Title

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Population Biology

*e-*X**tra***

Genotypic Diversity of *Phytophthora cinnamomi* and *P. plurivora* in Maryland's Nurseries and Mid-Atlantic Forests

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ABSTRACT

Genetic diversity of two *Phytophthora* spp.—*P. cinnamomi* (102 isolates), commonly encountered in Maryland nurseries and forests in the Mid-Atlantic United States, and *P. plurivora* (186 isolates), a species common in nurseries—was characterized using amplified fragment length polymorphism. Expected heterozygosity and other indices suggested a lower level of diversity among *P. cinnamomi* than *P. plurivora* isolates. Hierarchical clustering showed *P. cinnamomi* isolates separated into four clusters, and two of the largest clusters were closely related, containing 80% of the isolates. In contrast, *P. plurivora* isolates separated into six clusters, one of which included approximately 40% of the isolates. *P. plurivora* isolates recovered from the environment (e.g., soil and water) were genotypically more diverse than those found causing lesions. For both species, isolate origin (forest versus nursery or among nurseries) was a significant factor of heterozygosity. Clonal groups existed within *P. cinnamomi* and *P. plurivora* and included isolates from both forest and nurseries, suggesting that a pathway from nurseries to forests or vice versa exists.

The genus *Phytophthora* includes numerous species that are among the most significant plant pathogens worldwide. As a result, the genus has been the focus of many population biology studies (Bhat and Browne 2007; Bhat et al. 2006; Bienapfl and Balci 2014; Cooke et al. 2003, 2005; Dobrowolski et al. 2003; Donahoo and Lamour 2008; Duan et al. 2008; Dunn et al. 2010; Eggers et al. 2012; Gevens et al. 2008; Hantula et al. 2000; Hulvey et al. 2010; Ivors et al. 2004; Lamour and Hausbeck 2001; Linde et al. 1997, 1999). These studies have shown that population structures can vary considerably between different regions and species. For example, populations of *Phytophthora cactorum* in the United States, England, and Finland were shown to have lower genetic diversity (Bhat et al. 2006; Hantula et al. 2000) than populations of *P. citricola*, *P. capsici, P. nicotianae*, and *P. infestans* from agricultural or nursery systems (Bhat and Browne 2007; Dunn et al. 2010; Lamour and Hausbeck 2001; Shattock 1988).

In addition, relationships between genetic diversity and other variables such as host, pathogenicity, and fungicide sensitivity can differ. *P. citricola* isolates (at the time of analysis still considered a species complex) in California had high levels of genetic diversity, and much of the variation was associated with host and geography but not aggressiveness (Bhat and Browne 2007). In other instances, a population of *P. cactorum* isolated from rhododendron in Germany was found to be genetically similar to U.S. isolates from strawberry but were nonpathogenic on the fruit (Donahoo and Lamour 2008). Yet another study revealed differences where *P. capsici* isolates in Michigan displayed variation in phenotype and mefenoxam resistance but the isolates were similar within specific locations and sample times (Gevens et al. 2008).

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*The *e-*X**tra* logo stands for "electronic extra" and indicates that one supplementary figure and three supplementary tables are published online.

These studies demonstrate that *Phytophthora* populations can vary and spatial genetic diversity could improve management strategies. Incorporation of this knowledge into pathogen management plans can improve practices while streamlining fungicide use (the most widely utilized method in combating *Phytophthora*-related disease problems). This would be particularly useful for nurseries, where great plant diversity exists and new plant material is sourced regularly, facilitating movement of pathogens (Bienapfl and Balci 2014, Jung et al. 2016).

Among the *Phytophthora* spp. found in the United States, *P. cinnamomi* and *P. plurivora* are the most widespread (Bienapfl and Balci 2014; McConnell and Balci 2014). *P. plurivora* is one of eight species once considered part of the *P. citricola* complex (now delineated into *P. capensis*, *P. caryae*, *P. citricola*, *P. pachypleura*, *P. pini*, *P. plurivora, P. multivora*, and *P. acerina*) (Bezuidenhout et al. 2010, Brazee et al. in press, Ginetti et al. 2014; Henricot et al. 2014; Hong et al. 2011; Jung and Burgess 2009; Scott et al. 2009). Although both species have a worldwide distribution, *P. plurivora* is potentially of Asian origin and introduced to North America from Europe (Huai et al. 2013; Jung and Burgess 2009; Jung et al. 2017; Schoebel et al. 2014). *P. cinnamomi* is thought to have originated in Southeast Asia but became a globally well-established species in nursery, agricultural, and forest ecosystems, with records dating back over a century (Arentz and Simpson 1986; Balci et al. 2007; Brasier et al. 1993; Duan et al. 2008; Erwin and Ribeiro 1996; Jung et al. 2016, 2017; Zentmyer 1980).

Both species are widespread and differences exist in their pathogenicity, ecology, and mating behavior. Like *P. cinnamomi, P. plurivora* is known to cause disease on numerous plant species; however, *P. cinnamomi* is strictly causing root damage and, on susceptible hosts under conducive conditions, it commonly forms lesions extending toward the stem. Although the same pathology is true for *P. plurivora*, it also can cause damage on shoots and foliage. The reason behind this pathological difference is likely related to its ecological adaptations. Unlike *P. cinnamomi*, *P. plurivora* is adapted to aquatic environments (e.g., fresh stream water or irrigation water), and can spread to aboveground plant parts through rain splash or overhead irrigation (Brazee et al. 2016; Huai et al.

2013; Jung et al. 2016, 2017; Nechwatal et al. 2011). Ecologically, *P. plurivora* appears to be not restricted by cold winter temperatures whereas *P. cinnamomi* is limited in its pathology and spread (Bergot et al. 2004; McConnell and Balci 2014). *P. cinnamomi* is heterothallic and, interestingly, only its A2 mating type has successfully established itself globally (Arentz and Simpson 1986; Cahill et al. 2008; Zentmyer 1980). Unlike *P. cinnamomi, P. plurivora* is homothallic and reproduces via self fertilization.

Our objectives were to examine the population structure of these commonly encountered pathogens in Maryland nurseries or Mid-Atlantic forest. Data were examined to determine whether there was any association between genotypes and origin (nursery versus forest and among different nurseries), host type, isolation source (necrotic tissue versus environment including soil and water), and host symptom (asymptomatic versus symptomatic).

MATERIALS AND METHODS

Surveys for the incidence of *Phytophthora* spp. were conducted between 2010 and 2012 at seven Maryland ornamental nurseries, resulting in a collection of 680 *Phytophthora* isolates from various hosts and substrates (Bienapfl and Balci 2014). These isolates were recovered as a result of isolation from diseased plants (necrotic stem or root tissue), potting media, or irrigation water using direct plating and a rhododendron leaf-baiting technique (Bienapfl and Balci 2014). In total, 292 isolates (102 *P. cinnamomi* and 186 *P. plurivora* isolates) representing the two most common species was selected (Supplementary Table S1). These included 80 isolates from forests (76 *P. cinnamomi* and 4 *P. plurivora*) and 208 isolates from nurseries (26 *P. cinnamomi* and 182 *P. plurivora*). The forest isolates were collected over a decade (from 2002 and 2012) from rhizosphere soil associated with various mature oak trees with and without decline symptoms in mid-Atlantic oak forest ecosystems (Balci et al. 2007; McConnell and Balci 2014). Because these forests are not the subject of any sort of management (namely, fungicide applications, watering regimes, plant trade, and so on), isolates recovered from this system served as a baseline for comparison with isolates recovered from the intensively managed nurseries.

Isolates of each species were grouped based on variables such as origin (nursery versus forest and different nurseries), isolation source (necrotic tissue versus environment, including soil and water), host symptom (asymptomatic versus symptomatic), and host genus. Isolates included in the symptomatic host group were those that were recovered from a necrotic tissue or hosts with wilting symptoms. These isolates were collected from soil or soilless potting media using baits. Leaflets of *Quercus robur* or young *Rhododendron maximum* leaves served as baits. Approximately twice the amount of distilled water was added to 200 to 300 g of potting media in a plastic container. Any organic matter floating to the surface of the water was removed or cheesecloth was used to wrap soils with high organic matter to prevent debris from contacting the baits. Baits were then placed on the surface of the water and incubated for 3 to 5 days at 18°C. Leaves with discoloration or lesions were rinsed with tap water, followed by distilled water, then blotted dry and plated on clarified V8 (cV8) (10 g of $CaCO_3$ per 1 liter of V8 juice spun down at 4,000 rpm for 10 min) PARPNH (pimaricin at 10 µg/liter, ampicillin at 200 µg/liter, rifampicin at 10 µg/ml, pentachloronitrobenzene at 25 µg/liter, nystatin at 50 µg/liter, and hymexazol at 50 µg/liter) (Erwin and Ribeiro 1996; Jung et al. 1996; McConnell and Balci 2014). Emerging putative *Phytophthora* colonies were transferred to cornmeal agar slants and maintained at 8 or 15°C. Isolates included in the asymptomatic host group were those that were recovered from soil associated with asymptomatic plants or irrigation or stream water using rhododendron baits (*R. maximum*).

Mycelia preparation and DNA isolation. Isolates were grown on 10% buffered cV8 agar media (100 ml of cV8 juice in 900 ml of distilled H_2O with 10 g of agar) (Miller 1955). Two 1-cm² mycelial plugs were taken from 7- to 14-day-old isolates and placed in a 50-ml Falcon tube filled with 25 ml of 10% buffered cV8 broth (V8 agar media excluding agar). Tubes were placed on their sides at 23°C in the dark to produce mycelia. Approximately 200 mg of mycelia was produced in each tube (wet), harvested using a sterile dissecting needle under a bio hood, rinsed with sterile distilled water once, and dried on sterile filter paper. DNA was then isolated using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). DNA concentrations were adjusted to 14.0 ng/µl with nuclease-free water by averaging three measurements using a NanoDrop lite spectro-photometer (Thermo Scientific, Waltham, MA).

Amplified fragment length polymorphism analysis. Isolates were numbered sequentially and, to measure the replicability of peaks, 10 were randomly selected and repeated in each of the five plates. The location of these 10 isolates was determined by generating a set of 10 numbers between 1 and 96 for each plate. Five plates were created. Negative controls were included in plates 4 and 5. Randomization of plates was performed using random.org (https://www.random.org/).

The digestion and ligation steps were performed in the same reaction. Genomic DNA (approximately 0.42 µg per isolate) was incubated for 5 h at 37°C, then for 20 min at 80°C in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA) using an unheated lid. The solution consisted of 5 U of *Mse*I restriction enzyme (New England Biolabs [NEB], Ipswitch, MA) and 5 U of *Eco*RI-HF restriction enzyme (NEB) in 20 µl of nuclease free water, 10× CutSmart buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium zcetate, and bovine serum albumen at 100 µg/ml) (NEB), 10 mM ATP (NEB), 1 µl each of *Mse*I adapter and *Eco*RI adapter (Applied Biosystems, Foster City, CA), and 0.25 µl of T4 ligase (NEB). After the digestion or ligation, each reaction was diluted 1:10 with nuclease-free water.

A two-step amplification was used (Vos et al. 1995): an initial preamplification followed by a second round of amplification using selective primers. The preamplification step used 10 µM *Eco*RI and *Mse*I primers (NEB) in a 10-µl reaction of 10× Taq buffer [10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$) (NEB), 0.125 mM dNTP (NEB), 0.5 U of Taq DNA polymerase (NEB), and 2.0 µl of template DNA (the diluted digestion or ligation product). The preamplification cycling protocol was 95°C for 30 s; 25 cycles of 95°C for 30 s, 56°C for 1 min, and 68°C for 1 min; concluding with 68°C for 5 min and 4°C for 5 min (Hebert 2012). Preamplified products were diluted 1:40 with nuclease-free water and select amplification was performed using the same 10 µl of cocktail. However, for the select amplification, two bases (-AC) were added to the *Eco*RI primer (Integrated DNA Technologies [IDT], Coral-ville, IA), which was labeled with fluorescein amidite (Integrated DNA Technologies Coralville, IA), and three bases (-CAG) were added to the *Mse*I primer (IDT). A touchdown polymerase chain reaction (PCR) was used: 95°C for 30 s; eight cycles of 95°C for 30 s, 65°C for 1 min (lowering the annealing temperature by 1°C with each cycle), and 72°C for 1 min; 22 cycles of 95°C for 30 s, 58°C for 1 min, and 68°C for 1 min; concluding with 68°C for 5 min and 4°C for 5 min (Hebert 2012). To check quality, we visualized DNA by running final amplified fragment length polymorphism (AFLP) products on a 1.5% agarose gel (Fisher Scientific, Pittsburgh) in 1× sodium boric acid conductive medium (Brody and Kern 2004) for 12 min at 250 V, poststained with a 3× concentration of GelRed (Biotium, Hayward, CA) for 30 min.

AFLP fragment analysis. Selectively amplified PCR product was diluted 1:20 to eliminate off-scale peaking and fragment analysis was conducted by McLab (San Francisco, CA), which uses a 500 LIZ size standard (Life Technologies, Carlsbad, CA) and an ABI Genetic Analyzer (3730XL). Electropherograms were scored using GeneMapper (version 4.0; Applied Biosystems,). All samples were analyzed together to create the analysis panel. Blue dye color size range was set at 0 to 999, the marker repeat unit was set to 9, and the stutter ratio was left at 0.0. Peak detection was set to B = 1,000 and O = 75. Individual plates were scored separately using the same panel so that any peaks in the negative controls coinciding with

peaks in samples on the same plates could be removed. Resulting electropherograms with bad sizing quality or poorly resolved peaks were removed from the data set and fragment analysis was repeated. Occasionally, individual reactions resulted in poorly resolved AFLP profiles (e.g., low intensity of signal). Analyses were repeated for these isolates until quality improved.

All electropherograms were examined manually and compared with a matrix of presence or absence alleles between 57 and 489 bp. Those that displayed miscalled or unlabeled peaks were scored manually. Calls for peaks that did not reach or exceed the set threshold of 1,000 were removed. Peaks that appeared as "shoulders" of other peaks were only labeled if their height reached or exceeded 10% of the taller peak.

Selective primer optimization. Eleven different selective *Eco*RI and *Mse*I primer combinations (*Eco*RI-AA and *Mse*I-CAC and -CTA, *Eco*RI-AC and *Mse*I-CAA and -CAC, *Eco*RI-AG and *Mse*I-CTC, *Eco*RI-AT and *Mse*I-CAT, *Eco*RI-TA and *Mse*I-CTA and -CTC, *Eco*RI-TC and *Mse*I-CTC, and *Eco*RI-TG and *Mse*I-CAG and -CTC) were tested on five isolates representing each of the *Phytophthora* spp., with a positive control based on successful combinations in previous studies (Bhat and Browne 2007; Bhat et al. 2006; Lamour and Hausbeck 2001). *Eco*RI-AC was chosen based on number and resolution of bands produced on gels. Peak number and size in the resulting electropherograms for each of the isolate-primer pairs were compared and the primer combination *Eco*RI-AC/*Mse*I-CAG was selected.

Descriptive statistics, genetic diversity, and analysis of population structure. Parameters of genetic variability and population structure were estimated using GenAlEx 6.5 (Peakall and Smouse 2006). GenAlEx was also used to generate a clone-corrected data set by retaining one representative sample per clone in the data set. Analysis of molecular variance (AMOVA) was used to estimate the proportion of genetic variation within and among the isolates when defined as (i) source of isolation (environment versus necrotic tissue), (ii) origin of isolate (forest versus nursery or among different nurseries), (iii) host symptom (symptomatic versus asymptomatic host), and (iv) host–genus association. AMOVA was performed only for grouping variables that included more than five isolates. The parameter φ_{PT} (analogous to F_{ST} for binary data) was used to determine the effects of population subdivisions on genetic diversity. If the observed φ_{PT} value differed significantly from zero, the null hypothesis of no genetic differentiation could be rejected. Significance of fixation indices was tested using 1,000 nonparametric permutations.

A multivariate hierarchical clustering analysis using Ward's method was used to create a distance matrix, which was visualized with a heat map using JMP Genomics 6.0 (SAS Institute, Cary, NC). A dendrogram was built based on distance scales and clusters were then identified using the distance graph. Data without clone correction were utilized to visualize clonality within the population. In addition, a minimum spanning network using SplitsTree4 was generated to infer an implicit representation of evolutionary history of the two species analyzed (Huson and Bryant 2006). The default settings were used with 1,000 iterations.

Because prior information about the populations and an admixed model were lacking, evidence of clustering of genotypes within the population was assessed using the Bayesian analysis program STRUCTURE v.2.3.2 (Evanno et al. 2005; Pritchard et al. 2000). STRUCTURE determines the most likely number of differentiated clusters (K) represented by the sample and assigns the sampled genotypes to the inferred clusters. Using a random subset of 1,000 markers, we estimated the log likelihood of the data, given different numbers of genetic clusters K. An admixture model with correlated allele frequencies was utilized without sampling locations as priors. Other parameters were left at their default settings. For each K value of 1 through 15, we ran three replicates (100,000 burn-in cycles and 100,000 Markov chain Monte Carlo iterations), from which we calculated ΔK. The STRUCTURE Harvester software was used to identify the number of populations K with the best support (Earl and Vonholdt 2012).

RESULTS

AFLP analyses. The DNA banding pattern and polymorphisms detected by the AFLP experiment were consistent for control samples tested and no plate effect was found. The primer combination *Eco*RI-AC/*Mse*I-CAG resulted in a total of 168 clearly resolved fragments in the size range of 57 to 489 bp (Supplementary Table S2). *P. cinnamomi* had 44% of the polymorphisms while *P. plurivora* isolates had 83% (Table 1).

Genotypic diversity. The genetic diversity of each population was calculated based on the genotypes present using clone-corrected datasets and the genetic diversity parameters (i.e., the number of different alleles [N_A], number of effective alleles, and heterozygosis) (Table 1; Fig. 1). Overall, expected heterozygosity (H_E) was less for *P. cinnamomi* than for *P. plurivora*. Isolate origin (forest versus nursery) was a significant factor of genetic diversity for *P. cinnamomi* (Table 1; Fig. 1). Isolates recovered from forests had greater allelic richness and almost twice the number of polymorphisms. Isolates recovered from asymptomatic hosts, similar to *P. plurivora*, had greater genotypic diversity (Table 1; Fig. 1).

Separation of the *P. plurivora* population into origin or isolation source had interesting results: allelic richness (N_A) along with H_E and Shannon's Index indicated that, in *P. plurivora* populations, large differences exist among nurseries. In particular, isolates collected from nursery 6 had the greatest genotypic diversity (Table 1). When isolates were grouped based on other criteria, those that were recovered from lesions were less diverse than those isolated from the soil or environment. Similar to *P. cinnamomi*, genotypic diversity was higher among isolates recovered from asymptomatic hosts compared with those recovered from symptomatic hosts (i.e., isolates causing lesions). Interestingly, isolates associated with *Pieris* spp. had much greater allelic richness than *Rhododendron* and *Ilex* spp. (Table 1).

Population structure. Hierarchical clustering revealed distinctly different clades for both *Phytophthora* spp. (Fig. 2). *P. cinnamomi* isolates separated into four clusters, and two of the largest clusters were closely related (clusters 3 and 4), containing 80% of the isolates (isolate distribution [total 102]: cluster 1 = 15, cluster 2 = 5, cluster 3 = 43, and cluster 4 = 39). In contrast, *P. plurivora* isolates separated into six clusters, one of which included approximately 40% of the isolates (isolate distribution [total 186]: cluster 1 = 24, cluster 2 = 14, cluster 3 = 18, cluster 4 = 32, cluster 5 = 26, and cluster 6 = 72).

The minimum spanning networks were in agreement with genetic distances observed in hierarchical clustering. In spite of the wide range of *P. cinnamomi* isolate origins expanding over a 10-year period, they were closely related (Fig. 3). In contrast, *P. plurivora* isolates were more diverse and included clearly emerging separate lineages.

Clonal groups were detected in both species (Figs. 2 and 3; Supplementary Table S3). Interestingly, some clonal groups were present in both forests and nurseries and at different nurseries. Although all clonal groups within *P. cinnamomi* were located in clusters 3 and 4, those within *P. plurivora* were located in clusters 4, 5, and 6 (Fig. 2). Clonal *P. plurivora* isolates were found more frequently associated with symptomatic hosts (likelihood ratio: $\chi^2 = 44.4$, $P = 0.025$).

Four clusters were identified using STRUCTURE analyses for both *P. cinnamomi* and *P. plurivora* (Supplementary Fig. S1). These genetically distinct clusters primarily corresponded to hierarchical clustering.

P. cinnamomi isolates among different origins were the only source of a significant genetic variation in its overall population, although only at a relatively low level (3%) (Table 2). No genetic differences existed between or among isolates when grouped based on host symptoms or host associations. In contrast, with *P. plurivora*, Φ_{PT} values computed for the four categories (origin, isolation source, host symptom, and

host genus) were consistently significant (Table 2). The greatest genetic variation (24%) was detected among different nursery populations whereas approximately 8 to 13% of genetic variation were accounted among the isolation source, host symptom, or host association.

DISCUSSION

This study is the first to examine the genotypic diversity of the two most commonly encountered *Phytophthora* spp. in Maryland nurseries and forests. It also serves as a baseline for understanding the population biology of these pathogens for these systems.

Our findings support previous studies revealing the limited genetic diversity in *P. cinnamomi* regardless of the extremely diverse environments it colonized (Dobrowolski et al. 2003; Duan et al. 2008; Eggers et al. 2012; Linde et al. 1997, 1999; Old et al. 1984, 1988). Given the low genetic diversity seen in *P. cinnamomi* in our study, as well as in other regions where the population is primarily dominated by the A2 mating type, inbreeding becomes the means of reproduction (Lamour and Kamoun 2009). There are several explanations for why this might be occurring. First, sexual reproduction has costs and could be unfavorable if oospores fail to germinate or the progeny fail to survive (Dobrowolski et al. 2003).

TABLE 1. Population diversity measures for five *Phytophthora* spp. found in Maryland's nurseries and Mid-Atlantic forests[a]

Species, populations	N	B (n)	P (n)	P (%)	N_A	N_E	I	H_E	uH_E
P. plurivora									
Overall	113	N/A	142	82.74	1.655	1.071	0.109	0.056	0.056
Origin									
Nursery 2	61	20	…	46.43	0.929	1.073	0.091	0.051	0.051
Nursery 4	6	6	…	22.62	0.458	1.085	0.092	0.056	0.062
Nursery 6	37	43	…	66.07	1.321	1.074	0.117	0.060	0.061
Nursery 7	6	0	…	16.67	0.333	1.068	0.070	0.043	0.047
Source									
Environment	62	42	…	70.83	1.417	1.071	0.108	0.056	0.056
Necrotic tissue	51	20	…	57.74	1.155	1.076	0.097	0.053	0.053
Host symptom									
Symptomatic	71	41	…	58.33	1.167	1.074	0.103	0.056	0.056
Asymptomatic	42	24	…	68.45	1.369	1.068	0.111	0.056	0.056
Host genus									
Ilex	26	11	…	36.31	0.726	1.076	0.094	0.054	0.055
Pieris	35	49	…	63.69	1.274	1.073	0.112	0.058	0.058
Rhododendron	31	8	…	35.71	0.714	1.074	0.095	0.054	0.055
P. cinnamomi									
Overall	78	N/A	84	44.64	0.893	1.077	0.079	0.047	0.047
Origin									
Forest	58	39	…	39.29	0.786	1.075	0.079	0.047	0.047
Nursery 6	20	9	…	21.43	0.429	1.077	0.071	0.045	0.046
Host symptom									
Symptomatic	40	13	…	27.38	0.548	1.070	0.071	0.044	0.044
Asymptomatic	38	29	…	36.90	0.738	1.083	0.083	0.050	0.051
Host genus									
Pieris	14	8	…	19.05	0.381	1.077	0.071	0.045	0.047
Quercus	55	42	…	39.29	0.786	1.075	0.080	0.047	0.048

[a] Only populations >7 isolates were included. N = number of isolates tested, B = number of bands unique to a single population, P = number of polymorphic bands, P (%) = percentage of alleles that are polymorphic, N_A = number of different alleles, N_E = number of effective alleles, I = Shannon's information index H_E = expected heterozygosis, uH_E = unbiased expected heterozygosis, and N/A = not applicable.
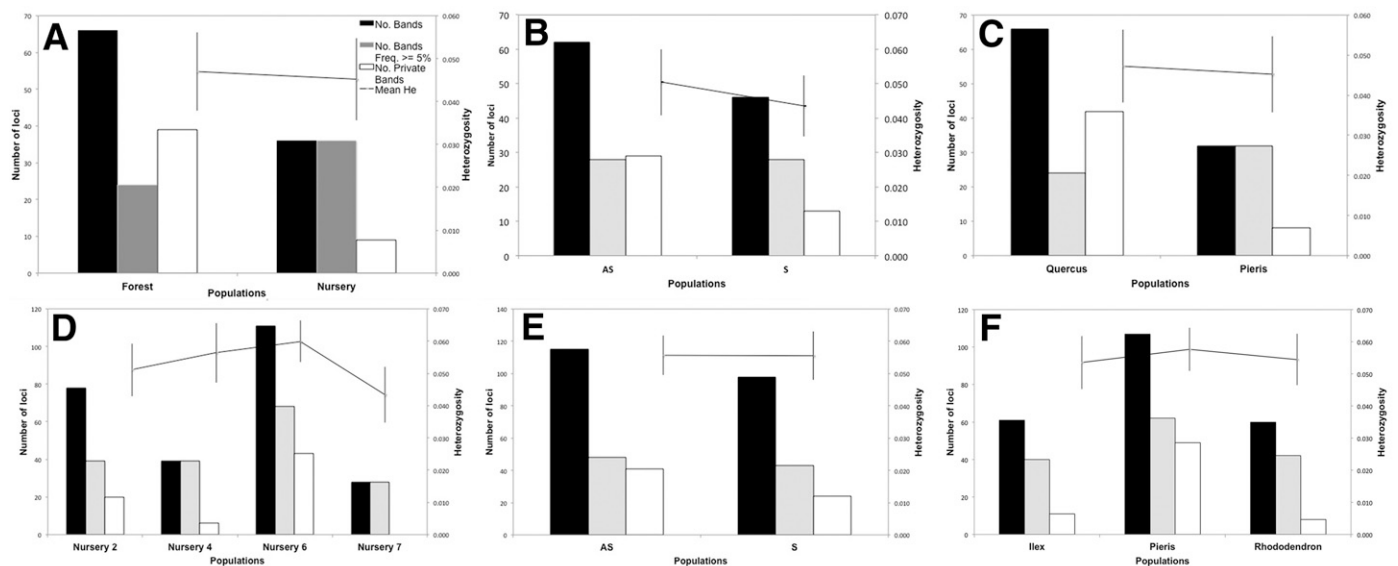

Fig. 1. Genetic variability of **A** to **C,** *Phytophthora cinnamomi* and **D** to **F,** *P. plurivora.*

Second, in environments where the pathogen is well established or where there are susceptible hosts, little to no selection pressure would exist and, thus, sexual reproduction would be unnecessary. The frequency with which this species is observed on a range of plant genera in nursery and forest systems demonstrates that *P. cinnamomi* is, indeed, well established, and can survive on and infect a wide range of hosts. Third, sexual reproduction can lead to the breakup of coadapted genes in clonal lineages, which would be unfavorable in low selection pressure environments (Dobrowolski et al. 2003). For these reasons, sexual reproduction is not always advantageous, which could explain why, even when both mating types of *P. cinnamomi* are present, outcrossing is limited (Dobrowolski et al. 2003; Jung et al. 2013).

Our study suggests that, although the difference was small, the forest population of *P. cinnamomi* is more diverse than the Maryland nursery population. A lower level of genetic diversity in the nursery population could have arisen from selection pressure created by nursery management practices; in particular, fungicide applications. However, when we screened most of the *P. cinnamomi* isolates for their sensitivity to five common fungicides, insensitive isolates were present in both forest and nursery populations (Beaulieu 2015). Alternatively, plant trade could be responsible for the difference between these two systems. It is possible that either particular genotypes are being repeatedly introduced into nurseries due to trade partnerships or particular genotypes were introduced early on as "founder populations" and have remained dominant over time. The possibility that particular genotypes are better adapted to the nursery environment due to nursery practices (i.e., soilless potting media, extreme high temperatures, watering regimes, or fungicide applications) and act as a limiting factor for genotypic diversity deserves further investigation.

Although *P. cinnamomi* appears to be well established in both forest and nursery systems, it is plausible that reproduction strategies differ in these systems as a result of nursery plant trade and pest management strategies. There is no barrier to alternative reproduction behaviors in heterothallic oomycetes because both
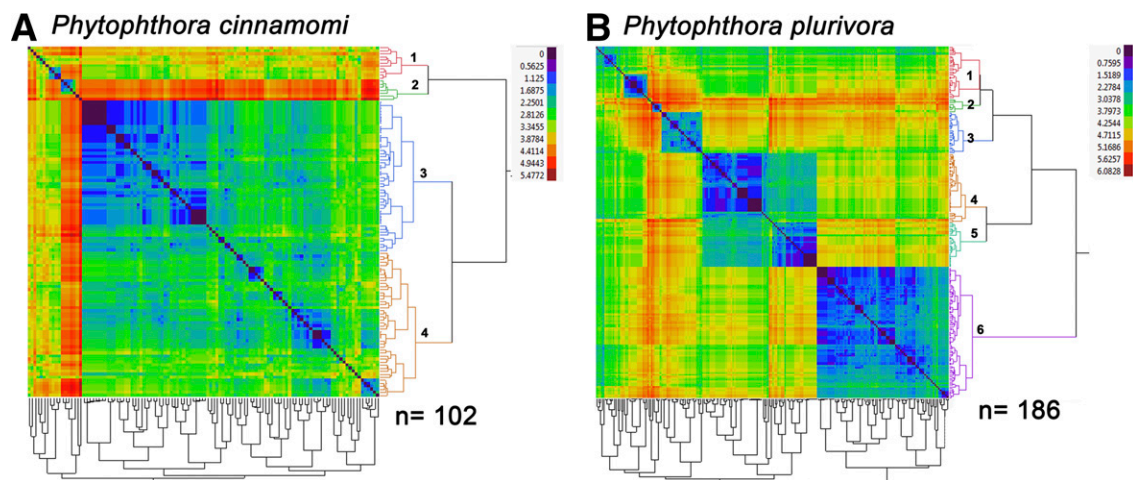

Fig. 2. Heat maps and dendrograms from hierarchical clustering generated in JMP Genomics using the non-clone-corrected amplified fragment length polymorphism data for *Phytophthora cinnamomi* and *P. plurivora*. Each line is represented by an individual.
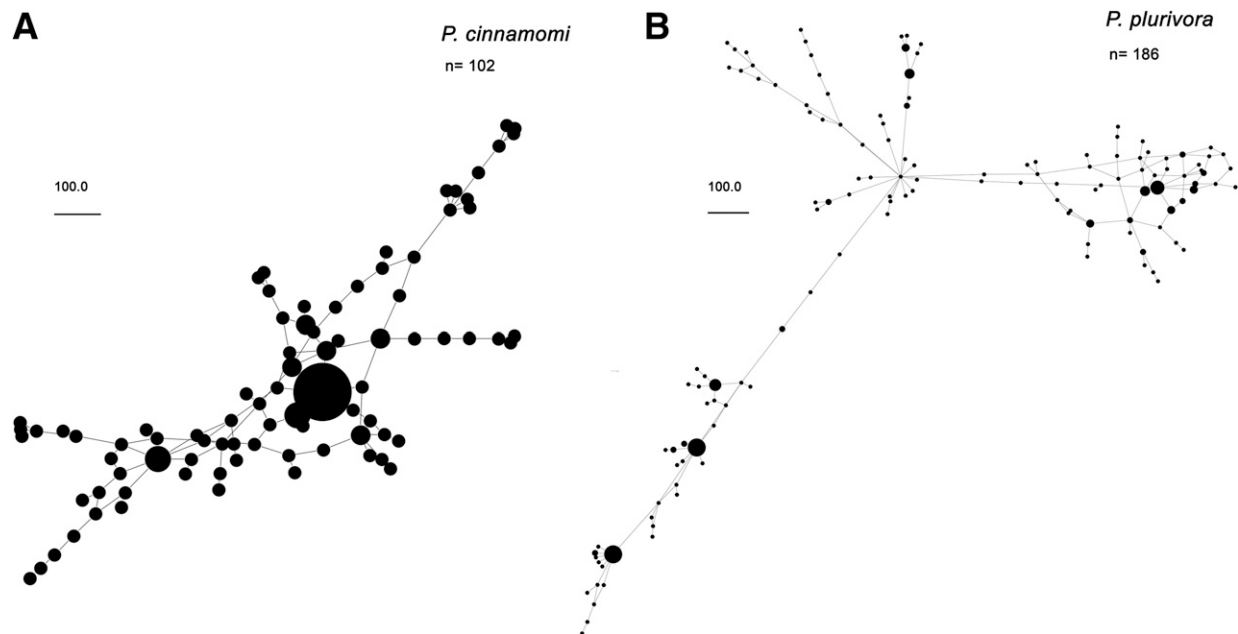

Fig. 3. Minimum spanning networks of **A,** *Phytophthora cinnamomi* and **B,** *P. plurivora.* Each circle represents a unique amplified fragment length polymorphism genotype, where the size of the circle is relative to the number of individuals represented in the data.

mating type hormones are consistent in all heterothallic *Phytophthora* spp. (Lamour and Kamoun 2009). Although less common, the A1 mating type of *P. cambivora* occurs in both mid-Atlantic forests and Maryland nurseries (Bienapfl and Balci 2014; McConnell and Balci 2014), which potentially could stimulate selfing and be responsible for population variations that we have observed in this study.

Environmental factors such as aging, physical damage, harsh environmental conditions, fungicides, host-plant exudates, and volatile compounds formed by *Trichoderma* spp. can also stimulate selfing within heterothallic oomycetes (Brasier 1971, 1978; Jayasekera et al. 2007; Jung et al. 2013; Lamour and Kamoun 2009). Differences between forest and nursery systems in terms of these environmental factors could cause different rates of selfing which, in turn, could affect genetic differences between the two systems. Selfing in heterothallic oomycetes, known as "secondary homothallism", may confer an evolutionary advantage by enabling heterothallic species to form oospores in adverse environmental conditions (Lamour and Kamoun 2009). In fact, the formation of oospores in A2 populations has been shown to occur regularly in natural forests as a strategy to survive adverse conditions (Crone et al. 2013; Jung et al. 2013). Another factor to consider is the stimulation of oospores in many water molds when salinity levels of the surrounding environment are consistent with those of their natural habitats (Lamour and Kamoun 2009). Perhaps differences in salinity levels between rainwater in forests and irrigation systems in nurseries resulted in different levels of oosporogenesis in the two systems.

In contrast to *P. cinnamomi*, the *P. plurivora* population was very diverse. The level of true genetic variability of both species is not clear because of their exotic origin in North America. The contrasting difference observed among the degree of polymorphism (*P. plurivora* = 83% versus *P. cinnamomi* = 44%) is probably related to their breeding systems. *P. quercina*, a species with a narrow host range (an average 39% polymorphism level) compared with *P. cinnamomi* and *P. plurivora*, was considered an invasive species for Europe (Cooke et al. 2005). Unlike *P. cinnamomi, P. plurivora* does not require another mating type to reproduce sexually (inbreeding), which perhaps can explain the differences in diversity observed between the two species. The only population study thus far for *P. plurivora* is by Schoebel et al. (2014), in which isolates representing Europe were characterized as moderately diverse. AFLP genotyping of *P. citricola* in California (Bhat and Browne 2007) and Tennessee (Donahoo and Lamour 2008; Hulvey et al. 2010) revealed high genetic diversity. However, it should be noted

that these studies were conducted before species delineation in the *P. citricola* complex and, hence, the population assessment is most likely a reflection of the fact that the isolates actually belonged to different species but could also point to diversity within each delineated species. *P. plurivora* is commonly found in both natural and managed environments (Bienapfl and Balci 2014; Jung and Burgess 2009; Jung et al. 2016; McConnell and Balci 2014; Schoebel et al. 2014); thus, it is possible that this species made up a significant proportion of the *P. citricola* populations genotyped in these studies.

Clonal isolates were commonly observed within the species investigated. Moreover, clonal groups that included isolates originating from both forests and nurseries were observed, providing evidence that a pathway exists between the two systems and also among nurseries. Both species have been reported in the United States for decades, thus potentially enabling the same clonal isolates, over time, to spread into both systems. Such pathways have been demonstrated between nurseries and forest, riparian, and horticultural plantings in Europe and, most notoriously, for *P. ramorum* in Europe and California (Ivors et al. 2004; Jung et al. 2016).

In *P. plurivora*, we found isolates causing lesions to be more likely of a clonal origin. This finding supports the hypothesis that soilborne populations of a *Phytophthora* spp. are more diverse than populations causing disease on host tissue. Isolates causing disease could be specifically adapted clones while the soilborne populations serve as a genetic reservoir, enabling the species to adapt to changes in environmental conditions and hosts (Brasier 1992). On the other hand, the findings might support the idea that introduced aggressive isolates are dominating the population over time. There are at least six genetically distinct clusters in the *P. plurivora* nursery population. A majority of these clusters contain clonal isolates. The ability of *Phytophthora* spp. to spread clonally has been demonstrated by the population structure of *P. ramorum* in the United States. AFLP profiles showed that 75% of U.S. *P. ramorum* isolates consisted of a single genotype (Ivors et al. 2004). Clonality was also found in both managed and natural populations of *P. citricola* sensu lato in Tennessee (Hulvey et al. 2010). Nevertheless, it remains unclear why some clonal groups are more common than others. Further study is needed to demonstrate whether fitness factors play a role in the success of these clonal populations.

The significant genetic variation found for *P. plurivora* on different host genera suggests that host selection may be occurring. This finding is indicative of a species that adapts quickly to its hosts.

TABLE 2. Summary of analysis of molecular variance of *Phytophthora* population based on amplified fragment length polymorphism

Source of variation	*P. cinnamomi*			*P. plurivora*		
	Variance	Total (%)	Φ_{PT}[a]	Variance	Total (%)	Φ_{PT}[a]
Origin (forest vs. nursery)						
Among populations	0.125	3	0.027*	…	…	…
Within populations	4.579	97	…	…	…	…
Origin (different nurseries)						
Among populations	…	…	…	2.083	24	0.240***
Within populations	…	…	…	6.604	76	…
Isolation source (environment vs. necrotic tissue)						
Among populations	…	…	…	1.088	13	0.130***
Within populations	…	…	…	7.259	87	…
Host symptom (symptomatic vs. asymptomatic)						
Among populations	0.037	1	0.008	0.613	8	0.075***
Within populations	4.609	99	…	7.521	92	…
Host association (different genera)						
Among populations	0.000	0	−0.005	0.838	10	0.103***
Within populations	4.506	100	…	7.261	90	…

[a] Analog of Wright's fixation index (F_{ST}). Asterisks indicate probability of the F_{ST} being significantly greater than 0, where * = $P \leq 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.

In contrast, we did not find any evidence that host selection is occurring in *P. cinnamomi*, despite its ability to infect thousands of plant species, including hundreds of plant genera (Cahill et al. 2008; Zentmyer 1980).

Differences in genetic diversity between *P. cinnamomi* and *P. plurivora* underscore the concept of diversity versus pathogenicity. Although worldwide populations of *P. cinnamomi* have been shown to be less diverse (Dobrowolski et al. 2003; Eggers et al. 2012; Linde et al. 1997, 1999), the pathogen causes severe damage to thousands of hosts, notoriously in Western Australian Jarrah forests and oak forests along the Mediterranean in Europe (Brasier et al. 1993; Cahill et al. 2008; Zentmyer 1980). Despite being widely prevalent on numerous woody plants in nursery and forest ecosystems both in Europe and North America, disease caused by *P. plurivora* has not been observed on the scale of the forest dieback caused by *P. cinnamomi*. One major difference is that *P. plurivora* appears to have even greater ecological plasticity than *P. cinnamomi*; it is present in cooler northern and warmer southern locations and colonizes diverse ecological niches (e.g., soil and aquatic environments) (Balci and Halmschlager 2003a,b; Balci et al. 2007; Jung and Burgess 2009; Jung et al. 2016; Nechwatal et al. 2011). This raises questions about whether or not genetic diversity is required for a pathogen to cause major losses or, rather, if it is more significant in adaptation to different environments and substrates.

Our isolate collection was an accumulation of intensive surveys of diverse ecosystems over a decade enabling the detection of *P. cinnamomi* population differentiation within the forest and nursery systems. Because our collection of isolates from nurseries was limited to a 2-year sampling, the same comparison for *P. plurivora* was not as strong. Although their population structures are quite different, both species were successful in adapting to various environments and becoming one of the more common species in Maryland. Due to shortcomings in the regulation of pathogens in nurseries and natural environments, we can expect to see continued introduction of exotic genotypes into new environments (Bienapfl and Balci 2014; Jung et al. 2016). The consequences of these introductions, however, remain unclear for these well-established and unregulated pathogens. Can the introduction of new genotypes significantly influence population structure and, if so, what is the timeline for such alterations? What are the driving forces that can lead a heterothallic species such as *P. cinnamomi* to have a different population? Answers to such questions will help us better understand the dynamics of shifting population structures in intensively managed plant systems.

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