Population biology analysis of *Pythium cryptoirregulare* in Rhododendron Nursery Soils in Oregon

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Abstract

Pythium cryptoirregulare (Garzon, Yanez and Moorman) is a recently described plant pathogenic species. *P. cryptoirregulare* was originally believed to belong to the species of *Pythium irregulare*, however, ITS and cox I-II sequencing, and colony morphology determined that this was actually a new species, with different characteristics from those of *P. irregulare* sensu stricto. *P. cryptoirregulare* causes major disease problems for nursery growers in the Pacific Northwest each year. Millions of acres suffer from root rots and damping off. Through the use of population biology studies, we can assess the movement of this pathogen as well as other epidemiological information on the species.

This study takes a deeper look into the population structure of this species. Using isolates from rhododendron nurseries in Oregon, we assess the genetic differentiation among populations of this species in order to determine patterns and gain information on genetic relatedness within and among populations of the species. Using population genetic analyses, we were able to visualize epidemiological patterns within four rhododendron nursery populations of *P. cryptoirregulare*. With this information, we will be able to provide better management recommendations to nursery growers who deal with the major damages that *P. cryptoirregulare* causes through root rots and damping off.

Introduction

Pythium (Pringsheim) (Oomycota, Pythiaceae) includes species that are economically important pathogens that affect many species of plants worldwide. *Pythium* species are the causal agents of major diseases such as damping-off and root rots. Damping off and root rots cause significant damage to the plants. Brown or grey roots with cankers and patches, poor or no seed emergence are often symptoms of *Pythium* diseases (Chase et al. 1995). *Pythium* can also appear as a white fluffy growth on plant surfaces if the plant is severely infected (Jones and Benson 2001). Estimates of 3.3% yield loss occurred in soybeans due to seedling blights (Jardine 2014). In woody ornamentals, the situation can be much worse. According to the USDA Forest Service, root diseases caused damage to over 5.8 million forest acres in northern Idaho and western Montana (Lockman et al. 2015). *Pythium* diseases are often found in extremely wet soils, which is particularly troublesome for the Pacific Northwest where some parts of the region receive over 200 inches of precipitation a year.

Not only are there problems in the Pacific Northwest due to rainfall, but the systems that crops are grown in are often different than most of the continental U.S. Oregon is a huge player in nurseries and greenhouses, not only in the number of nurseries in the state, but also in sales and output. This makes *Pythium* diseases that much harder to control. Hansen et al. (1979) found that *Phytophthora* (Oomycota, Pythiaceae) species that were found in one nursery, also occurred in two other nurseries. Root rot was then found in all three locations. *Pythium* diseases may also be spread in this manner, which makes identification and understanding of *Pythium* species that much more necessary.

It is essential to control diseases so as to reduce economic damage whenever possible. According to Canker and Moore (1998), landscape and nursery businesses were part of an $842 million industry in the state of Washing-

ton alone. Nurseries and landscaping are major economic factors in the Pacific Northwest, and *Pythium* diseases are known to cause damage to the crops produced by this industry. The epidemiology of *Pythium* in woody ornamentals is not as well researched and more information on this subject is essential in determining the best management strategies for diseases.

Simple sequence repeats (SSRs), also known as microsatellites, are one set of genetic markers that we can use to determine genetic variation. SSRs are 1 to 6 base pair tandem repeats associated to specific loci (Karaoglu et al. 2005). SSRs are often used in population genetic studies as they are easily located via PCR amplification and are readily abundant. SSR markers have now been developed for some *Pythium* species, including *P. cryptoirregulare*, the focus of this study (Lee and Moorman, 2008)

Pythium cryptoirregulare Garzon, Yanez & Moorman is a recently discovered species that is still being taxonomically studied. The species was originally identified as *Pythium cryptoirregulare* in 2007, as part of the *Pythium irregulare* species complex. Its genus was renamed *Globisporangium* in 2010 (Uzuhashi et al., 2010), however this name has not been extensively adopted and is currently being disputed (Abad, pers. comm.). *P. cryptoirregulare* in the past was included within *Pythium irregulare* which is one of the most prevalent species of *Pythium* found in regards to seedling blight and other diseases (Jardine, 2014). More information on *P. cryptoirregulare* is necessary in determining the impact this species has on nursery diseases and to make management recommendations.

The purpose of this study was to determine the population structure among populations of *P. cryptoirregulare* to determine the movement of this species geographically and identify potential sources origin of inoculum. The study used SSR markers to determine genetic diversity and population structure across multiple nurseries, varieties, and years.

Materials and Methods

Isolates.—Fifty-one *P. cryptoirregulare* isolates were obtained from four nurseries in Oregon by Dr. Jerry Weiland (USDA-ARS, Corvallis) and shipped to Oklahoma State University for genotyping. The nurseries were varied in the type of production, including propagation, container and field facilities. All isolates were directly sampled from rhododendron roots and/or stems. All samples were received as on PARP selective medium (Weiland, 2011). *P. cryptoirregulare* isolates were found across all four nurseries (nursery A = 12, nursery B = 10, nursery C = 21, nursery D = 8). ITS sequences and colony morphology were used to confirm the isolates were *P. cryptoirregulare.* All isolates were stored on water agar plugs at room temperature for the duration of the study.

Mycelial plugs were taken from the original isolates in PARP media and were transferred to Potato Dextrose Agar (PDA). Isolates were incubated for two days, then transferred to Water Agar with antibiotics (WA+). The isolates were grown on WA+ for three days. Plugs were transferred from the WA+ media to Potato Dextrose Broth (PDB) and were left on the shaker for 3-4 days. The mycelia was then lyophilized for DNA extraction.

DNA purification.—DNA extractions were performed using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), DNA concentration was insured to be at least 35 µg/mL and quality was assessed to be between a ratio of 1.7 and 2.0.

SSR screening.—In a previous study, 22 SSR markers were found for use in population genetics studies for *P. cryptoirregulare* (Lee and Moorman, 2008). Primers were assessed to determine if they were informative for the population under study.

Table 1~~1~~. Information on SSR microsatellite markers used in *PC*. *cryptoirregulare* phylogenetic study.

Locus	Repeat motif	Primer sequences (5' to 3')	T_a/PCR cycles	Size (bp)
P50CT1-58	(CT)15	F: TCTACCAACACTGAGCGCTAGCAA R: TCGAATGCGCCAGTCAAGCTC	60°C/30	177
P50AG1-61	(AG)16	F: AATGTTCAGAAGCGTGGGAAGCAG R: CTCACATTGCTCCACAACCAGTCA	60°C/30	246
P50CA1-68	(CA)17	F: GCTGATCTGCAGTGCACCTA R: GGTAAGGCGATGATGATGCT	56°C/30	138
P50TC2-23	(TC)20	F: CCTGGCTGGTTCATTAGTCTCT R: TGGCTATCTGGATTGGTTTGTA	55°C/35	121
P50TG2-93	(TG)18	F: GCGTGGCTCGCGTCCTTAAA R: TGGGAACTCACACGGAAATGGCTA	60°C/30	113
P50GA3-20	(GA)13(GT)11	F: AGATCCGAAAGGCGATAAGC R: ATCACGCTCGAATAGTTCCTGT	55°C/30	179
P50AG3-30	(AG)18	F: CGAGCGACGATTTGTAATGCCAGT R: TCAAGGACGGAAACCCTTGTGGAA	60°C/30	117

PCR amplification was performed under the following conditions. Each 20 µL reaction was performed using 35 µg of DNA, 10 µL of 2X GoTaq Green (Promega, Madison, WI), 6 µL of nuclease-free water, and 1 µL each of the respective forward and reverse primers (5µM). PCR reactions were performed on a PTC-200 DNA engine thermal cycler (Bio-Rad, Hercules, CA). PCR cycles as follows: 94°C (2 minutes) followed by 35 cycles of 94°C (30 seconds), annealing temperatures were primer specific (25 seconds), and extension at 72°C (1 minute) at end of each cycle. There was an additional extension for 10 minutes at 72°C at the end of the PCR program. PCR products were visualized on a 2% agarose gel using an electrophoresis chamber. Each gel was run at 95 V cm^{-1} for 1 hour. The gels were photographed using a GelDoc-It® Imaging System (UVP, Upland, CA) and the imaging software VisionWorks® LS (UVP ,Upland, CA).

SSR analysis.—Seven SSR markers were informative in the studied populations (Table 1). For fragment sizing, each SSR product underwent a second round of PCR amplification using M13-tailed SSR primers. M13-tailed SSR markers are universal primers, making them much more cost efficient, while still giving us reliable data. Each 20 µL reaction consisted of 9 µL of 2X GoTaq Green (Promega, Madison, WI), 3.2 µL of 5 µM FAM or HEX M13, 3.2 µL of 5 µM reverse primer (locus specific), 2 µL of sterile water, and 1.6 µL of 5 µM M13 forward primer. We used 1 µL of the PCR product as template in the 20 µL reaction. PCR reactions were performed on a PTC-200 DNA engine thermal cycler (Bio-Rad, Hercules, CA) and the conditions were completed as follows: 94°C (5 minutes); followed by 20 cycles of 94°C (30 seconds), 56°C (45 seconds), and extension temperatures at 72°C (45 seconds) to end out the first round of cycles. The second round of 5 cycles was at 94°C (30 seconds), 53°C (45 seconds), and 72°C (45 seconds) per cycle; and a final extension at 72°C for 10 minutes. PCR products were visualized as described above. These gels were looked at to ensure PCR amplification with the labeled primers was successful. Color labeled PCR products were sent for fragment analysis at OSU's Biochemistry and Molecular Biology Recombinant DNA and Protein Core Facility.

Labeled PCR products were diluted based on the intensity of the bands. These samples were then mixed with 9 µL of Hi-Di™ Formamide (Thermo Fisher Scientific, Waltham, MA) and 0.4 µL of Size standard – Liz600 (Thermo Fisher Scientific, Waltham, MA) per 2 µL sample. This mixture was then sent off for fragment analysis and the resulting data was scored to generate SSR databases for the statistical analysis that followed.

Statistical analysis.— Statistical analysis was completed using diverse software. Fragment sizes were scored using Peak Scanner Software v1.0. Using this information, Analysis of Molecular Variance (AMOVA) and Principal Coordinate Analysis (PCoA) tests were completed and Unweighted Pair Group Method of Arithmetic Mean (UPGMA) trees and Minimum Spanning Networks (MSNs) were constructed using PoppR.

Results

Statistical analysis.—Based on the analyses completed in this study, two distinct clades of *P. cryptoirregulare* were found. These results can be seen in every analysis we ran to at least some extent.

AMOVA looks at the proportion of genetic differentiation between individuals in a population and between different populations (Kim and Sappington 2013). F_{ST}, *Nm*, and Φ_{PT} values were obtained. F_{ST} is a fixation index that assesses genetic drift between populations (Kim and Sappington 2013). An F_{ST} value of 1 would mean that there is not a single allele similar between the two populations; while a value of 0 would mean that every allele is the exact same between them. Overall, AMOVA revealed low genetic differentiation (F_{ST}=0.024) among populations.

~~Table 2. F_{ST} values of each nursery compared to the other nurseries.~~

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A	B	C	D	
0	-	-	-	A
0	0	-	-	B
0.021	0.039	0	-	C
0	0	0.048	0	D

However, significant population differentiation was observed between Nursery C and the other nurseries, as seen in Table 2. AMOVA also revealed an *Nm* value of 10.162, ~~c~~concluding that there is frequent gene flow between the populations. Φ_{PT} is another F statistic used to evaluate genetic differentiation. Φ_{PT} values are important as it is a measurement that suppresses intra-individual variation (Texiera et al. 2015) from the analysis. Our results displayed a Φ_{PT} value of 0.093, with a p value of 0.018, which means the population differentiation was statistically significant (Dracatos et. al 2009).

~~Figure 1. Scatter plot of Principal Coordinate Analysis (PCoA) for each of the four populations of *P. cryptoirregulare*.~~

~~P~~PCoA is a scatter diagram used to help visualize genetic differentiation amongst populations. The two PC axes account for the most variation, and the isolates are compared to those extremes (Kim and Sappington 2013). PCoA tests show two distinct groups among our isolates, as shown in Figure 1.

UPGMA is a clustering analysis that produces one of the more familiar types of genetic relationship trees in intra-specific population studies. UPGMA trees have the purpose of displaying genetic relatedness through branching. Bootstrap analysis tests the robustness of dendrogram topologies. A higher percentage value means that a higher proportion of bootstrap trees support the topology of each node

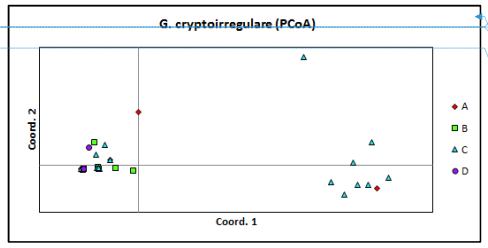

Figure 1. Scatter plot of Principal Coordinate Analysis (PCoA) for each of the four populations of *P. cryptoirregulare*.

(Highton 1993). UPGMA tree confirmed the

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two clusters observed in PCoA (Figure 2). One of the clusters is 92.5% supported.

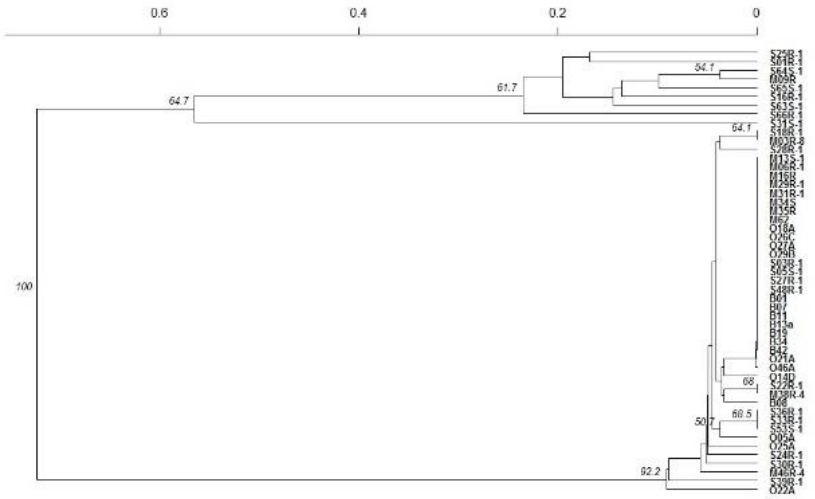

Figure 2. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) tree of *P. cryptoirregulare* isolates using SSR microsatellite markers.

MSNs were generated using two types of genetic distances, Bruvo and Nei distances. Bruvo distance take into account the repetitive nature of microsatellites when making genetic distance calculations (Bruvo et al. 2004). Nei distances into account single nucleotide mutations (Nei, 1972). Bruvo and Nei plots confirmed the two clusters identified by PCoA and UPGMA (Figures 3 and 4). We are able to clearly visualize the populations that are separated. In one of the groups, all of the populations are represented; however, almost of the isolates in the other cluster came from nursery C.

Discussion

Statistical analyses.—Our results clearly outline two distinct groups within the populations. MSNs are trees that show genetic distance between nodes as a function of length and thickness of the lines. This allows easy visualization of the relatedness of individuals and populations.

As shown in Figures 3 and 4, we can see that in one group, all of the populations are represented. However, in the other group, we see mostly isolates from nursery C. What we could speculate from this information is the possibility of movement of isolates from the main cluster between nurseries on infected plant materials or a common source of inoculum. To explain further, the less genetic differentiation between populations, the greater the possibility that these isolates originated from a the same source, or that the inoculum is frequently moved between facilities. However, in the case of nursery C, it could be stated that this nursery may have a local source of inoculum, that persist in this nursery but has not been moved to the other facilities. Since Nursery C had a larger sample with a total of 21 isolates, the most out of any of the studied populations, it is pos-

sible that this nursery received infected plant materials from a different provider than the other ornamental operations.

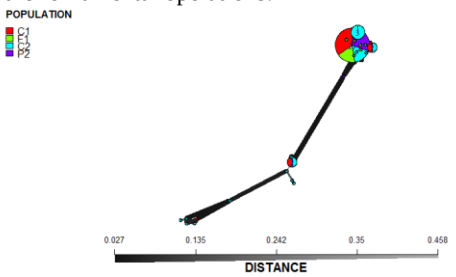

Figure 3. Minimum Spanning Network (MSN) of *P. cryptoirregulare* using Bruvo's distance.

From the *Nm* values, frequent migration can be inferred. This high number of individuals that move between populations each generation, means that as time goes on, populations will generally become more genetically similar because they share the same alleles. However, this also proves that each population can change over time. The problem with populations becoming more similar is that the best traits for the survival of the species are carried over into the next generation. Therefore, if one isolate of the species has resistance to a certain type of fungicide, this resistance trait can be carried across nurseries through infected seedlings or equipment.

Management strategies.— With the knowledge that *P. cryptoirregulare* may be mobilized between woody ornamental nurseries, it is important for nursery managers to remain vigilant in reducing host susceptibility. One of the best ways to prevent *P. cryptoirregulare* growth is by using current-year or fresh seed (Paulitz et al. 2002). Using fresh seed will prevent contamination by infected seedlings. However, this can be costly to nursery managers, so it may not be as feasible. It is still preferred to limit the amount of seedling movement from nurseries when possible. A much more practical way to combat *P. cryptoirregulare* movement is to thoroughly clean any shared equipment. With proper sterilization techniques, the number of any pathogen on the equipment will be reduced.

Other management strategies to help

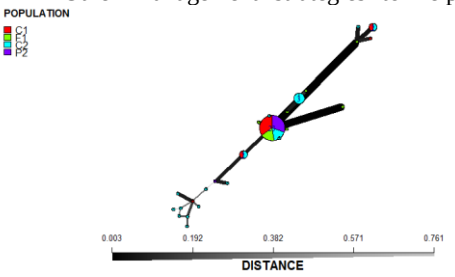

Figure 4. Minimum Spanning Network (MSN) of *P. cryptoirregulare* using Nei's distance.

prevent Pythium diseases are to utilize seed treatments and fertilizer placements (Paulitz et al. 2002). Seed treatments are effective in controlling damping-off that occurs in seedlings. Fertilizer placement is important for plant growth when dealing with any root related disease. Ensuring the plant has any nutrient necessary is essential for growth and resistance to diseases. Proper fertilizer placement makes nutrients more available to the root, and limits the amount of energy roots must spend growing to find the nutrients.

Future research.— As described by Highton (1993), as the number of loci in a study increases, the support of the topology at those nodes increases. In order to have a better grasp on what the genetic relatedness of the isolates and the populations, testing more loci will be necessary. It can also be said that testing more isolates within each loci could also help create more supported phylogenetic branching, however, due to the collection process of *P. cryptoirregulare*, finding more isolates per nursery, is not always feasible. For example, not every type of root disease is related back to *P. cryptoirregulare*. Other species of *Pythium* can be found just as easily. Therefore, in order to continue to receive better data for the epidemiological analysis of this species, using more SSR markers may be useful.

This study was part of a larger multistate study being researched. The testing of

SSR markers against the four nurseries listed is just one phase of the study. More isolates have come in from separate years, including some from the same nurseries in this study, as well as additional nurseries in Oregon. At the completion of the study, we will have better understanding of the movement of this pathogen between nurseries its survival over time.

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