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Variation among *Phytophthora cinnamomi* isolates from oak forest soils in the eastern United States

Jordan E. Eggers
West Virginia University

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Variation Among *Phytophthora cinnamomi* Isolates from Oak Forest Soils in the Eastern United States

by

Jordan E. Eggers

Thesis submitted to the
Davis College of Agriculture, Natural Resources and Design
at West Virginia University
in partial fulfillment of the requirements
for the degree of

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in
Plant and Soil Science
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William L. MacDonald, Ph.D., Chair
Yilmaz Balci, Ph.D.
Daniel G. Panaccione, Ph.D.
Joseph B. Morton, Ph.D.
Mannon E. Gallegly, Ph.D

Division of Plant and Soil Sciences

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Abstract

Variation Among *Phytophthora cinnamomi* Isolates from Oak Forest Soils in the Eastern United States

Jordan E. Eggers

Phytophthora cinnamomi isolates, collected from eastern US forest soils, were studied to determine the extent of phenotypic, genotypic, and pathogenic variation present. Four microsatellite loci were targeted for genetic analysis. Phenotypic characteristics measured were sporangia length, width, and length:width ratio; chlamydospore, oogonia, and oospore diameter; antheridia width, colony morphology on PDA; and colony growth rate on clarified V8 agar at 20°C, 24°C, 28°C, and 32°C. Red oak logs were inoculated with select isolates to determine relative pathogenicity. Microsatellite analysis showed that the genetic variability of the eastern US oak forest *P. cinnamomi* isolates was low. Isolates were grouped into two microsatellite fingerprint groups (MFG). Isolates in microsatellite group one (MFG1) were characterized by DNA fragment lengths of 120 and 122 bp at locus d39, 169 and 170 bp at locus e16, and 254 and 255 bp at locus g13. Microsatellite Fingerprint Group 2 (MFG2) isolates were characterized by marker sizes of 122 and 124 bp at locus d39, 161 and 163 bp at locus e16, and 247 and 248 bp at locus g13. Similar microsatellite profiles have been found in *P. cinnamomi* isolate populations from Australia and other regions of the world. The low level of genetic variability and microsatellite marker similarities to *P. cinnamomi* isolates from other continents supports the theory that *P. cinnamomi* is not indigenous to the U.S. and may provide clues as to the origin of US *P. cinnamomi*. Asexual and sexual spore dimension varied greatly within the populations, but were similar to previous descriptions. Differences in phenotypic characteristics among isolates were most pronounced when data were grouped by MFG, the most significant being colony morphology and growth rate. Isolates expressed either rosaceous or stellate colony morphology. There was some correlation between colony morphology and MFG, but colony morphology alone could not be relied upon to predict the genotype of an isolate. Differences in growth rates of microsatellite fingerprint groups were observed with MFG1 being less tolerant of increased incubation temperatures. No variation in pathogenicity on red oak logs was observed.

Dedication

I dedicate this work to my wonderful mother and father, my sisters, my grandparents and my extended family. I thank them for their support and encouragement.

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1. Introduction

There has been increasing concern about the role species of *Phytophthora* play in forest health. Decline of oak and chestnut forests has been a recurring problem in Europe; *Phytophthoras* are thought to be an important component in the decline complex of both species. As a result numerous studies have been undertaken in Europe to investigate this issue. They have included surveys of soil and trees in oak and chestnut forests in France, England, Scotland, Sweden, Germany, Spain, Italy, Turkey, and Austria (Jung and Blaschke 2000, Balci and Hamschlager 2003a, Balci and Hamschlager 2003b, Vettraino *et al.* 2005, Jönsson *et al.* 2005, Chavarriaga 2007). In these studies, a diverse population of *Phytophthora* species including *P. cinnamomi* Rands, *P. citricola* Sawada, *P. cactorum* (Leb. & Cohn) Schroet, *P. gonapodyides* (Peterson) Buisman, *P. cambivora* (Petri) Buis, *P. megasperma* (Drechsler), *P. quercina* Jung, *P. syringae* (Klebahn) Klebahn, *P. europaea* Hansen & Jung, *P. cryptogea* Pethib & Laff, and previously unclassified species were found associated with soil and diseased plant tissues. Studies by Balci and Halmschlager (2003a and 2003b) and Jung *et al.* (2000) showed a possible correlation between the presence of *Phytophthora* species and the occurrence of oak decline in some oak forests.

In the US, *Phytophthora ramorum* Werres, a newly described species, has been recognized since the 1990's as the cause of mortality of tanoak (*Lithocarpus densiflorus* (Hook. & Arn.) Rehd.), coast live oak (*Quercus agrifolia* Née), California black oak (*Q. kelloggii* Newb.), and Shreve's oak (*Q. parvula* Greene var. *shrevei* (C.H. Mull.) Nixon) in California (Rizzo *et al.* 2002). The impact of *P. ramorum* on oak ecosystems in California has been the driving force behind many

of the recent investigations addressing the relationship between *Phytophthora* species and forest ecosystems in the eastern US.

Until recently, the occurrence of *Phytophthora* populations in northcentral and northeastern US predominantly oak forest ecosystems was unknown. Beginning in 2004, a survey of oak forest soils in Maryland, Pennsylvania, West Virginia, Ohio, Indiana, Illinois, Michigan, Wisconsin, and Minnesota was conducted by researchers at West Virginia University and the USDA Forest Service to investigate whether *Phytophthora* species are present in these areas and their role in the health of the hardwood forest if present. This effort resulted in the isolation of seven *Phytophthora* species, two of which were previously unknown. Among the species found, *Phytophthora cinnamomi* was most frequently isolated, recovered from 69% of 171 sites where of *Phytophthora* species were present (Balci *et al.* 2007). The common presence of *P. cinnamomi* and its known ability to cause disease warranted further investigation into its role in eastern oak forests. The focus of this study is to evaluate basic biological characteristics of *P. cinnamomi* isolates recovered by Balci *et al.* (2007).

2. Literature Review

2.1 *Phytophthora cinnamomi*

Phytophthora cinnamomi is a cosmopolitan plant pathogen associated with root diseases of numerous woody plants. Since its description as a new species by R.D. Rands in 1922, the list of plants susceptible to *P. cinnamomi* infection has grown to include over 1000 different species, the majority of which are native to Australia (Zentmyer 1980). More plant species likely will be

added in the future (Hardham 2005). Because of *P. cinnamomi*'s broad host range, in favorable conditions the pathogen can rapidly spread and infect many plant species. For this reason, the diseases caused by *P. cinnamomi* are important concerns in agricultural and forest settings as well as in the ornamental trade. A dramatic example of *P. cinnamomi* as a forest pathogen is in Australia, where Eucalyptus and many native plant species are being infected and killed, with losses of large stretches of Eucalyptus forest (Zentmyer 1980). *Phytophthora cinnamomi* is involved in the decline of *Quercus suber* L. and *Q. ilex* L. in the Mediterranean regions of Portugal, Spain and France, and has been observed causing stem cankers on *Q. rubra* L. in southwest France (Braiser 1994, Robin 1998) and it is the cause of oak mortality in Colima, Mexico (Tainter *et al.* 2000).

Phytophthora cinnamomi was probably introduced into North America in the Gulf coast and southern Atlantic states region from the late 1700's to the early 1800's through importation of exotic plants (Crandall *et al.* 1945). Inland spread was most likely facilitated by nursery and horticultural plantings. The first isolation of *P. cinnamomi* in the US was made by White (1930) from diseased rhododendron in New Jersey. Impact of *P. cinnamomi* has been greatest in eastern US forests where American chestnut, chinquapin, Ozark chinquapin and shortleaf pine serve as hosts (Crandall *et al.* 1945). *Phytophthora cinnamomi* was first isolated from dying chestnut in 1932, but there are accounts of American chestnut (*Castanea dentata* (Marsh.) Borkh.) mortality in the US as early as 1824. Disease symptoms of the dying American chestnuts were similar to those of the "ink disease" that was plaguing European chestnut on the Iberian Peninsula around the same time; *P. cinnamomi* was determined to be the cause of the European chestnut mortality (Crandall 1945). Chestnuts and chinquapins in low lying areas of Mississippi, Alabama,

Georgia, Tennessee, Virginia, Maryland, and North and South Carolina would either wilt and die or suffer branch dieback over several years, culminating in death. This affliction was originally attributed solely to high water tables and heavy soils, but it is now believed that these trees may have succumbed to *P. cinnamomi* infections. The Ozark chinquapin growing at lower elevations in its native range in Arkansas, Missouri, and Oklahoma began dying around the turn of the century of a similar malady, presumably caused by *P. cinnamomi* (Crandall *et al.* 1945). Crandall suggested that the native range of American chestnut, chinquapin, and Ozark chinquapin was greatly altered due to the activity of *P. cinnamomi*.

Phytophthora cinnamomi also is associated with diseases of conifers in the US and is a component of the little leaf disease complex that afflicts shortleaf pine growing on poorly drained clay soils in the southern US (Oak and Tainter 1988). During periods of heavy moisture, soils become waterlogged and predispose tree roots to increased *P. cinnamomi* infection.

Phytophthora cinnamomi infects and destroys the feeder roots, reducing their capability for nutrient and water uptake. Infected trees gradually decline and die, and are often attacked by insects in the interim (Oak and Tainter 1988, Zentmyer 1980 and Crandall *et al.* 1945).

Phytophthora cinnamomi also has been shown to cause root rot of other conifers such as Fraser fir in Christmas tree plantations and ornamental and nursery plantings of Douglas-fir (Pratt *et al.* 1976, Benson and Grand 2000, Kenerley *et al.* 1984, and Kenerley and Bruck 1983).

Phytophthora cinnamomi is highly pathogenic on seedlings of Nobel fir, pacific silver fir, shasta red fir, white fir, and western hemlock and moderately pathogenic on Ponderosa and sugar pine seedlings (Hamm and Hanson 1982).

The association of *P. cinnamomi* with oak has received less attention in the United States because the frequency and severity of disease caused by *P. cinnamomi* is lower than that in Europe and Mexico. There are, however, scattered reports of *P. cinnamomi* infections of oak in forest and landscape settings across the country. *Phytophthora cinnamomi* has been recovered from bark lesions found on declining *Quercus agrifolia*, and *Q. suber* in California (Garbelotto and Huberli 2006 and Mircetich *et al.* 1977). Wood and Tainter (2002) isolated *P. cinnamomi* in central Florida from the margins of bleeding cankers on *Q. laurifolia* Michx. with chlorotic leaves, sparse canopies and little new growth. Barnard (2006) recovered *P. cinnamomi* from basal cankers on *Quercus hemisphaerica* Bartram ex Willd. in northcentral Florida. *Phytophthora cinnamomi* commonly is found in soils of the southeast US and pathogenicity studies with isolates from this region has resulted in the successful infection of white oak (*Quercus alba* L.), southern red oak (*Q. falcata* Michx.), and scarlet oak (*Q. coccinea* Muenchh) (Jordan and Tainter 1996). While no natural infections have been observed on these oak species, the ability of *P. cinnamomi* to cause infections and its common occurrence in forest soils has lead some to propose that *P. cinnamomi* may be an important component of the decline syndrome of eastern oak forests (Jordan and Tainter 1996, Balci *et al.* 2007).

2.2 Phenotypic and Genotypic Variation

To better understand the relationship between *P. cinnamomi* and oak forest health, knowledge of existing *P. cinnamomi* populations is necessary. Determining genetic variability is important for understanding the organism's pathogenic potential. Phenotypic characteristics of *P. cinnamomi* differ among isolates in colony growth (Zentmyer *et al.* 1976, Shepherd and Pratt 1974), colony morphology (Huberli *et al.* 2001), sexual and asexual spore characteristics (Erwin and Ribeiro

1996, Huberli *et al.* 1997), and heat tolerance (Zentmyer *et al.* 1976). Variations in sensitivity to chemicals used to control *P. cinnamomi* also have been observed among isolates. Wilkinson *et al.* (2001) observed three levels of sensitivity to *in vitro* phosphite treatments among Australian *P. cinnamomi* isolates and Benson and Grand (2000) found slight variations in sensitivity to metalaxyl.

Genetic analysis of *P. cinnamomi* populations worldwide has shown that the species consists of multiple genotype groups. Isozyme analysis of *P. cinnamomi* isolates from Australia revealed four multilocus genotype groups (Old *et al.* 1984). Both the A1 and A2 mating types have two of the four genotype groups (Old *et al.* 1984). Isozyme analysis of *P. cinnamomi* isolates from Papua New Guinea showed even greater intraspecific diversity (Old *et al.* 1988). Studies in South Africa using isozyme (Linde *et al.* 1997), RAPD and RFLP (Linde *et al.* 1999) analysis also showed genetic diversity within *P. cinnamomi* populations. The most recent genetic analysis, using microsatellite markers, showed that Australian *P. cinnamomi* populations were represented by three genotype groups and as were most *P. cinnamomi* populations from elsewhere (Dobrowolski *et al.* 2003). Caetano *et al.* (2007) found that *P. cinnamomi* infecting Mediterranean *Quercus* spp. in southern Portugal and southwest Spain clustered into two groups based on AFLP fingerprints and that the isolate clusters closely aligned with the geographic origin. They were able to show that growth rate, optimum growth temperature, and hyphal patterns varied among isolates and that these variations were correlated to isolate geographic origin as well. No work to date has evaluated the genetic diversity of *P. cinnamomi* isolates from forest soils in the eastern US.

2.3 Pathogenicity

Pathogenic variation among *P. cinnamomi* isolates has been well documented. Zentmyer and Guillemet (1981) found that an A1 *P. cinnamomi* isolate recovered from diseased camellia tissue caused a reduction in growth and root health in both camellia and avocado plants when introduced into the soil. Conversely, when an A2 *P. cinnamomi* isolate recovered from diseased avocado tissue was introduced into soils of avocado and camellia plants, only the avocado showed a reduction in growth and root health. No significant change in the health of the camellia plants was observed. *Phytophthora cinnamomi* isolates collected from many different tree species in the eastern and southern US states were used by Crandall *et al.* (1945) to conduct pathogenicity tests on common forest species. Crandall showed that isolates recovered from *Juglans nigra* and *J. regia* in Maryland were able to infect potted black walnut seedlings when soil inoculated, but *J. nigra* was not susceptible to *P. cinnamomi* isolates recovered from *Pinus resinosa* in Maryland or *Castanea dentata* in Georgia. Hoitink and Schmitthenner (1974) found that *P. cinnamomi* isolates from rhododendron and other ericaceous plants were more virulent on the rhododendron hybrid Purple Splendor than *P. cinnamomi* isolates from nonericaceous plants. These differences may indicate that there are different strains of *P. cinnamomi* in the US (Crandall 1945). Robin *et al.* (1998) observed significant variation in pathogenicity of *P. cinnamomi* isolates from different hosts and different regions of the world on different tree species, but could not correlate host origin or isolate origin to this variation. Whether differences in pathogenicity can be attributed to genotypic variation, host specificity, or geographic origin of isolates has yet to be investigated for *P. cinnamomi* isolates in the US.

2.4 Variation in other *Phytophthora* Species

Within species variation is common among species of *Phytophthora*. Cohen *et al.* (2003) divided a population of *P. citrophthora* isolates from Corsica into two distinct genetic groups based on RFLP of mtDNA and variability in microsatellite alleles and ITS sequences. European populations of *P. quercina* were divided into two general groups based on AFLP fingerprint patterns (Cooke *et al.* 2005). Lamour and Hausbeck (2002) found that *P. capsici* isolates from Michigan differed in AFLP patterns as well as sensitivity to mefenoxam. Research also has shown that variation within a species - be it phenotypic, genetic, or pathogenic - may be related to host or geographic origin of the isolate. Bhat *et al.* (2006) reported that 30.8% of the AFLP variation observed among *P. cactorum* isolates was associated with host origin and 24.5% was associated with geographical sources. *Phytophthora cactorum* isolates from strawberry crown rot are pathogenic on multiple hosts whereas *P. cactorum* from other hosts have limited ability to cause crown rot on strawberry (Hantula *et al.* 1997, Hantula *et al.* 2000, Eikemo *et al.* 2004, Huang *et al.* 2004, Bhat *et al.* 2006). Intraspecific genetic and phenotypic variation reported in the genus *Phytophthora* and the correlation of host and geographic origin to these variations also may be exhibited by the US *P. cinnamomi* population studied in this work.

3. Objectives

The goal of this research was to evaluate the variation among a population of *P. cinnamomi* isolates recovered from soils of eastern US forests dominated by oaks by comparing phenotypic and genotypic characteristics.

Specific objectives of this study were to:

1. evaluate intraspecific diversity by comparing phenotypic characteristics among isolates;
2. evaluate genotypic variation of isolates by comparing DNA fingerprints obtained through the amplification of four length-polymorphic microsatellite loci; and
3. compare the relative pathogenicity of selected isolates within this collection.

4. Materials and Methods

4.1 Isolates

Phytophthora cinnamomi isolates used in this study were collected by Balci *et al.* (2007) in a survey of rhizosphere soil surrounding oak trees in Maryland, Pennsylvania, West Virginia, Ohio, and Indiana during the fall and spring of 2004. Isolates of *Phytophthora* were recovered from soil samples by flooding the soil and baiting them with 3-5 day old leaves of *Quercus robur*. Foliage that became discolored was examined under light microscope (200X) for the presence of typical sporangia of *Phytophthora*. Positive leaves were rinsed, patted dry with sterile filter paper, cut into 5 mm pieces, and plated onto a selective media (PARPNH).

PARPNH consists of V8 agar [100 ml V8 vegetable juice (Campbell Soup Company Camden, NJ), 3 g CaCO₃, 20 g agar (Now Foods Bloomingdale, IL), 900 mL distilled water] amended with 10 µg mL⁻¹ pimaricin, 200 µg mL⁻¹ ampicillin, 10 µg mL⁻¹ rifampicin, 25 µg mL⁻¹ pentachloronitrobenzene (PCNB), 50 µg mL⁻¹ nystatin and 50 µg mL⁻¹ hymexazol (Balci & Hamschlager 2003). Mycelium characteristic of *Phytophthora* was then transferred to V8 agar (V8A). Isolates were identified as *P. cinnamomi* by morphological characteristics and DNA sequencing (Balci *et al.* 2007). Additional *Phytophthora cinnamomi* isolates were recovered from Kentucky and Missouri oak forest soils using the same protocol. Study isolates were subcultured on to V8A from stock cultures. Prior to investigation of morphological, molecular, and pathogenic variation, isolates were inoculated onto live plant tissue as a precaution against development of atypical morphology and the loss of pathogenicity, which can occur after long periods of storage in culture (Huberli *et al.* 2001, Erwin & Ribeiro 1996). Examples of atypical morphology are slow growth on agar media at optimum temperatures and colonies that do not

produce aerial hyphae. Microscopic structures, such as sporangia, could be of irregular shape and size for stored isolates. To minimize the risk of these problems, peppers were inoculated with an agar plug from the edge of a *P. cinnamomi* colony growing on V8A. Peppers were washed with dish soap and water and surface sterilized by spraying them with 70% ethanol and patted dry with paper towels. A 5×10 mm rectangle was cut from the pepper approximately 5 cm from the stem and 5 cm from the tip of the pepper using a sterilized scalpel. A similar size agar block taken from the margin of a 3-5 day old *P. cinnamomi* colony growing on V8A was placed into the hole cut in the pepper. Inoculated peppers were incubated for 3-4 days in a moist chamber at room temperature until an area of necrotic tissue developed. Ten to fifteen, 3-5 mm pieces of pepper tissue then were cut from margin of the necrotic area with a sterile scalpel, transferred to PARPNH selective medium, and incubated for 2-3 days at 20°C in the dark. Mycelium growing from pepper tissue into the agar was transferred to V8A and incubated at 20°C in the dark.

Single zoospore isolates (SZI) were produced for each isolate after they were recovered from the peppers. This was to ensure that each isolate had only one reproducible microsatellite fingerprint. Microsatellite analysis of an isolate composed of more than one genetically distinct individual could possibly result in a composite DNA fingerprint for that isolate. This composite pattern would make analysis of fingerprints difficult. Single-spore isolates have commonly been used to produce genetically homogenous cultures of *P. cinnamomi* in similar studies (Robin and Desprez-Loustau 1998, Dobrowolski 2003). To obtain single zoospore isolates, a zoospore suspension was produced by washing sporangia (produced as described in section **4.3.1 Sporangia**) present on mycelia mats three times with sterile distilled water, chilling mycelium

and sporangia at 16°C for 30 minutes, and incubating them in sterile distilled water at 20°C under light. Most zoospores are released from the sporangia after 1 hour (Hwang and Ko 1975). Upon release, 0.5 ml of the zoospore suspension was spread on potato dextrose agar (PDA) (Becton, Dickinson and Co.; Sparks, MD) with a sterile glass rod and incubated in the dark at 20°C. Plates were inspected for germinating zoospores using a compound microscope 4 hrs after plating. Three germinating zoospores of each isolate were transferred to V8A and incubated at 20°C. One SZI of each isolate was selected for the study. Stock cultures of SZI were maintained on corn meal agar (Becton, Dickinson, and Company Sparks, MD) slants and corn meal agar slants under mineral oil. Cultures being used for the study were transferred bimonthly on V8A.

4.2 Genetic Analysis

All isolates used for the study were analyzed for genetic variability by comparing microsatellite DNA fingerprints. Four microsatellite loci were amplified from DNA extracts of isolates by PCR. PCR products were separated by capillary electrophoresis to produce microsatellite fingerprints.

4.2.1 DNA Extraction

Mycelium for DNA extraction was produced by placing one small (approximately 1 mm³) agar block taken from the margin of a 3-day-old *P. cinnamomi* SZI colony into a 1.5 ml Eppendorf microcentrifuge tube containing 750 µl of potato dextrose broth (Difco Laboratories, Detroit, MI). The tubes were incubated in the dark at 20°C for 5-7 days. The mycelium in the tubes was separated from the remaining media by centrifuging at 14000 rpm for 2 minutes. The residual

potato dextrose broth was decanted and the mycelial pellets were frozen at -20°C, vacuum freeze dried, and stored at -20°C (Linde *et al.* 1999).

To extract DNA, 500 µl of CTAB extraction buffer (100 mM Tris-HCl pH 8.0, 1.4 NaCl, 20 mM EDTA and 2% w/v hexadecyltrimethylammonium bromide) was added to the 1.5 ml microcentrifuge tubes containing freeze dried mycelium, incubated at 65°C, and pulverized periodically with a sterile plastic pestle. Five hundred microliters of chloroform were added to the tube, mixed thoroughly, and centrifuged at 14500 rpm for 5 minutes (Taylor and Powell 1982). A GeneClean Spin Kit (MP Biomedicals, Solon, OH) then was used to purify the DNA from the supernatant following the instructions in the kit. The recovered DNA was stored in sterile distilled water at -20°C.

4.2.2 Microsatellite PCR

Fingerprints of each *P. cinnamomi* isolate were produced through amplification of four length polymorphic microsatellite loci. Specific primers were used for each of four loci; d39, g16, and g10; each has a different primer pair specific for that locus. In previous analyses locus g13 was analyzed with two primer pairs, g13f and g13r1 (g13 primer pair) and g13f and g13r4 (g13(4) primer pair), but only the g13(4) primer set was used and was sufficient for this present analysis (Dobrowolski *et al.* 2002). Primers d39 and g16 amplify (AC)_n microsatellite motifs while primers g10 and g13 amplify (AG)_n microsatellite motifs. Fluorescent labels were attached to the 5' end of one of the primers in each primer set so that each locus was represented by a different color dye (Applied Biosystems, Foster City, CA). Primer sequences and label locations are listed in Table 1.

Table 1. Primer sequences 5' to 3' and fluorescent labels of each primer set.

Locus	Primer #	Primer Sequences
d39	Primer 1	6FAM-GAGAGGTCTTCAGCACGAATG
	Primer 2	ACCACTTTGGTTTCTCGCC
e16	Primer 1	VIC-AGATCTCGCACCTCCCTAAAG
	Primer 2	AGGCAAGATTTCGTAGAAGGC
g10	Primer 1	PET-GAACTCCAGCTCCTCCTGC
	Primer 2	AAGTTCCCATTTTCGCAACAC
g13r4	Primer 1	NED-ATGAGCTCACACAAGGTCTCG
	Primer 2	CTTAGGCGTCAAAGTGTGTCAGC

Reaction mixtures consisted of 3 μ l of DNA solution (approximately 20 ng genomic DNA), 0.1 mM dNTPs, 2 mM $MgCl_2$, 0.1 μ M forward and reverse primers, 0.5 U GoTaq Flexi DNA polymerase (Promega Corporation, Madison, WI), and 1X reaction buffer in a 10 μ l volume. PCR reactions were carried out using a PTC-100 thermocycler (MJ Research, Waltham, MA) with a heated lid and a touchdown program of 1 cycle of 96°C for 2 minutes, then 1 amplification cycle of 96°C for 30 seconds, 66°C for 30 seconds, 72°C for 30 seconds, followed by 5 cycles similar to the first amplification cycle except that the annealing temperature was lowered 1°C every cycle, then 20 cycles of 96°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final cycle of 72°C for 7 minutes (Dobrowolski 2003). Microsatellite products were separated by capillary electrophoresis at the Pennsylvania State University Biotechnology Institute's Nucleic Acid Facility using standard protocols. Electrophoresis results were analyzed using GeneMapper 4.0 (Applied Biosystems, Foster City, CA). Isolates were assigned to DNA fingerprint groups based on length polymorphisms of microsatellite markers (Lasker and Ran 2004).

4.3 Morphological Analysis

Characteristics of asexual (sporangia and chlamydospores) and sexual (oospores, oogonia, and antheridia) reproductive structures, and colony growth patterns and growth rates of each isolate were measured. Variations among isolates in the aforementioned morphological characteristics were evaluated to distinguish phenotypic subpopulations among the studied *P. cinnamomi* population.

4.3.1 Sporangia

Sporangia were produced by subculturing single zoospore isolates on to fresh V8A and incubating them at 20°C for 3-4 days in the dark. Ten, 5 mm agar plugs were taken from the colony margin and placed on a sterile 90 mm cellophane disc covering 15 ml of V8A and incubated at 20°C for 24 hrs or until the colonies grew to form a solid mat of mycelium covering the cellophane. The mycelium-covered cellophane was transferred to a sterile Petri dish containing 15 ml of 5% clarified V8 broth (50 ml V8 juice, 1 g CaCO₃, centrifuged at 1500 rpm for 5 minutes, 50 ml supernatant brought up to 1 L by adding distilled water and sterilized). After incubation at 20°C for 24 hrs, the V8 broth was removed from the dish and the mycelium washed twice with 20 ml of sterile mineral salt solution and incubated in 20 ml of mineral salt solution at 20°C in light conditions (Hwang and Ko 1975). The mineral salt solution was composed of calcium nitrate (0.01 M), potassium nitrate (0.005 M), magnesium sulfate (0.004 M), distilled water to make one liter, and 1 ml filter sterilized (0.22 µm pore size) chelated iron (13.05 g ethylene-dinitrilotetraacetic acid, 7.5g KOH, 24.9 g FeSO₄ • 7H₂O, distilled water to 1 L) added after autoclaving (Chen and Zentmyer 1970). A similar protocol was described by Hwang and Ko (1975) who found that abundant sporangia were produced by incubating

mycelium in mineral salt solution for 9 hrs, removing most of the mineral salt solution, and incubating the mycelium for an additional 12 hrs in moist conditions. The length and width (μm) of fifty randomly selected sporangia of each isolate were measured at 200X. Sporangia length and width were used to calculate sporangia length width ratio (Huberli *et al.* 2001).

4.3.2 Chlamydospores

Chlamydospores were produce using a protocol similar to that used for sporangia production. Fifteen 5 mm plugs from a SZI *P. cinnamomi* culture growing on V8A were transferred to a V8A plate covered with a 90 mm cellophane disc and incubated for 2-3 days at 20°C in the dark. The mycelium covered cellophane then was transferred to a sterile empty 100 mm diameter Petri dish. Fifteen milliliters of clarified V8 (CV8) broth were poured over the mycelium and the Petri dish was incubated in the dark for 2 days at 20°C. The CV8 broth was decanted and the mycelium was rinsed three times with sterile water and incubated in 5 ml of sterile water under lights for 2 days at room temperature (Huberli 1997, Ribeiro 1983, and Hwang and Ko 1975). The diameters of fifty chlamydospores were measured per isolate at 200X.

4.3.3 Sexual Reproductive Structures

Phytophthora cinnamomi is heterothallic and requires two different mating types for oospore production (Zentmyer 1980). All of the isolates collected during the 2004 survey were determined to be the A2 mating type (Balci *et al.* 2007). Therefore, production of oospores from these isolates was accomplished by pairing isolates with a known A1 mating type of *P. cinnamomi*. The isolates were grown on V8A for 5 days at which time a 5 mm agar plug was taken from the colony margin and place near the margin of a 60 mm diameter Petri dish

containing 10% clarified V8A (100 ml V8 juice, 2 g CaCO₃, centrifuged for 5 minutes at 1500 rpm, 100 ml of supernatant brought up to 1 L with distilled water and autoclaved after adding 20 grams of agar). An agar plug from the margin of an A1 *P. cinnamomi* colony growing on V8A was placed directly across the Petri dish from the isolate to be tested. A mark on the underside of the Petri dish was made to designate the test isolate. Pairings were incubated at 20°C in the dark for 3 weeks or until sex bodies formed. Agar blocks taken from the area of mycelium closest to the test isolate were scanned for sex bodies at 400X magnification. The diameter of twenty-five oogonia and oospores as well as the width of ten antheridia were measured for each isolate.

4.3.4 Growth Rate and Colony Morphology

Colony growth rate was determined by growing each isolate on 5% clarified V8A (5% clarified V8 broth with 20g per liter agar added before autoclaving) at 20°C, 24°C, 28°C, 32°C, 34°C, and 37°C. Five mm diameter agar plugs taken from the margins of 5-day-old *P. cinnamomi* SZI colonies growing on V8A were transferred to Petri dishes containing 15 ml of 5% clarified V8A. Three replicates of each isolate were grown at each temperature. Cultures were initially incubated at room temperature for 48 hours and examined for growth. The margin of the colony was traced with a fine tip marker on the bottom of the Petri dish and the diameter of the colony was measured in millimeters on two axes ninety degrees to each other. The plates were returned to the incubator for an additional 48-72 hours. Duration of the second incubation was varied because some isolates grew slower and required more incubation time for measureable growth. All isolates grown at 28°C and 32°C also received an extended incubation time. The margin of the colony was traced and the diameter measured again using the same two axes. Total diameter

growth along the two axes was calculated by subtracting the first measured diameter from the second measured diameter for both axes. To calculate diameter growth per day, the total growth for each axis was divided by the number of incubation days between measurements. The colony growth per day of each axis were added together and divided by two to get the mean diameter growth rate for the plate. Growth rate for the three replicates were summed and divided by three to get mean growth rate per day per isolate.

For comparison of colony morphology, SZI were transferred from V8A cultures to 100 mm Petri dishes containing 15 ml PDA and incubated at 24°C for 10 days. Digital pictures were taken of each isolate. The isolate was recorded as having either a rosaceous or stellate colony pattern (Huberli 2001 and Erwin & Ribeiro 1996).

4.4 Pathogenicity

Isolates were selected for pathogenicity testing based on microsatellite fingerprint grouping. The *P. cinnamomi* isolates from the study area had microsatellite fingerprint patterns, which allowed them to be divided into two groups. Isolates from each microsatellite group were chosen that encompassed distribution across the study area and host type. Twenty-three isolates were used in the pathogenicity study; with twelve from microsatellite fingerprint group one and eleven isolates from group two (Table 2).

Relative pathogenicity of *P. cinnamomi* isolates was determined by inoculation of cut red oak logs using a protocol adapted from Brasier and Kirk (2001) and Hansen *et al.* (2005). Red oak logs were cut from live trees on September 25th, 2007, 48 hours before inoculation. The ends of

the logs were sealed with paraffin wax to retain moisture. Logs were approximately 1 meter in length and ranged 9.75-20 cm in diameter with an overall mean log diameter of 12.7 cm.

Eleven of the 23 *P. cinnamomi* isolates tested were selected at random for inoculation into each log. Therefore, each log was inoculated with eleven different *P. cinnamomi* isolates and each of the 23 isolates being tested was inoculated into at least eleven different logs. A flame sterilized cork borer was used to punch 5 mm holes through the bark to the cambium of each log. Three sets of 4 holes were punched in each log with one set encircling the log at 20 cm, the second at 50 cm and the third at 80 cm from one end of the log. Agar plugs 5 mm in diameter from isolates actively growing on V8A were randomly inserted into eleven of the holes in each log. A 5 mm uncolonized V8A plug was inserted into the remaining hole in each log to serve as a control. The bark plugs were replaced and covered with moistened cotton wool. A 5 cm² piece of aluminum foil was placed over the cotton wool and secured with duct tape. Inoculated logs were incubated at 20°C for 4 weeks. After incubation, the outer bark of each log was removed with a wood chisel; the revealed lesions were outlined with a marker and traced onto paper. The tracings were digitally scanned and the area of each lesion was measured using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA).

4.5 Statistical Analysis

Isolate spore characteristics, colony pattern and growth rate data were grouped based on microsatellite fingerprint pattern, host oak type, isolate geographic origin, and colony morphology.

Isolates were recovered from the rhizosphere soil of five different oak species. In this study the “host” refers to the oak group of the oak tree from around which the soil was taken, and is designated as either red or white. The oak species from which *P. cinnamomi* was recovered in the white oak group were *Q. alba* (white oak) and *Q. prinus* (chestnut oak) and the oak species in the red oak group were *Q. rubra* (northern red oak), *Q. coccinea* (scarlet oak) and *Q. velutina* (black oak).

Isolate geographic origin groups were formed with respect to the north-south distribution of isolates in the study area. According to Balci *et al.* (2007), the 40th latitude appears to be the northern boundary for *P. cinnamomi* in eastern US oak forests. Therefore, isolate origin group one consisted of isolates recovered from sites south of the northern limit (between the 37th and 39th latitude) and isolate origin group two isolates were from sites near the suspected northern limit (39th latitude and above) of *P. cinnamomi*.

In each grouping, morphological characteristics such as sporangia length, width, length:width ratio, chlamydospore diameter, oogonia diameter, oospore diameter, and antheridia width were compared. Mean colony growth rate at 20°C, 24°C, 28°C and 32°C of isolate groups also were compared (Huberli *et al.* 2001). Growth rates were log transformed to achieve a normal distribution. The mean differences of each isolate group were tested using ANOVA and Tukey-Kramer HSD comparison.

A hierarchical cluster analysis was performed using Ward’s Method with the variables mean sporangia length, width, and length:width ratio; mean chlamydospore diameter; mean oogonium

and oospore diameter; mean antheridia width; colony morphology; and mean growth rate at 20°C, 24°C, 28°C, and 32°C (Huberli *et al.* 2001). Isolates in each phenotypic cluster were analyzed with respect to DNA fingerprint pattern, colony morphology, oak type and geographic origin. Fishers Exact Test was used to analyze microsatellite fingerprint, colony morphology, host oak type, and geographic origin characteristics of the isolates in one phenotypic cluster to isolates grouped together in other clusters.

Lesion areas were log transformed to achieve normal distribution and then tested using ANOVA and Tukey-Kramer HSD based on microsatellite fingerprint groups, isolate geographic origin groups and host oak type groups. Fishers Exact Test was used to analyze the interaction of microsatellite fingerprint, colony morphology, host oak type, and isolate geographic origin groups. All statistical analysis was carried out using JMP 5.0 (SAS Institute Inc., Cary, NC).

5. Results

5.1 Microsatellite Fingerprints

Analysis of the four microsatellite loci showed that the 61 *P. cinnamomi* isolates could be grouped into two microsatellite fingerprint groups (MFG) (Table 2). Isolates from each group were distinguished from one another using three of the four microsatellite loci. Locus g10 was excluded because fragment lengths were too variable, although there was some fragment length specificity for each group. Microsatellite fingerprint group 1 (MFG1) was characterized by marker sizes of 120 and 122 base pairs at locus d39, 169 and 170 base pairs at locus e16, and 254 and 255 base pairs at locus g13 (Table 2). The most common fragment lengths at the g10 locus for MFG1 were 117 and 120 base pairs, followed by 119 and 120 base pairs and lastly 117, 119, and 120 base pairs. Microsatellite fingerprint group 2 (MFG2) was characterized by marker sizes of 122 and 124 base pairs at locus d39, 161 and 163 base pairs at locus e16, and 247 and 248 base pairs at locus g13. Isolates in MFG2 had an even greater amount of fragment length variability at the g10 locus. The most frequently observed fragments were 115 and 117 base pairs in length (Table 2). Not all isolates from each group had the exact same fingerprint at loci d39, e16, and g13. Some isolates contained extra fragments or fragments with different lengths at one locus, but were similar at the other two loci. Isolates from MFG1 with slightly different fingerprints were IN 5/5 and MOA5j. Isolates in MFG2 with slightly different fingerprints include PA 1/6 and WVE-UF 5/3.

Table 2. DNA fragment lengths in nucleotides of each microsatellite locus for all isolates.

Isolate	d39	e16	g13	g10	Microsatellite Group
IN5/5	120/124/126	169/171	249/254	-	1
KYMCNP3E	120/122	169/171	254/255	117/120	1
KYWC2E	120/122	169/171	254/255	117/120	1
KYWC4W	120/122	169/171	254/255	117/120	1
MD6/3	120/122	169/171	254/255	117/120	1
MOA5j	120/122	169/171/173/176	254/255	117/120	1
MOE1k	120/122	169/171	254/255	117/120	1
OH1/3	120/122	169/171	254/255	117/120	1
OH13/1	120/122	169/171	254/255	117/120	1
OH2/2	120/122	169/171	254/255	117/120	1
ST 6-12	120/122	169/171	254/255	117/119/120	1
ST1-1	120/122	169/171	254/255	119/120	1
ST11-22	120/122	169/171	254/255	119/120	1
ST11-23	120/122	169/171	254/255	119/120	1
ST20-40	120/122	169/171	254/255	119/120	1
ST24-48	120/122	169/171	254/255	117/120	1
ST30-61	120/122	169/171	254/255	-	1
ST39-79	120/122	169/171	254/255	117/120	1
ST48-86	120/122	169/171	254/255	119/120	1
ST48-96	120/122	169/171	254/255	117/120	1
ST50-101	120/122	169/171	254/255	117/119/120	1
ST5-9	120/122	169/171	254/255	117/120	1
WV1/3	120/122	169/171	254/255	117/120	1
WV10/3	120/122	169/171	254/255	117/120	1
WV2/2	120/122	169/171	254/255	117/119/120	1
WV3/1	120/122	169/171	254/255	117/120	1
WV4/2	120/122	169/171	254/255	117/120	1
WV7/8	120/122	169/171	254/255	117/120	1
WVE-UF13/3	120/122	169/171	254/255	117/120	1

Table 2 (Cont.)

Isolate	d39	e16	g13	g10	Microsatellite Group
IN 1/2	122/124	161/163	247/248	115/117	2
IN10/3	122/124	-	247/248	114/115/116/117	2
IN11/1	122/124	161/163	247/248	115/117	2
IN14/5	122/124	161/163	247/248	114/115/116/117	2
IN2/1	122/124	161/163	247/248	114/115/116/117	2
IN2/5	122/124	161/163	247/248	115/117	2
IN3/2	122/124	161/163	247/248	115/117	2
IN4/4	122/124	161/163	247/248	115/117/119	2
IN6/3	122/124	161/163	247/248	114/115/116/117	2
MD 15/2	122/124	161/163	247/248	115/117	2
MD1/4	122/124	161/163	247/248	114/115/116/117	2
MD1/5	122/124	161/163	247/248	115/117	2
MD11/6	122/124	161/163	247/248	115/117	2
MD2/1	122/124	161/163	247/248	114/115/116/117	2
MD3/2	122/124	161/163	247/248	114/115/116/117	2
MD4/3	122/124	161/163	247/248	115/117	2
MD5/2	122/124	161/163	247/248	115/117	2
MOE2i	122/124	161/163	247/248	115/116/117	2
PA1/6	122/124	161/163/169/172	247/248	103/115/117	2
PA7/4	122/124	161/163	247/248	115/117	2
WV8/3B	122/124	161/163	247/248	115/117	2
WV9/1	122/124	161/163	247/248	115/117	2
WVE-SL2/2	122/124	161/163	247/248	115/117	2
WVE-UF10/1	122/124	161/163	247/248	115/117	2
WVE-UF2/4	122/124	161/163	247/248	114/115/116/117	2
WVE-UF6/3	122/124	161/163	247/248	115/117	2
WVE-UF7/3	122/124	161/163	247/248	115/117	2
WV-GR1/4	122/124	161/163	247/248	115/116/117	2
WV-SC3/3	122/124	161/163	247/248	114/115/116/117	2
WVE-UF 5/3	122/124	169/171	247/248	115/116/117	2
WVE-UF15/5	122/124	161/163	247/248	115/117	2
WVE-UF4/3	122/124	161/163	247/248	115/116/117	2

Only one isolate from Indiana and one isolate from Maryland were in MFG1, with the remainder from these states in MFG2. All Ohio and Kentucky isolates grouped in MFG1 and all Pennsylvania isolates grouped in MFG2. One of two Missouri isolates was in MFG1 while the other was in MFG2. Of nineteen West Virginia isolates, seven were in MFG1 and twelve were in MFG2. All but one of the isolates recovered from soil from the West Virginia University forest (isolate ID's WVE-UF or WV-UF) were in MFG2.

5.2 Characteristics of Colony Morphology and Asexual and Sexual Spore Structures

Two colony morphologies were exhibited by the study isolates (Table 3). Among the 61 *P. cinnamomi* isolates, 40 formed a rosaceous colony pattern (Figure 1) and the remaining 21 formed a stellate pattern (Figure 2) when grown on PDA.

Variation in sporangia length, width and length:width ratio of was observed. However, little variation existed in diameters of chlamydospores, oogonia, and oospores and antheridia width among the studied *P. cinnamomi* isolates (Table 3).

Sporangia of all MFG1 isolates were significantly greater in length ($P<0.001$, $R^2=0.01$) and width ($P<0.001$, $R^2=0.23$) than those of MFG2, but the length:width ratios ($P<0.001$, $R^2=0.1$) were significantly smaller (Table 4). No significant differences among the diameters of chlamydospores ($P=0.19$), oogonia ($P=0.82$), oospores ($P=0.95$) or the width of antheridia ($P=0.62$) were found between the isolates in MFG1 and MFG2 (Table 4).

Table 3. Mean spore measurements, colony morphologies, mean diameter growth rates, isolate geographic origin and host oak type of *Phytophthora cinnamomi* isolates.

Isolate	State ^a	Spore Measurement Means								Mean Growth Rate (mm/day)				Isolate groups	
		Sporangia			Chlamydospore Diameter (µm)	Oogonia Diameter (µm)	Oospore Diameter (µm)	Antheridia Width (µm)	Colony Morphology on PDA	20°C	24°C	28°C	32°C	Geographic Origin ^b	Oak Type ^c
		Length (µm)	Width (µm)	Length to Width Ratio											
IN1/2*	IN	65.5±12	35.9±3	1.8±0.3	47.9±5	42.3±3	37.9±3	16.2±2	Rosaceous	18.8	12.9	11.8	8.6	1	White
IN10/3	IN	65.1±10	35.6±5	1.9±0.3	42.6±4	40.9±4	37.4±4	17.7±2	Rosaceous	23.3	12.8	13.4	7.8	1	White
IN3/2	IN	73.7±12	39.4±4	1.9±0.3	48.3±6	42.4±4	38.3±4	16.4±1	Rosaceous	12.9	12.9	11.9	8.6	1	White
IN4/4	IN	59.5±11	35.5±4	1.7±0.2	41.8±4	40±4	36.6±3	17.7±2	Rosaceous	18.6	12.4	11.3	8	2	White
IN5/5*	IN	62.9±11	39.2±5	1.6±0.2	51.9±6	39.4±3	35.6±3	17.2±2	Rosaceous	17.3	17.9	12.7	4.4	2	White
IN6/3*	IN	63.5±10	35±3	1.8±0.3	42.4±4	41.9±4	37.9±3	17.6±2	Rosaceous	17.3	13.9	12.6	8.1	1	White
IN11/1	IN	61.1±8	35.2±3	1.7±0.2	45.9±4	42.3±4	38.7±3	17.8±3	Stellate	14.4	12.9	12.7	7.1	1	Red
IN14/5	IN	62.6±12	36.1±5	1.7±0.3	46.2±5	39.9±3	36.5±3	16.6±2	Stellate	17.1	13.1	12.9	5.9	1	Red
IN2/1	IN	79.1±17	37.3±7	2.1±0.4	49.7±6	41±5	37.5±4	18.6±1	Stellate	12.3	14.1	14.9	8.3	1	Red
IN2/5*	IN	72.7±10	39.1±4	1.9±0.3	46.2±6	41.2±4	37.8±4	17.1±2	Stellate	12.8	12.3	13.7	6.7	1	White
KYMCNP3E*	KY	71.3±9	43.6±5	1.6±0.2	44.9±6	41.8±6	36.7±7	17.3±2	Rosaceous	19.9	19.6	17.5	3.8	1	White
KYWC2E*	KY	67.6±10	43.4±5	1.6±0.1	47.1±6	41.9±3	38.1±3	17.9±1	Rosaceous	18.3	17.8	12.5	4.1	1	White
KYWC4W	KY	71.3±13	39.8±7	1.8±0.3	46±7	41.3±4	37.5±3	17.2±1	Rosaceous	16.1	11.9	17.5	4.4	1	White
MD2/1*	MD	71.4±7	39±4	1.8±0.2	42.1±4	41.1±3	37.4±3	16.9±2	Rosaceous	13	11.4	12.2	9.2	2	White
MD3/2	MD	61.9±10	35.4±4	1.8±0.2	44.5±4	41±3	37±3	17.7±2	Rosaceous	15.5	14.9	16.6	5.6	2	Red
MD5/2	MD	64.7±9	34.9±3	1.9±0.3	47.4±7	41.4±4	37.4±4	16.3±1	Rosaceous	13.6	10.8	12.6	7.7	2	White
MD6/3*	MD	59.6±9	38.9±5	1.5±0.2	49.3±8	41.7±3	38.1±3	16.3±1	Rosaceous	15.8	11.8	12.8	3.5	2	White
MD1/4	MD	60.2±8	33.1±2	1.8±0.2	49±5	40.5±5	36.4±4	18.7±3	Rosaceous	22	10.8	13.4	8.1	2	NA
MD1/5	MD	63.5±10	37.4±5	1.7±0.2	48.3±7	42.8±4	38.3±3	15.7±1	Stellate	26.7	14.2	13.5	7.8	2	Red
MD11/6	MD	61.1±10	35.1±4	1.7±0.2	44.6±5	39.6±4	36.4±4	16.6±2	Stellate	18.7	11.9	11.8	6.8	2	Red
MD15/2*	MD	71.2±11	35±3	2±0.3	43.1±5	40.9±5	37.1±4	17.4±2	Stellate	14.5	11.8	12.2	6.1	2	NA
MD4/3	MD	58.6±8	36.2±3	1.6±0.2	47.7±6	43.3±4	38±3	16.7±1	Stellate	16	14.2	11.2	7.3	2	Red

Table 3 (Cont.)

Isolate	State ^a	Spore Measurement Means								Mean Growth Rate (mm/day)				Isolate groups	
		Sporangia			Chlamyospore Diameter (µm)	Oogonia Diameter (µm)	Oospore Diameter (µm)	Antheridia Width (µm)	Colony Morphology on PDA	20°C	24°C	28°C	32°C	Geographic Origin ^b	Oak Type ^c
		Length (µm)	Width (µm)	Length to Width Ratio											
MOA5j*	MO	69.2±8	42.1±5	1.7±0.2	48.3±5	40.2±3	36.7±3	16.4±1	Rosaceous	17.9	17.9	10.8	3.2	1	NA
MOE1k	MO	82±9	50.6±4	1.6±0.4	43.9±7	44.2±3	40.3±3	17.4±2	Rosaceous	18	17.8	11.3	4.1	1	NA
MOE2i*	MO	70.3±16	36.5±3	1.9±0.5	44.4±4	40.2±4	36.6±4	15.2±1	Stellate	16.1	15.9	13.7	8.1	1	NA
OH1/3	OH	66.1±11	39.9±6	1.7±0.2	48.8±6	39.2±2	35.5±2	16.5±1	Rosaceous	17.2	13.1	9.8	5.4	2	White
OH2/2*	OH	71.1±12	43.6±6	1.6±0.2	47.6±6	40.8±3	36.8±3	16.9±1	Rosaceous	17.3	15.9	10.9	5.1	2	White
OH13/1	OH	67.7±13	40.3±5	1.7±0.2	45.7±7	40.8±3	36.8±3	18.4±2	Rosaceous	17.6	14.5	16.6	5.1	2	Red
ST 1-1*	OH	68.1±14	40.3±5	1.7±0.2	46.8±8	39.5±4	36.1±3	19.4±6	Rosaceous	15.8	16.2	10.3	5.3	2	White
ST 11-22*	OH	72.6±15	40.9±4	1.8±0.3	40.2±5	41.1±3	37.4±2	16.6±1	Rosaceous	16.8	15.7	10.4	4.2	2	White
ST 11-23	OH	81.1±21	43.8±7	1.9±0.4	39.2±5	41.5±4	38±4	16.7±3	Rosaceous	15.5	14.4	11.5	4.5	2	White
ST 20-40	OH	66.3±15	42.1±6	1.6±0.3	39.1±7	40.4±4	36.4±4	17.7±2	Rosaceous	16.8	16.9	11	5.5	2	White
ST 24-48	OH	63.3±11	41.1±6	1.6±0.2	35.4±6	39.6±3	35.8±3	17.1±2	Rosaceous	15.3	14	11.6	5.5	2	White
ST 30-61	OH	58.7±10	42.1±5	1.4±0.1	46.4±8	34.7±4	31.4±4	17.2±2	Rosaceous	13	15.9	10.5	5.4	2	White
ST 39-79	OH	64.3±10	40±5	1.6±0.2	49.9±5	41.9±3	37.2±6	17±2	Rosaceous	16.3	15.8	12.2	5.7	2	White
ST 43-86*	OH	64.5±13	41.3±5	1.6±0.2	39.7±6	42.2±3	38.8±3	17.2±2	Rosaceous	17	16.9	11.3	5	2	White
ST 48-96	OH	69.4±16	41.3±6	1.7±0.2	47.6±7	40.8±3	36.9±2	17.7±2	Rosaceous	16.5	17.4	11.7	8	2	White
ST 50-101	OH	65.1±11	40.4±5	1.6±0.2	45.7±5	39.7±3	35.6±4	16.5±1	Rosaceous	15.9	15.3	11.1	3.5	2	White
ST 5-9	OH	53.8±10	36.7±5	1.5±0.2	47.6±5	39±3	35.9±3	17.7±2	Rosaceous	15.9	15.3	11.2	5.9	2	White
ST 6-12	OH	64.7±13	43.5±7	1.5±0.2	49±7	37.9±3	34.9±2	17.2±2	Rosaceous	15.9	16.5	10.4	4	2	White
PA1/6*	PA	61.9±8	36.6±4	1.7±0.3	51.7±7	37.3±4	33.9±4	18.8±3	Rosaceous	14.3	11.1	13.1	7.9	2	Red
PA7/4*	PA	60.7±9	36.5±3	1.7±0.2	44.6±5	38.6±4	34.6±4	16.3±2	Stellate	14.6	11	12.4	8	2	White

Table 3 (Cont.)

Spore Measurement Means															
Isolate	State ^a	Sporangia			Chlamydospore Diameter (μm)	Oogonia Diameter (μm)	Oospore Diameter (μm)	Antheridia Width (μm)	Colony Morphology on PDA	Mean Growth Rate (mm/day)				Isolate groups	
		Length (μm)	Width (μm)	Length:Width Ratio						20°C	24°C	28°C	32°C	Geographic Origin ^b	Oak Type ^c
GR1/4	WV	65.5±8	34.9±3	1.9±0.2	41.9±4	38.3±5	35.3±4	17.1±2	Rosaceous	15.8	14.1	16.9	6.4	2	Red
WV10/3*	WV	71.8±13	40.6±6	1.8±0.3	47.1±5	41±3	36.8±3	17.7±2	Rosaceous	18.8	18	11	5.5	1	Red
WV2/2*	WV	71.4±12	43±5	1.7±0.2	45±6	42.9±3	37.5±6	17.4±1	Rosaceous	27.3	17.9	13.6	6.5	1	White
WV3/1	WV	69.4±11	42.4±4	1.6±0.2	44.6±5	41.1±6	37.2±6	18.9±2	Rosaceous	28.3	18.5	18.5	5.8	1	White
WV4/2	WV	68.9±13	41.4±6	1.7±0.2	49.7±6	42.8±3	39±3	20.8±6	Rosaceous	29.5	20.4	12.7	6.9	1	Red
WV7/8	WV	68±13	40.9±6	1.7±0.2	46.9±7	41.2±3	37.9±2	17.1±1	Rosaceous	18.6	17.1	12.9	5.7	1	White
WVE-SL2/2	WV	65.7±11	36.8±4	1.8±0.3	45.9±7	39.4±6	35.2±5	17.6±3	Rosaceous	15	12.7	14.7	7	1	Red
WVE-UF10/1	WV	66.6±9	36.3±4	1.9±0.3	45.3±7	40.1±4	35.4±3	15.8±2	Rosaceous	15.1	12.6	14	6.4	2	Red
WVE-UF13/3*	WV	67.7±7	44.5±5	1.5±0.2	40.8±5	39.8±2	36.4±2	16.8±1	Rosaceous	15.7	14.5	9.6	4.1	2	Red
WV-UF15/5*	WV	58.1±10	34.8±3	1.7±0.2	43.7±5	39±5	35±4	17±2	Rosaceous	14.3	15.7	15.3	4.2	2	Red
WV-UF5/3*	WV	55.9±9	34.1±4	1.7±0.3	35.3±4	36.6±4	33.5±3	16.1±2	Rosaceous	21.3	14	11	2.6	2	White
WV1/3	WV	81.4±11	45.4±5	1.8±0.2	49±5	39.4±4	34.9±4	16±1	Stellate	16	16.8	9.9	3.4	1	White
WV8/3B*	WV	62.3±13	35.4±4	1.8±0.3	47±6	41.8±3	38±3	17.3±2	Stellate	15	13.4	11.5	6.9	1	Red
WV9/1	WV	67.7±10	38.7±4	1.8±0.2	44.2±5	39.3±6	36±5	18.5±2	Stellate	13.9	11.4	12	6.1	1	White
WVE-UF2/4	WV	65.1±12	36.3±4	1.8±0.3	46.8±5	39.5±4	35.4±4	16.8±2	Stellate	14.6	13	13.6	5.5	2	Red
WVE-UF6/3	WV	69.4±7	37.6±3	1.9±0.2	49.7±5	39±4	35.1±4	17.6±3	Stellate	15.8	13.3	13.2	5.9	2	White
WVE-UF7/3	WV	68.5±11	37.2±4	1.9±0.3	46.1±6	42.1±4	38.4±4	18.3±3	Stellate	14.3	13.1	13.5	6.5	2	White
WV-SC3/3	WV	60.1±8	36.3±4	1.7±0.1	44.5±3	39±3	35.4±3	19±8	Stellate	15.3	12.4	11.8	5.9	1	NA
WV-UF4/3	WV	69.7±13	36.4±4	1.9±0.3	43.4±5	41.2±5	37±5	17.9±3	Stellate	15.2	13.2	13.7	6	2	White
Average		66.6±13	38.9±6	1.7±0.3	45.3±7	40.6±4	36.8±4	17.3±2		17.0	14.5	12.7	6.0		
Max		137	78	3.56	70	52	47	40		30.5	20.5	19	10.5		
Min		37	24	0.75	22	19	13	12		12	9.8	9	2.4		

^a IN=Indiana, KY=Kentucky, MD=Maryland, MO=Missouri, OH=Ohio, PA=Pennsylvania, WV=West Virginia.

^b 1=Isolates collected from sites between the 37 and 39° latitude, 2=Isolates collected from sites north of the 39th latitude.

^c White= Isolates collected from the rhizosphere soil of either *Q. alba* (white oak) or *Q. prinus* (chestnut oak), Red=Isolates collected from the rhizosphere soil of either *Q. rubra* (northern red oak), *Q. coccinea* (scarlet oak) or *Q. velutina* (black oak).

* Isolates used in pathogenicity tests.

Figure 1. *Phytophthora cinnamomi* isolate exhibiting the rosaceous colony morphology

Figure 2. *Phytophthora cinnamomi* isolate exhibiting the stellate colony morphology

Isolate means organized by latitude of site origin showed that isolates obtained between the 37th and 39th latitude had significantly greater mean sporangia length ($P<0.001$, $R^2=0.03$), width ($P<0.001$, $R^2=0.01$), and length:width ratio ($P<0.001$, $R^2=0.01$) as well as chlamydospore ($P<0.001$, $R^2=0.007$), oogonia ($P<0.001$, $R^2=0.02$) and oospore diameter ($P<0.001$, $R^2=0.01$) (Table 4) than isolates from above the 39th latitude. Antheridia width ($P=0.22$) did not differ between groups (Table 4).

Mean sporangia length ($P<0.001$, $R^2=0.01$), width ($P<0.001$, $R^2=0.04$), and length:width ratio ($P<0.001$, $R^2=0.005$) and chlamydospore diameter ($P<0.001$, $R^2=0.008$) differed significantly between isolates recovered from the rhizosphere of red versus white oaks. Sporangia of isolates recovered from white oak rhizosphere soil averaged 2.7mm longer and 2.5mm wider than isolates from red oak rhizosphere soil. However, the average chlamydospore diameter of isolates from red oak soil was 1.3mm greater than chlamydospores of isolates from white oak soils (table 4). Mean oogonium ($P=0.81$) and oospore ($P=0.91$) diameter and antheridium width ($P=0.17$) did not differ significantly (Table 4).

When spore characteristics of isolates based on colony morphology were compared, sporangia of isolates with a rosaceous colony pattern were slightly wider ($P<0.001$, $R^2=0.01$). In contrast, sporangium length:width ratio ($P<0.001$, $R^2=0.05$) and chlamydospore diameter ($P<0.01$, $R^2=0.003$) of isolates with a stellate colony appearance were larger (Table 4). Sporangia length ($P=0.19$), oogonia diameter ($P=0.28$), oospore diameter ($P=0.22$), and antheridia width ($P=0.23$) did not differ significantly.

Table 4. Means and standard deviations of morphological characteristics of isolates grouped by microsatellite fingerprint pattern, geographic origin, host, and colony morphology.^a

Group	Group Category	Sporangia Length (μm)	Sporangia Width (μm)	Sporangia Length:Width Ratio	Chlamydospore Diameter (μm)	Oogonia Diameter (μm)	Oospore Diameter (μm)	Antheridia Width (μm)
MFG ^b	1	68.3 ± 13.5 a	41.8 ± 6.0 a	1.63 ± 0.23 a	45.6 ± 7.2 a	40.6 ± 3.9 a	36.76 ± 3.8 a	17.4 ± 2.3 a
	2	65.2 ± 11.7 b	36.2 ± 4.1 b	1.80 ± 0.29 b	45.3 ± 6.0 a	40.6 ± 4.3 a	36.78 ± 3.9 a	17.3 ± 2.5 a
Isolate Origin (Latitude)	37 to 39	69.2 ± 12.7 a	39.6 ± 6.1 a	1.76 ± 0.28 a	46.5 ± 5.9 a	41.28 ± 4.10 a	37.4 ± 4.0 a	17.4 ± 2.5 a
	N of 39	65.0 ± 12.3 b	38.4 ± 5.5 b	1.70 ± 0.27 b	45.0 ± 7.0 b	40.05 ± 4.05 b	36.3 ± 3.7 b	17.2 ± 2.3 a
Host Oak Type	White	67.2 ± 12.9 a	39.5 ± 5.7 a	1.71 ± 0.28 a	45.0 ± 6.9 a	40.5 ± 4.1 a	36.7 ± 4.0 a	17.3 ± 2.1 a
	Red	64.5 ± 11.6 b	37.0 ± 5.1 b	1.75 ± 0.27 b	46.3 ± 6.1 b	40.6 ± 4.2 a	36.7 ± 3.7 a	17.6 ± 3.0 a
Colony Morphology	Rosaceous	66.4 ± 12.6 a	39.7 ± 6.1 a	1.68 ± 0.26 a	45.2 ± 7.0 a	40.5 ± 4.0 a	36.7 ± 3.8 a	17.4 ± 2.3 a
	Stellate	67.1 ± 12.8 a	37.1 ± 4.6 b	1.81 ± 0.30 a	46.0 ± 5.5 a	40.7 ± 4.3 a	36.9 ± 3.9 a	17.2 ± 2.6 a

^a Means within a group followed by the same letter are not significantly different (Tukey-Kramer HSD).

^b MFG = Microsatellite Fingerprint Group

5.3 Colony Growth Rate

Colony diameter growth rates varied among isolates (Table 3). Average growth rate usually was fastest at 20°C and slowest at 32°C. The fastest growth rate at any temperature was recorded with isolate WV 4/2 at 20°C. The slowest growth rate at 20°C was recorded with isolate IN 2/1. The slowest recorded growth rate for 24°C was with isolate MD 1/4 and the fastest growth at this temperature was with isolate WV 4/2. The slowest growth rate at 28°C was recorded with WVE-UF 13/1 and the fastest was recorded with WV 3/1. The slowest growth rate at 32°C was recorded with WV-UF 5/3, which also was the slowest average growth rate recorded overall. The fastest growth at 32°C was recorded with isolate MD 2/1. No growth was observed for any isolate at 34°C or 37°C.

Significant variations in growth rate were observed when isolates were grouped based on microsatellite fingerprints, geographic origin, oak host type, and colony morphology (Table 5). When grouped by microsatellite fingerprints, MFG1 isolates had significantly greater growth rates at 20°C ($P<0.001$, $R^2=0.07$) and 24°C ($P<0.001$, $R^2=0.47$) than MFG2 isolates. Conversely, MFG2 isolates grew significantly faster at 28°C ($P<0.001$, $R^2=0.07$) and 32°C ($P<0.001$, $R^2=0.25$) than MFG1 isolates (Table 5). Isolates from between the 37th and 39th latitude had significantly greater growth rates at 20°C ($P=0.006$, $R^2=0.04$), 24°C ($P=0.006$, $R^2=0.04$), and 28°C ($P<0.01$, $R^2=0.04$) than isolates from south of the 37th parallel. There was no difference in growth rate at 32°C of isolate geographic origin groups (Table 5). Isolates recovered from the rhizosphere of the different host groups showed no significant differences

Table 5. Mean colony diameter growth rates of isolates grouped by microsatellite fingerprint, isolate geographic origin, host oak type, and colony morphology.

Isolate Groups	Group Categories	Colony Growth Rate (mm/day) ^a			
		20°C	24°C	28°C	32°C
MFG ^b	1	17.9 ± 3.9 a	16.3 ± 2.1 a	12.2 ± 2.4 a	5.0 ± 1.4 a
	2	16.2 ± 3.4 b	12.9 ± 1.4 b	13.1 ± 1.5 b	6.9 ± 1.8 b
Isolate Origin (Latitude)	37 to 39	18.0 ± 4.7 a	15.2 ± 2.8 a	13.2 ± 2.2 a	6.2 ± 1.9 a
	N of 39	16.4 ± 2.7 b	14.1 ± 2.1 b	12.4 ± 1.9 b	5.8 ± 1.8 a
Oak Type	White	17.0 ± 4.4 a	14.2 ± 2.2 a	13.5 ± 2.1 a	6.4 ± 1.6 a
	Red	16.8 ± 3.6 a	14.6 ± 2.3 a	12.0 ± 1.7 b	6.1 ± 1.9 a
Colony Morphology	Rosaceous	17.6 ± 3.8 a	15.1 ± 2.5 a	12.7 ± 2.3 a	5.7 ± 1.9 a
	Stellate	15.7 ± 3.2 b	13.2 ± 1.5 b	12.7 ± 1.2 a	6.6 ± 1.6 b

^a Means within a group followed by the same letter are not significantly different (Tukey-Kramer HSD)

^b MFG = Microsatellite Fingerprint Group

in growth rate at 20°C, 24°C, and 32°C. Isolates from white oak soils had a significantly greater growth rate at 28°C than isolates from red oak hosts (Table 5). When growth rates of isolates with different colony morphologies were compared, isolates with a rosaceous colony pattern had significantly greater growth rates at 20°C ($P=0.002$, $R^2=0.05$) and 24°C ($P<0.001$, $R^2=0.12$) while stellate isolates had greater average growth rate at 32°C ($P=0.004$, $R^2=0.04$). There was no significant difference in growth rate of the colony morphology groups at 28°C (Table 5).

Growth rate trends also were observed that varied across the microsatellite fingerprint and colony morphology groups. One trend was the difference in the change in growth rate from one incubation temperature to the next among isolate groups. Growth rates of MFG1 isolates decreased as incubation temperatures increased from 24°C to 28°C, while MFG2 growth rates

stayed relatively the same (Table 5). This also was observed for isolates with different colony patterns. The rosaceous colonies had a mean reduction in diameter growth rate of 2.4 mm/day between 24°C and 28°C. The stellate colonies were much less affected and only had a mean growth rate reduction of 0.5 mm/day between the 24°C and 28°C incubation temperatures. At 32°C growth rates of both microsatellite fingerprint groups decreased, but the growth rates of MFG1 isolates were more negatively affected than MFG2 isolates. This also was observed in isolates with different colony patterns where the decrease in growth rate from 28° to 32°C was greater for isolates with the rosaceous colony pattern than for isolates with the stellate colony pattern. Additionally, the difference in mean diameter growth rate from the lowest to the highest incubation temperature was significantly greater ($P<0.0001$, $R^2=0.2$) for MFG1 isolates (13 mm/day) than for MFG2 isolates (9 mm/day). The difference in mean diameter growth rate from the lowest to the highest incubation temperature was significantly greater ($P<0.0001$, $R^2=0.09$) for isolates with rosaceous colony morphology (12 mm/day) than for isolates with stellate colony morphology (9 mm/day). These trends were not observed to occur in the other two isolate groups where the isolates in each category of each isolate group behaved similarly with regards to growth rate. As incubation temperatures increased, growth rates of isolates in isolate geographic origin and host groups decreased

5.4 Cluster Analysis of Phenotypic Characteristics

A hierarchal cluster analysis was constructed using the variables of mean sporangia length, sporangia width, sporangia length:width ratio; chlamydospore, oogonia, and oospore diameters;

Figure 3. Cluster analysis of isolates based on tested phenotypic variables constructed using Ward's Method. Variables include sporangia length, width, and length:width ratio; chlamydospore, oogonia, and oospore diameters; antheridia width; colony morphology on PDA and colony growth rate at 20°C, 24°C, 28°C, and 32°C. Cluster 1 is highlighted in red and Cluster 2 is highlighted in green.

Figure 4. Number of isolates from each phenotypic cluster group (Figure 3) when grouped by microsatellite fingerprint, colony morphology, host oak type, and isolate geographic origin. (Fisher's Exact Test $P < 0.001$, $R^2 = 0.65$).

antheridia width; and growth rates on CV8A at 20°C, 24°C, 28°C, and 32°C for each isolate (Figure 3). The only isolates that did not cluster with isolates of the same MFG were KYWC4W, OH 13/1, and WV-UF 5/3. The analysis corresponded similar to microsatellite DNA fingerprint groups (Figure 4). Ninety three percent of the isolates in MFG1 grouped in phenotype cluster two and 97% of the isolates in MFG2 grouped in phenotype cluster one. Phenotype clusters and colony morphology also correlated. Ninety-four percent of the isolates with a stellate colony pattern grouped in phenotype cluster 1 (Fisher's Exact Test $P < 0.001$,

$R^2=0.25$). A significant relationship between isolate host oak type and phenotype cluster was measured. The majority of isolates from red oak rhizosphere soil (78%) grouped in the phenotype cluster 1 and the remainder in cluster 2. Conversely, 62% of the isolates from white oak soil grouped in phenotype cluster 1 and the remainder were in cluster 2. These correlations were significant based on a 2-tailed Fishers Exact Test ($P=0.009$, $R^2=0.11$). The relationship between phenotype clusters and isolate geographic origin were not significant (Fisher's Exact Test $P=0.6$).

5.5 Relationship among groups defined by Microsatellite Fingerprint Patterns, Colony Morphology Geographic Origin, and Host.

Isolates with a rosaceous colony pattern predominated MFG1 and the majority of the stellate isolates were in MFG2 (Figure 5). These relationships were significant (2-Tail Fisher's Exact Test $P=0.0002$, $R^2=0.18$). Similarly, a greater number of isolates from white oak rhizosphere soil were in MFG1 and most of the isolates from red oak rhizosphere soil were in MFG2 (2-Tail Fisher's Exact Test $P=0.009$, $R^2=0.11$)(Figure 5). Both geographic origin groups were composed of nearly equal numbers of isolates from each microsatellite fingerprint group (Figure 5).

When isolates were grouped based on colony morphology, isolates in the MFG1 more often formed rosaceous colonies (Fisher's Exact Test $P=0.0002$, $R^2=0.20$) (Figure 6). However, an equal number of isolates with rosaceous or stellate colony morphologies were in MFG2 (Figure 5). When colony morphology of isolates was compared across isolates from different oak types, isolates recovered from white oak rhizosphere soil more frequently formed the rosaceous

Figure 5. Number of isolates in microsatellite fingerprint group 1 (MFG1) and microsatellite fingerprint group 2 (MFG2) in each category of colony morphology, host oak type, and isolate geographic origin groups. Some isolates were not included in all of the groups due to missing host and/or origin data.

Figure 6. Number of isolates with rosaceous and stellate colony morphology in each category of microsatellite fingerprint, host oak type, and isolate geographic origin groups. Some isolates were not included in all of the groups due to missing host and/or origin data.

colonies (Fisher's Exact Test $P=0.04$, $R^2=0.07$) (Figure 6). The relationship between the geographic origin of isolates and their colony morphology was not significant, although a greater number of isolates from sites north of the 39th latitude formed rosaceous rather than stellate colonies (Figure 6).

5.6 Pathogenic Variation

No significant difference was detected among lesion sizes of all isolates (Table 6). All isolates except PA 1/6 and WVE-UF 5/3 produced significantly larger lesions on bark and cambial tissues of red oak logs than wounds inoculated with sterile agar plugs ($P<0.05$) (Table 6). Mean lesion areas of isolates grouped based on microsatellite fingerprint ($P=0.99$), isolate geographic origin ($P=0.94$), host oak type ($P=0.24$), or colony morphology ($P=0.91$) did not differ significantly (Figure 7).

Table 6. Ranked lesion area resulting from inoculations of *Phytophthora cinnamomi* isolates on to red oak (*Quercus rubra*) logs.

Isolates	Mean Lesion Area (mm)*
ST1-1	5616 a
IN2/5	4326 a
OH2/2	4364 a
MOA5j	4594 a
PA7/4	3579 a
KYMCNP3E	3691 a
MD2/1	3333 a
IN6/3	3901 a
MD6/3	3337 a
WVE-UF13/3	3073 a
IN1/2	3382 a
WV10/3	3208 a
IN5/5	2944 a
ST43-86	3541 a
ST11-22	3623 a
MD15/2	2637 a
WV2/2	2972 a
KYWC2E	2314 a
WV8/3B	2483 a
WV-UF15/5	2284 a
MOE2i	1925 a
PA1/6	2191 ab
WV-UF5/3	1156 ab
Control	428 b

* Values followed by the same letter are not significantly different (Tukey-Kramer HSD).

Figure 7. Comparison of mean lesion areas on red oak logs caused by *Phytophthora cinnamomi* isolates grouped by microsatellite fingerprint group (MFG), isolate geographic origin, host oak type, and colony morphology. Bars with a different pattern within each isolate group category are significantly different (Tukey-Kramer HSD).

6. Discussion

The goal of this study was to investigate the phenotypic and genotypic characteristics of *P. cinnamomi* population originating from eastern US oak forest soils. These isolates were collected as part of a study to determine the species composition found in oak forest ecosystems. Their role in oak decline still remains to be explored and multiple questions still remain about their biology and impact. The work conducted provided insights on the characteristics of *P. cinnamomi* isolates from U.S. forests.

6.1 Genetic Variation

Microsatellite analysis of US *P. cinnamomi* isolates indicated low levels of genetic diversity among isolates collected from the study area. Isolates in this study were divided into two populations based on microsatellite fingerprint patterns (MFP) and the majority of isolates within each Microsatellite Fingerprint Group (MFG) were identical at loci e16, g13 and d39. This low level of genetic diversity has been shown to occur in other *P. cinnamomi* populations. The most extensive study of *P. cinnamomi* population genetics using the same molecular markers was conducted by Dobrowolski *et al.* (2003). The authors showed that isolates from Australia also had very low levels of genetic diversity. Genetic analysis of U.S. *P. cinnamomi* isolates from South Carolina using amplified fragments polymorphisms (AFLP) provided additional evidence of low genetic variability in *P. cinnamomi* from the US (Duan, Riley, and Jeffers 2008). Dobrowolski *et al.* (2003) and Huberli *et al.* (2001) showed that the majority of A2 *P. cinnamomi* isolates in Australia could be divided into two groups using the same microsatellite markers which divided US isolates into the two groups identified in this study. Additionally,

some of the allele fragment lengths are shared between the groups established by Dobrowolski and the US MFGs.

Comparisons of microsatellite fingerprints of US isolates to the fingerprints of *P. cinnamomi* isolates from Dobrowolski *et al.* (2003) and Huberli *et al.* (2001) indicated similarities in *P. cinnamomi* isolates from different continents (Table 7). Isolates of MFG1 were identical to the predominant fingerprint pattern of Dobrowolski's Australian A2 type 1 isolates at the e16 locus (169/171) and have the same marker size on one allele at locus d39 (122). The other d39 allele of the US MFG1 isolates matches one allele at the d39 locus (120) of the Australian A1 type 1 *P. cinnamomi* isolates. Locus g10 of US MFG1 isolates have markers not found in the three predominant genotypes identified by Dobrowolski *et al.* (2003). The 120 base pair marker consistently found in the US MFG1 isolates also was present in an A1 *P. cinnamomi* isolate from the western US included in the Dobrowolski *et al.* (2003) analysis. The US MFG2 isolates appear to have originated from a greater number of *P. cinnamomi* isolates characterized by Dobrowolski *et al.* (2003). The markers at e16 of US MFG2 match those of the Australian A2 type 2 (163) and markers found in *P. cinnamomi* isolates from Papua New Guinea (161). The d39 locus of US MFG2 isolates matches markers found in both the d39 locus of Australian A1 type 1 isolates and the d39 locus of Australian A2 type 1 isolates (Dobrowolski *et al.* 2003). The markers of the g10 locus of MFG2 do not match any of the predominant genotypes found in Australia. The most frequent markers found in the US isolates were 115 and 117 base pairs in length. These markers could be the result of nucleotide deletion on both alleles at the g10 locus of the Australian *P. cinnamomi* A1 type 1 (116 and 118) or nucleotide insertion on both alleles at the g10 locus of the Australian *P. cinnamomi* A2 type 2 (114 and 116). The less frequently

Table 7. Most commonly observed allele sizes at microsatellite loci d39, e16 and g10 of *P. cinnamomi* isolates from Australia, Papua New Guinea, and the eastern United States.

<i>P. cinnamomi</i> Microsatellite Group	Isolate Origin ^c	Common DNA Fragment Lengths of Microsatellite Locus Alleles (nucleotides)		
		Locus d39	Locus e16	Locus g10
MFG1 ^b	US	120/122	169/171	117/120
MFG2 ^b	US	122/124	161/163	115/117
A1 type 1 ^a	AUS, PNG	120/124	169/169	116/118
A2 type 1 ^a	AUS, PNG	122/136	169/171	118/154
A2 type 2 ^a	AUS, PNG	126/162	163	114/116

^a As described by Dobrowolski *et al.* (2003)

^b *P. cinnamomi* isolates from eastern US forest soils

^c AUS=Australia, PNG=Papua New Guinea, US=United States

observed markers at the g10 locus for the MFG2, 116 and 114, also are found at the g10 locus of the Australian A1 type 1 (116 only), Australian A2 type 2 (both) and some of the other world isolates including isolates from Papua New Guinea and the US (Dobrowolski *et al.* 2003). Other *P. cinnamomi* populations distributed worldwide also are represented predominantly by two genotypes. Linde *et al.* (1999) found South African A2 mating type *P. cinnamomi* isolates were one of two RFLP genotypes. They also determined that genotypes of *P. cinnamomi* isolates from Australia and Papua New Guinea were identical to those of the South African isolates. Comparison of other populations to Australia and US *P. cinnamomi* microsatellite groups is difficult due to the variety of molecular methods used to distinguish the different genotypes (Linde *et al.* 1999, Huberli *et al.* 2001, Dobrowolski *et al.* 2003). All methods previously used provide reliable data but unless isolates from multiple populations are included as controls, genetic similarities among all dual A2 populations is difficult to prove. The dissimilarities between microsatellite fingerprint patterns of US *P. cinnamomi* isolates and those studied by Dobrowolski *et al.* (2003) suggests that while the *P. cinnamomi* population from our study can be divided into two groups based on microsatellite fingerprints, these populations are not direct clonal descendents of the predominant *P. cinnamomi* genotypes described by Dobrowolski *et al.*

(2003) and Huberli *et al.* (2001). This probably holds true for A2 *P. cinnamomi* isolates from other parts of the world. Conversely, microsatellite marker similarities among isolates from the US, Australia and Papua New Guinea does suggest that they all have a common ancestor, possibly of Austral-Asian origin. Microsatellite fingerprint characteristics of the US *P. cinnamomi* isolates give clues to possible origins of these two lineages. The shared allele of the two MFGs at locus d39 suggests that all US isolates had a common ancestor. The absence of the A1 mating type reduces the likelihood of sexual recombination with A2 isolates as a source of the shared allele. In Australia, where two mating types were found in close proximity, evidence of sexual recombination could not be found (Dobrowolski *et al.* 2003). Variation in microsatellite fingerprints of Australian isolates was attributed to mitotic recombination. However, the data suggest that the shared allele in the US *P. cinnamomi* isolates most likely did not arise from such mitotic recombination or through genetic exchange between two individuals of different fingerprint groups. If mitotic recombination or exchange between isolates from each MFG had occurred, variability in fingerprint patterns of recovered isolates would have been greater. Presumably, if mitotic recombination were occurring, there would have been isolates that were homozygous at d39 and there would have been greater marker variability at locus g13 because the two loci are linked (Dobrowolski *et al.* 2002). Isolates from each MFG were found coexisting at two of the sites from which *P. cinnamomi* was isolated. One site was the West Virginia University forest, which was intensively sampled. The data from this site as well as data including all sites demonstrate that isolates in each MFG are heterozygous at both d39 and g13 and that neither group shares alleles at g13. Additionally, the consistency of the MFG1 and MFG2 across a very large sample area suggests that these genotypes were formed prior to the establishment of these populations. Thus, the common allele probably was the result of sexual

recombination with a *P. cinnamomi* A1 mating type isolate before these populations were established at the sites from which isolates were collected and possibly prior to their introduction to North America.

Determining the origin of the US *P. cinnamomi* populations will require further investigation of *P. cinnamomi* present in the US along with analysis of *P. cinnamomi* isolates from other continents using a standardized method such as analysis of the same microsatellite markers or DNA sequencing. The microsatellite markers developed by Dobrowolski *et al.* (2003) can consistently differentiate genetic differences among *P. cinnamomi* populations, even those that are geographically distant from the populations used to develop the markers.

6.2 Phenotypic Variation

The variations in phenotypic characteristics observed in the *P. cinnamomi* populations studied here were consistent with variations described by other researchers. Average sporangia size is somewhat larger than that first described by Rands (1922), but other detailed descriptions have reported similar ranges and averages for sporangia length, width and length-width ratio (Royal and Hickman 1964, Ho and Zentmyer 1977, Gerrettson-Cornell 1983, Gallegly and Hong 2008). The largest sporangium recorded in this study (14 μm longer and 15 μm wider) appears to be the largest recorded for *P. cinnamomi* when compared to the largest sporangia measured by Royle and Hickman (1964). Average chlamydospore diameter and range of variation among isolates in this study were slightly greater than those described by Rands (1922). The dimensions of antheridia, oogonia, and oospores were within maximum and minimum measurement ranges and within the range of means described previously (Royal and Hickman 1964, Ann and Ko 1985,

Garretson-Cornell 1989, Huberli *et al.* 2001, Gallegly and Hong 2008). Likewise, colony morphology did not deviate greatly from previous descriptions (Huberli *et al.* 2001, Zentmyer 1980). Growth rates were slightly higher than reported by Huberli *et al.* (2001) but were similar to that of the *P. cinnamomi* type culture (Zentmyer 1980).

6.3 Relationship of Phenotype, Microsatellite Fingerprint, Host and Geographic Origin

This is the first study where large numbers of *P. cinnamomi* isolates from the US compared interspecific phenotypic and genotypic characteristics relative to geographic and host plant origin. Asexual and sexual spore features differed most with regard to geographic origin suggesting a geographical effect on expression of phenotype. Huberli *et al.* (2001) also observed differences in sporangia dimensions of isolates from different study sites. Because of considerable variation in sporangia size, however, the reliability of this trait to identify subspecies is limited (Zentmyer 1980). In this study, chlamydospore, oogonia, and oospore diameters also differed significantly between isolates from different latitude zones, but all means still fell within the ranges reported in past descriptions (e.g. Zentmyer 1980). Sporangia dimensions were significantly different when isolates were grouped and compared based on host, MFP and colony morphology. Chlamydospore dimensions of isolates grouped by host type also were significantly different. The level of significance (P) when comparing spore means from different isolate groupings was relatively similar and the R^2 of all spore dimensions comparisons in relation to MFP, origin, host, or colony morphology was small. Sporangia, chlamydospores, oospores, oogonia, and antheridia dimensions were weakly correlated to MFP, geographic origin, host, or colony morphology because standard deviations was high for all means and often overlapped. The large variation in spore characteristics observed for each isolate further

supports that the variation of spore dimensions between isolate groups is explained by the variation inherent in the species and not by the factors used to separate the isolates in this study.

Colony morphology of these US *P. cinnamomi* isolates could not be predicted by genotype, host, or geographic origin, even though each MFG tended to exhibit one colony morphology more frequently than the other. Varying results on the linkage between genotype groups and colony morphology have been reported by others. Colony morphology of Australian *P. cinnamomi* isolates also grouped with a particular genotype (Dobrowolski *et al.* 2003). In contrast, colony morphology of Australian A2 type 1 isolates from a single site varied considerably (Huberli *et al.* 2001). Environment may have contributed to this variation, since colony morphology of some isolates changed with incubation temperature. Despite some consistency of correlation between colony morphology and MFP, evidence also indicates colony morphology of *P. cinnamomi* isolates depends on other factors, so that colony morphology alone is not diagnostic of genotype differences.

The relationship between isolate colony morphology and host was only evident with isolates recovered from soil around white oaks. This result may be attributed in part to the grouping of these isolates in the MFG1 and predominant rosaceous colony pattern. Conversely, isolates from sites north of the 39th latitude grouped with similar frequency in each MFG, but isolates from northern sites had a higher frequency of rosaceous colonies. Possibly, isolates with this colony morphology have a survivability advantage at lower temperatures, or they are more sensitive to higher temperatures. The latter hypothesis is supported by growth rate data where isolates with

rosaceous colony patterns were less tolerant of temperature increases than isolates with stellate colony morphologies.

6.4 Pathogenic Variation

One value of discriminating among subgroups of a particular species of a plant pathogen, be it by their physical characteristics or their genetics, is to determine if these variations translate into variations in each group's ability to cause disease and whether such variation might influence pathogenicity. The *P. cinnamomi* isolates studied in this work varied phenotypically and genetically. The differences in MFG or phenotype of isolates do not appear to translate into pathogenic variation when inoculated to red oak. This does not rule out the possibility that *P. cinnamomi* isolates belonging to each MFG do not differ in their pathogenic potential to hosts other than red oak. Variation in pathogenicity of *P. cinnamomi* on other oak and other tree genera has been reported, although the genetics of the isolates were not characterized (Crandle *et al.* 1945, Jordan and Tainter 1996, Robin and Desprez-Loustau 1998). Linde *et al.* (1999) reported correlations between *P. cinnamomi* isolate multilocus isozyme genotypes and the level of pathogenicity in eucalyptus, suggesting genotype variation may indicate pathogenic variability. Inoculation data from the present study, however, suggest that microsatellite fingerprint groups do not differ in pathogenicity. Additional pathogenicity tests on other *P. cinnamomi* hosts from different genera may differentiate pathogenicity among the different MFG found in the US. Balci *et al.* (2008) showed that one *P. cinnamomi* isolate from eastern forest soils varied in pathogenicity when inoculated into the stems of four different oak species. An isolate from the same site was used in this study and it was found to belong to the MFG2. An isolate belonging to MFG1 may therefore exhibit similar variation. Whether or not the variation

would be consistent with that of MFG2 isolates is unknown. The different *P. cinnamomi* microsatellite fingerprint groups may even have similar pathogenicities on oaks but each fingerprint group may react differently to other known hosts. Knowing the pathogenicity of these MFG on a wide range of *P. cinnamomi* hosts would be useful in developing plant cultivars resistant to *P. cinnamomi*. If different microsatellite fingerprint groups did turn out to have different pathogenicities on different hosts, the microsatellite markers would be useful in distinguishing which isolates were pathogenic on which hosts.

The presence of this fungus-like organism in ecosystems across the globe and the diversity of its hosts is testament to the versatility of this pathogen. This study has shown that the pathogenic versatility and cosmopolitan nature of *P. cinnamomi* arise from a rather discrete set of characters that have changed little despite its spread across the globe. Questions still remain about the impact of *P. cinnamomi* on health of northeastern US forests and why *P. cinnamomi* is not having a greater impact on tree species known to be susceptible to infection. The extensive phenotypic evaluation would suggest that physical differences are not vastly different among *P. cinnamomi* isolates and the same can be said for genetic variation. This leads one to believe that there are other external factors influencing the ability of the *P. cinnamomi* populations in eastern forests to cause disease. One such factor could be climate. As previously stated, the northern limit of *P. cinnamomi* in the eastern US is around the 40th latitude. Isolates were collected from regions with an average annual minimum temperature range of -12°C to -23°C (USDA Plant Hardiness Zone). These low winter temperatures probably prevent a build up of inoculum (Brasier and Scott 1994), which may be the reason why a greater disease impact is not seen. Balci *et al.* (2008) demonstrated in field inoculation experiments that lesions development of

stem inoculated oak seedlings varied between spring and fall inoculations. When inoculated in the fall, lesion development was decreased, probably due to lower temperatures. Even if natural stem infections were to occur, winter temperatures would slow the progression of the disease possibly allowing the tree to overcome the infection. These are important considerations when looking to the future of these forests. While *P. cinnamomi* was not observed causing above ground infections in the northeast-central US, decreased periods of temperatures that limit *P. cinnamomi* growth in the soil could result in increased *P. cinnamomi* inoculum, leading to more root infections. Warmer or shorter winters also could result in the progression of *P. cinnamomi* from the roots into the main stem of trees instead of being limited to the roots (Balci *et al.* 2008). Increasing temperatures could lead to an increase incidence of drought, thus weakening trees, leaving them more susceptible to *P. cinnamomi* infection (Brasier and Scott 1994). *Phytophthora cinnamomi* has already been linked to white oak decline in southern Ohio (Balci *et al.* 2009) and could contribute to further decline or outright disease could be more commonplace if warming trends continue.

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