baiting sessions in 2010 and 2011. Average temperature for spring and fall are listed in brackets.

Phytophthora citricola is another species recovered from four different locations. This species was also reported in similar stream and nursery surveys in Tennessee (Donahoo and Lamour 2008; Hulvey et al. 2010). *Phytophthora citricola* has very wide host range, which include economically important fruit trees such as avocado, walnut, and almond, and forest trees (Bhat and Browne 2007). *P. citricola* was also recovered previously from irrigation water (MacDonald et al. 1994; Hong and Moorman 2005). The previous study showed that *P. citricola* recovered in

Tennessee are genetically diverse, are transported to different places through nursery stocks (Donahoo and Lamour 2008; Hulvey et al. 2010), and might be a threat to more forest trees.

Another *Phytophthora* species recovered from streams was *Phytophthora hydropathica*. This species was isolated from irrigation reservoirs and first described as a new species in 2010 (Hong et al. 2010; Ghimire et al. 2011). In previous research, *Phytophthora hydropathica* has also been isolated from the watersheds and nurseries of eastern Tennessee (Hulvey et al. 2010). *Phytophthora hydropathica* is an important pathogen, common in rivers and irrigation water, of various ornamental species, which causes leaf blight and leaf necrosis of *Rhododendron*, and root rot of Kalmia (mountain laurel) (Hong et al. 2008b; Hong et al. 2010). Since *Phytophthora hydropathica* is widespread in watersheds of eastern Tennessee, this may rise as a threat to the nursery ornamentals as well as forest hosts.

Phytophthora gonapodyides was recovered in two locations during this stream survey. *Phytophthora gonapodyides* has also been recovered from stream, reservoir, soil and stem samples of declining oak stands (Jung et al. 1996; Balcì and Halmschlager 2003; Bush et al. 2003; Ghimire et al. 2011). Previous studies have shown that *Phytophthora gonapodyides* is pathogenic to conifer trees (Hamm and Hansen 1982) and many other ornamentals (Erwin and Ribeiro 1996).

One undescribed taxon, *Phytophthora* "Pgchlamydo" (Brasier et al. 2003) was also recovered from two locations. *Phytophthora* "Pgchlamydo" was also recovered previously from ornamentals plants, forest watershed and stream baiting (Moralejo et al. 2009; Reeser et al.

2011a; Reeser et al. 2011b). Artificial inoculation of *Phytophthora* "Pgchlamydo" in *Rhododendron* has shown that this new taxon can cause leaf lesions (Schwingle and Blanchette 2008). During this survey one newly described genus *Phytopythium* was recovered from one isolate (Bala et al. 2010)

Most species recovered in this survey were present year round regardless of the season (Figure 3.3) *Phytophthora cryptogea* and *P. irrigata* were more common in fall while *P. citricola* was only present in spring (Figure 3.3). Although *P. ramorum* was not detected at these sites over the course of two years, the presence of diverse known and unknown *Phytophthora* species may present a threat to plants grown commercially or present in the environment. More work is needed to formally describe the unknown species and to test their potential host range.

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Chapter Four

Conclusions

The genus *Phytophthora* contains a number of important pathogens that attack a wide range of hosts worldwide. Taro is a staple crop in Asia, Africa and Pacific regions and disease caused by *P. colocasiae* destroys the edible leaves and significantly reduces the production of corms. Our investigation of *P. colocasiae* populations in Hawaii, Vietnam and Hainan Island, China suggests clonal lineages are widely dispersed and shared among geographically separated countries. Although our study does not provide insight into how the isolates are dispersed, it may prove useful for taro breeding. In this scenario, breeding to produce resistant taro varieties able to defend against the most widely distributed clonal lineages may be the best way to limit disease.

The Sudden Oak Death pathogen *P. ramorum* poses a significant threat to natural woodlands in eastern Tennessee. Many of the understory trees (*Rhododendron* and mountain laurel) are known hosts and in epidemic areas, often serve as disease amplifiers with high levels of spore production from foliar lesions but not necessarily succumbing to disease. *Phytophthora ramorum* has been recovered from nursery plants in Tennessee on two different occasions and although surveys are useful and may help limit spread, clearly they cannot stop all movement and *P. ramorum* has made it into the environment in multiple states. The stream monitoring reported here provides a useful baseline of *Phytophthora* and *Pythium* species diversity, indicating there is diversity currently unknown to science. These species may pose a threat to valuable plants or may play an important role in the natural ecosystem and further research is warranted.

Vita

Sandesh Kumar Shrestha was born in Narayangarh, Chitwan and raised in Dumre, Tanahun in the central part of Nepal. He completed his elementary school at the Fishtail English Boarding School and high school at the Orchid Science College. He received his B.Sc in Agriculture from the Institute of Agriculture and Animal Science at Tribhuvan University in 2008. In 2008, he joined the Nepal Agricultural Research Council and worked in the Plant Pathology laboratory for about a year. He started his Master's degree in the Department of Entomology and Plant Pathology at the University of Tennessee in fall 2010 and completed his M.S in August 2012. He will continue his Ph.D. in plant breeding and work in the field of agriculture.